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Chemical Composition of Different Botanical Origin Honeys Produced by Sicilian Black Honeybees (*Apis mellifera* ssp. *sicula*)

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ABSTRACT: In 2008 a Slow Food Presidium was launched in Sicily (Italy) for an early warning of the risk of extinction of the Sicilian native breed of black honeybee (*Apis mellifera* L. ssp *sicula*). Today, the honey produced by these honeybees is the only Sicilian honey produced entirely by the black honeybees. In view of few available data regarding the chemical composition of *A. mellifera* ssp. *sicula* honeys, in the present investigation the chemical compositions of sulla honey (*Hedysarum coronarium* L.) and dill honey (*Anethum graveolens* L.) were studied with a multimethodological approach, which consists of HPLC-PDA-ESI-MS*n* and NMR spectroscopy. Moreover, three unifloral honeys (lemon honey (obtained from *Citrus limon* (L.) Osbeck), orange honey (*Citrus arantium* L.), and medlar honey (*Eriobotrya japonica* (Thunb.) Lindl)), with known phenol and polyphenol compositions, were studied with NMR spectroscopy to deepen the knowledge about sugar and amino acid compositions.

KEYWORDS: *honey, Apis mellifera ssp sicula, HPLC-PAD-ESI-MSn, NMR, chemical characterization*

INTRODUCTION

Honey is produced by honeybees (*Apis mellifera* L.) from plant nectars, plant secretions, excretions from plant-sucking insects, and glandular secretions and is a complex mixture that contains nutrients and bioactive compounds such as carbohydrates, proteins and enzymes, amino acids, minerals, vitamins, and polyphenols. The main honey components are sugars and polyphenols, which influence its sensorial and biological properties.[1−3] In fact, honey consists of a supersaturated solution of carbohydrates, which represent about 95% of its dry weight and among which the main constituents are glucose (31% w/w) and fructose (38% w/w), followed by disaccharides and oligosaccharides (3−4% w/w), which are formed when nectar and honeydew are converted to honey.[4] Moreover, honey contains polyphenols, the concentration of which strictly depends on its botanical and geographical origins, seasonal and environmental factors, and processing, packaging, and storage conditions. Flavonoids are considered naturally occurring markers for honey botanical origin due to the correlation between floral origin and flavonoid profiles demonstrated by some studies.[5−7]

Sicilian black honeybees (*Apis mellifera* ssp. *sicula*) are a subspecies of the more common *A. mellifera* ssp. *ligustica*, from which they differ in color, wing dimensions, and other characteristics such as better resistance to high temperature, higher ability of impollination, and higher physical and immunological resistance. These features are due to the fact that Sicilian black honeybees have common origins with African bees, which have some genes in common with *A. mellifera* ssp. *sicula*.[8] After living in Sicily for thousands of years, during the 1970s, the Sicilian black honeybees were threatened by extinction when Sicilian apiarists started to import *A. mellifera* ssp. *ligustica* from northern Italy, considering the latter more productive and docile and thus triggering a contamination of the native species of honeybees. Sicilian black honeybees were saved from extinction thanks to the interest of some entomologists who moved some of their hives to the Aeolian Islands, where the black honeybees could reproduce without the risk of contamination by other species. In 2008 a Slow Food Presidium was launched in Sicily (Italy) for an early warning of the risk of extinction of *A. mellifera* ssp. *sicula*. Today, this Sicilian breeding is the only one dedicated specifically to the black Sicilian honeybees. and the honey produced by these honeybees is the only Sicilian honey produced entirely by black honeybees.[9]

Little is known about the chemical composition and properties of unifloral honeys indigenous to Sicily, so a few years ago we started an investigation of these honeys. Our previous research determined the phenolic and polyphenolic contents and the antioxidant and antimicrobial properties of lemon honey (obtained from *Citrus limon* (L.) Osbeck), orange honey (*Citrus arantium* L.), prickly pear honey (*Opuntia littoralis* var. *vaseyi* (J.M. Coult.) L.D. Benson & Walk.), medlar

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honey (*Eriobotrya japonica* (Thunb.) Lindl.), and almond honey (*Prunus dulcis* Mill. ex Rchb.).[10] The results showed that the tested honey samples presented higher phenolic content and antioxidant capacity than those produced by other honeybee subspecies from Sicily, other different Italian regions, and abroad.[10−14] Moreover, all tested honeys showed good antimicrobial activity against a broad spectrum of microorganisms.

Honey chemical characterization is generally studied with high-performance liquid chromatography coupled with diode array detection or electrospray ionization tandem mass spectrometry (HPLC-PDA or HPLC-ESI-MS/MS). These techniques are useful to reveal the presence of botanical biomarkers, α-dicarbonyl and phenolic compounds,[3,15] and contaminants.[16] Nevertheless, matrix interference, especially in the analysis of carbohydrates and peptides, and low sensitivity require the application of solid phase extraction (SPE) sample treatment before the analysis of the sugar- and peptide-derived fractions. Therefore, in most investigations carbohydrates are not determined or are evaluated as total sugar content according to spectrophotometric methods with a lack of specific knowledge on these components.[17−19] Given this scarce information about the chemical composition of *A. mellifera* ssp. *sicula* honeys, especially regarding carbohydrates and polyphenols, the present investigation represents an extension of our previous research[10] and is aimed to (1) investigate the chemical composition of two further honeys, sulla honey (*Hedysarum coronarium* L.) and dill honey (*Anethum graveolens* L.), with a multimethodological approach that consists of HPLC-PDA-ESI-MS^n and NMR spectroscopy; and (2) to extend the study of lemon, orange, and medlar honeys with NMR spectroscopy, to deepen the knowledge about their sugar and amino acid compositions. The combination of these techniques combines the advantages of liquid chromatography coupled with MS to those of NMR spectroscopy, which is a technique that has been recently employed in the field of food analysis, because it can lead both to "high-throughput" and simultaneous structural information on a wide range of metabolites and to the identification of specific metabolites or their classes. This approach allowed us to describe the phytochemical composition of each type of honey and identify many compounds that were found in these types of honeys for the first time. In addition, we could compare the metabolic profiles obtained by our analysis with those already reported in the literature and provide a first phytochemical composition for that kind of honey for which no chemical data are available in the literature.

MATERIALS AND METHODS

Chemicals and Materials. Millipore grade water was obtained with a Milli-Q water purification system (Millipore Corp., Billerica, MA, USA); formic acid, (−)-catechin, and luteolin were purchased from Sigma-Aldrich (Milan, Italy); LC-MS grade methanol was obtained from VWR International PBI srl (Milan, Italy). D_2O 99.9%D was purchased from EURISO-TOP (Saint-Aubin, France).

Honey Samples. *A. mellifera* ssp. *sicula* honey samples of different botanical origin, that is, sulla (*H. coronarium*), dill (*A. graveolens*), lemon (*C. limon*), orange (*C. arantium*), and medlar honeys (*E. japonica*), were obtained from an Italian independent apiarist (Carlo Amodeo, Termini Imerese, Palermo, Italy). Honey samples were harvested in 2014 by individual apiarists in the area of Termini Imerese (37°98′ N, 13°70′ E, Palermo, Italy). Samples were classified following the melissopalynological analysis[10] according to which the floral source was confirmed if the pollen content in the honeys was not lower than 10% (this percentage ranged between 27 and 61%). During the experiments, three samples for each botanical source were kept at 5 °C in the dark in airtight containers for <5 months until the analysis.

Sample Preparation for RP-HPLC-PDA-ESI-MS^n Analysis. One gram of each honey sample was added to 2 mL of Millipore grade water acidified with 0.1% formic acid. Each sample was purified with SPE, using an RP Sep-Pak Vac 6 cm^3 (1g) tC18 cartridge (Waters, Milford, MA, USA). After wetting the cartridge with methanol (10 mL) and conditioning it with 0.1% formic acid (10 mL), the polyphenol-enriched fraction (SPE_2 fraction) was eluted with 2 mL of a solution composed of methanol and 0.1% formic acid, 90:10% v/v, at a flow rate of 0.4 mL/min. The SPE_2 fraction was concentrated under a nitrogen stream until the volume of 130 μL, corresponding to a concentration of 7.69 mg/mL. After filtration with 0.45 and 0.20 μm nonsterile syringe filters, the concentrated SPE_2 fraction was submitted to RP-HPLC-PDA-ESI-MS^n analysis.

RP-HPLC-PDA-ESI-MS^n Analysis. All experiments were performed using a Thermo Finnigan Surveyor Plus HPLC, equipped with a quaternary pump, a Surveyor UV−vis diode array detector, and a LCQ Advantage Max ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), connected through an ESI source.

The separation was carried out on a Gemini C18 110 Å (150 × 2 mm, 5 μm), equipped with an Ultra Cartridge C18 Peptide Security Guard Column, both from Phenomenex (Torrance, CA, USA). The mobile phase consisted of water acidified with 0.1% of formic acid (A) and methanol (B) and was eluted with the following gradient: from 2 to 5% B in 10 min, from 5 to 40% B in 50 min, from 40 to 60% B in 10 min, from 60 to 100% B in 10 min, followed by a 10 min isocratic run of 100% B. Total run time was 115 min, including column reconditioning. The flow rate was maintained at 0.3 mL/min, the autosampler and column temperatures were maintained at 4 and 25 °C, respectively. Chromatograms were monitored at 210, 254, and 280 nm; spectral data were collected within the range of 200−800 nm for all peaks. The ion trap was operated in data-dependent, full scan (m/z 100−1000), zoom scan, and MS^n mode. To obtain MS^2 data, a 35% collision energy and an isolation width of 2 m/z were applied. ESI source parameters had previously been optimized by flow injection analysis using (−)-catechin (10 ppm, in 0.1% formic acid and methanol, 50:50, v/v) and luteolin (5 ppm, in 0.1% formic acid and methanol, 50:50, v/v), for negative and positive ionization, respectively, to a ionization voltage of 3.5 kV, a capillary temperature of 200 °C, a sheath gas flow of 45 arbitrary units, and an auxiliary gas flow of 20 arbitrary units.

Sample Preparation for NMR Analysis. The samples were prepared dissolving 20 mg of honey in 700 μL of D_2O containing 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP, 2 mM) as internal standard. Once a homogeneous solution was achieved using a vortex rotor, 700 μL of honey solution was transferred into a standard 5 mm NMR tube and subjected to NMR analysis.

NMR Analysis. The NMR spectra were recorded at 27 °C on a Bruker AVANCE 600 NMR spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear z-gradient inverse probehead capable of producing gradients with a strength of 55 G cm^{-1} in the z-direction. ^{1}H spectra were referenced to methyl group signals of TSP (δ = 0.00 ppm) in D_2O. ^{1}H spectra were acquired by adding 64 transients with a recycle delay of 3 s. The residual HDO signal was suppressed using presaturation with a single soft pulse on the HDO resonance frequency applied during the relaxation delay followed by a strong observation pulse.

2D NMR experiments, namely, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC, were performed using the same experimental conditions previously reported.[20] The mixing time for the ^{1}H−^{1}H TOCSY was 80 ms, and the spin lock field was 6250 Hz. The HSQC experiments were performed using a coupling constant $^1J_{C-H}$ of 150 Hz, and the ^{1}H−^{13}C HMBC experiments were performed using a delay for the evolution of long-range couplings of 80 ms.

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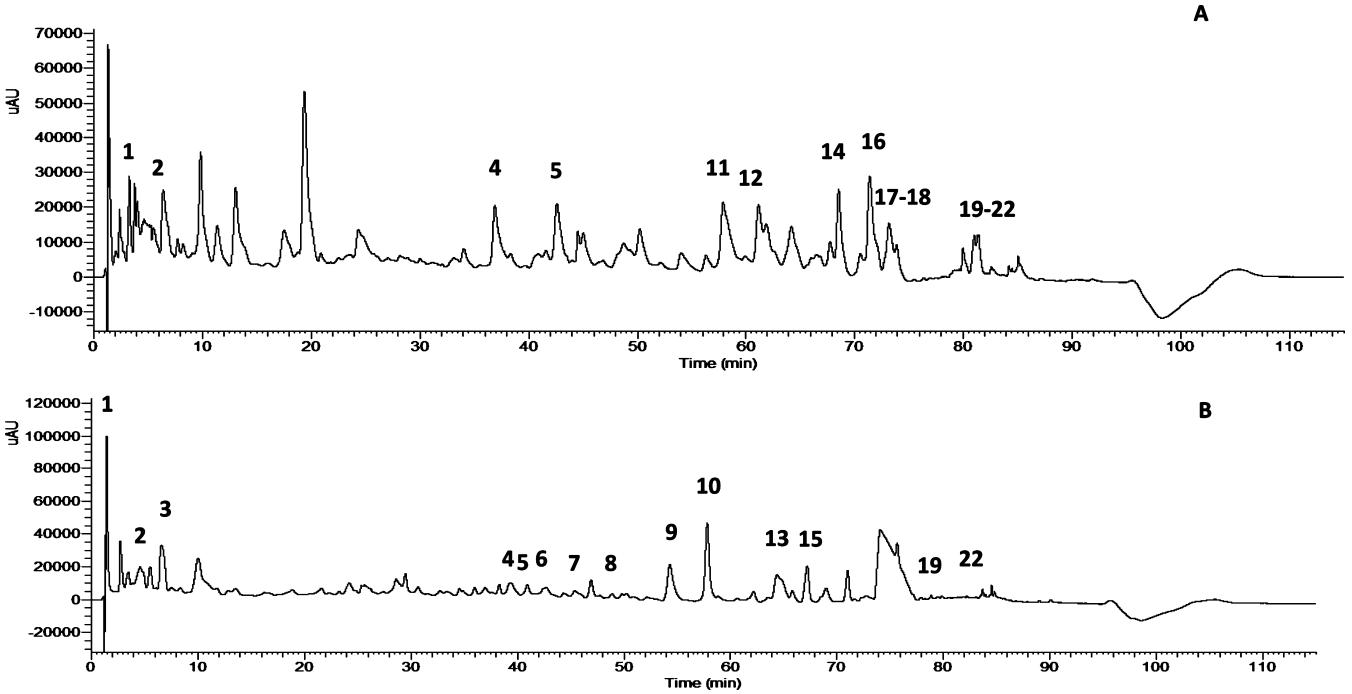

Figure 1. Chromatographic profile of SPE_2 fraction of sulla (A) and dill (B) honeys.

Table 1. Chromatographic Behavior and MS and MS^n Data of the Compounds Identified in Sulla and Dill Honeys

peak	RT (min)	λ_{max} (nm)	m/z	HPLC-ESI-MS^n m/z (% of base peak)	compound
1	2.29	260	195	177 (100), 49 (50)	4-methoxyphenyllactic acid[a,b]
2	6.86	260	191	111 (100), 129 (20)	citric acid[a,b]
3	6.96	257	166[c]	120 (100)	phenylalanine[b]
4	39.80	260	165	147 (100)	phenyllactic acid[a,b]
5	42.60	230	223[c]	205 (100), 121 (15)	dehydrovomifoliol[a,b]
6	42.80	230	223[c]	205 (100), 135 (22)	3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione[b]
7	44.80	260	137	93 (100)	*p*-hydroxybenzoic acid[b]
8	49.81	270	377[c]	243 (100), 172 (2)	riboflavin[b]
9	52.80	240	190[c]	162 (30), 173 (40)	kynurenic acid[b]
10	57.98	260	213[c]	181 (100), 154 (20), 121 (2)	methyl siringate[b]
11	58.39	257, 346	771	609 (100), 463 (10), 301 (5)	quercetin hexosyl rutinoside[a]
12	60.36	260, 350	755	300 (100), 301 (30), 609 (20)	quercetin rhamnosyl-hexosyl-rhamnoside[a]
13	65.70	250	243[c]	172 (65), 200 (60), 216 (70), 198 (5)	lumichrome[b]
14	65.96	255, 351	609	301 (100)	quercetin rutinoside[a]
15	67.22	250	265[c]	247 (100), 229 (20)	abscisic acid[b]
16	70.15	280	285	267 (100), 139	pinobanksin methyl ether[a]
17	71.09	270	593	285 (100)	kaempferol rutinoside[a]
18	71.51	287	271	253 (100), 225 (25)	pinobanksin[a]
19	79.65	290	255	213 (35), 151	pinocembrin[a,b]
20	79.93	255, 287	247	179 (100), 135 (20)	caffeic acid isoprenyl ester[a]
21	80.28	290, 326	313	253 (100), 271 (20)	pinobanksin acetate[a]
22	83.38	240	343[c]	240 (100)	pinobanksin butyrate[a,b]

[a]Compound identified in sulla honey. [b]Cmpound identified in dill honey. [c]Compound revealed by positive ionization mode.

RESULTS AND DISCUSSION

RP-HPLC-PDA-ESI-MS^n Analysis. RP-HPLC-PDA-ESI-MS^n analysis of sulla and dill honeys allowed the identification of 22 compounds, some of which were present in both honey samples. The chromatograms obtained from sulla and dill honey analyses are reported in Figure 1. The identification of these compounds was performed on the basis of their chromatographic behaviors; UV−vis, MS, and MS/MS spectra; and comparisons with literature data. The results obtained are summarized in Tables 1 and 2. For each compound, the retention time, the wavelength of maximum absorbance, m/z values of molecular ion, and its fragment ions are reported.

Nine flavonoids, among which were four flavonols (quercetin hexosyl rutinoside, quercetin rhamnosyl-hexosyl-rhamnoside, quercetin rutinoside, and kaempferol rutinoside), four flavanonols (dihydroflavonols, i.e. pinobanksin methyl ether, pinobanksin, pinobanksin acetate, and pinobanksin butyrate), and one flavanone (pinocembrin), were identified. Moreover, the analysis showed the presence of four organic acids (citric

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Table 2. Presence of Nutrients and Bioactive Compounds in Sulla and Dill Honeys

compound	sulla honey[a]	dill honey[a]
flavonols		
quercetin hexosyl-rutinoside	+	−
quercetin rhamnosyl-hexosyl-rhamonoside	+	−
quercetin rutinoside	+	−
kaempferol rutinoside	+	−
flavanonols		
pinobanksin	+	−
pinobanksin methyl ethere	+	−
pinobanksin acetate	+	−
pinobanksin butyrate	+	+
flavanones		
pinocembrin	+	+
organic acids		
citric acid	+	+
lactic acid	+	+
succinic acid	+	+
acetic acid	+	+
4-methoxyphenyllactic acid	+	+
phenyllactic acid	+	+
kynurenic acid	−	+
phenolic acids and derivatives		
p-hydroxybenzoic acid	−	+
methyl siringate	−	+
caffeic acid isoprenyl ester	−	+
amino acids		
phenylalanine	−	+
proline	+	+
alanine	+	+
vitamins and metabolites		
riboflavin	−	+
lumichrome	−	+
norisoprenoid		
abscisic acid	−	+
other compounds		
dehydrovomifoliol	+	+
3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione	−	+

[a]+, found; −, not found.

acid, kynurenic acid, phenyllactic acid, and 4-methoxyphenyllactic acid), three phenolic acids and derivatives (*p*-hydroxybenzoic acid, caffeic acid isoprenyl ester, and methyl syringate), one vitamin and its main metabolite (riboflavin and lumichrome), one norisoprenoid (abscisic acid), one amino acid (phenylalanine), dehydrovomifoliol ((±)-1′-hydroxy-4′-keto-α-ionone), and 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione.

As far as flavonoids are concerned, RP-HPLC-PDA-ESI-MS^n analysis of sulla honey showed the presence of flavonols, flavanonols, and a flavanone. In contrast, in dill honey just a flavanonol and a flavanone were detected. Peaks 11, 12, and 14 were identified as quercetin derivatives, because all of their MS^2 spectra showed the presence of quercetin aglycone at m/z 301. Peak 11 was assigned to quercetin hexosyl-rutinoside (MW 772) because the parent ion at m/z 771 produced fragment ions at m/z 609, due to the loss of a hexosyl moiety $[M - 162]^-$, and at m/z 463, corresponding to a hexosylated quercetin, due to the loss of the rutinoside group. Peak 12 was identified as quercetin-rhamnosyl-hexosyl-rhamnoside (MW 756) because its MS^2 spectrum showed the presence of a significant fragment ion at m/z 609, due to the loss of 146 Da that corresponded to a rhamnosyl unit. Moreover, the produced fragment ion corresponded to quercetin linked to a rhamnosyl moiety and a hexosyl one, $[301 + 146 + 162]^-$. Peak 14 was assigned to quercetin rutinoside (MW 610) on the basis of a comparison with literature data.[21] In fact, the pseudomolecular ion at m/z 609 produced a fragment ion at m/z 301, corresponding to the aglycone, due to the loss of the rutinoside moiety, $[M - 308]^-$. Peak 17 was assigned to kaempferol rutinoside (MW 594) because it produced a parent ion at m/z 593 and a fragment ion at m/z 285, corresponding to kaempferol aglycone and obtained by the loss of the rutinoside group.[21]

Of the flavanonols, peak 18 was identified as pinobanksin (MW 272) because the parent ion at m/z 271 produced the typical MS^2 spectrum already known in the literature,[22] characterized by the presence of fragment ions at m/z 253, due to the loss of H_2O, at m/z 243, corresponding to $[M - H - CO]^-$, at m/z 165, corresponding to $[M - H - H_2O - 2CO_2]^-$, at m/z 151 corresponding to the ion $[^{1,3}A]^-$, which derives from the cleavage of the C ring of the aglycone that undergoes retro-Diels–Alder scission. Peaks 16, 21, and 22 were assigned to pinobanksin derivatives. In more detail, peak 16 was identified as pinobanksin methyl ether (MW 286) because its MS/MS spectrum showed the presence of ions at m/z 252, due to the loss of the methyl group and a water molecule, at m/z 271, corresponding to the aglycone obtained by the loss of the methyl group, and at m/z 239 and 224 as previously reported.[23] Peak 21 was identified as pinobanksin acetate (MW 314). The assignment was possible by comparing the MS^2 spectrum with data already available in the literature.[23] The parent ion at m/z 313 produced fragment ions at m/z 271, corresponding to pinobanksin aglycone, obtained by the loss of the acetate (− 42 Da), and at m/z 253, due to the loss of a water molecule from the aglycone. Pinobanksin methyl ether and pinobanksin acetate were detected only in sulla honey, whereas pinobanksin butyrate, identified as peak 22, was found in both honey samples. The assignment was possible due to the fact that pinobanksin butyrate (MW 342) parent ion at m/z 343 produced a MS/MS spectrum as already reported by Chua et al.[24]

The flavanone, peak 19, was detected in both sulla and dill honeys and was identified as pinocembrin because the molecular ion at m/z 255 produced fragment ions at m/z 213, due to the loss of $[M - C_2H_2O^-]^-$, and at m/z 151, corresponding to the ion $[^{1,3}A]^-$, which derives from C ring cleavage by RDA scission, as reported by Ristivojević et al.[23]

The chemical structures of flavonoids detected in sulla and dill honeys are reported in Figure 2.

The presence of flavonoids in sulla honey is in agreement with earlier studies carried out on an Italian sulla honey sample in which quercetin and kaempferol aglycones were identified.[11] For the other flavonoids, this is the first report on the isolation and identification of flavanonols and a flavanone in sulla honey, to date. With regard to the absence of flavonols in dill honey, Ortan et al.[25] showed the presence of quercetin in *A. graveolens* flowers, leaves, and fruits, but no literature data have been reported on the potential content of these compounds in nectar and honey. Also in this case, this is the first report on the isolation and identification of pinobanksin butyrate and pinocembrin in dill honey.

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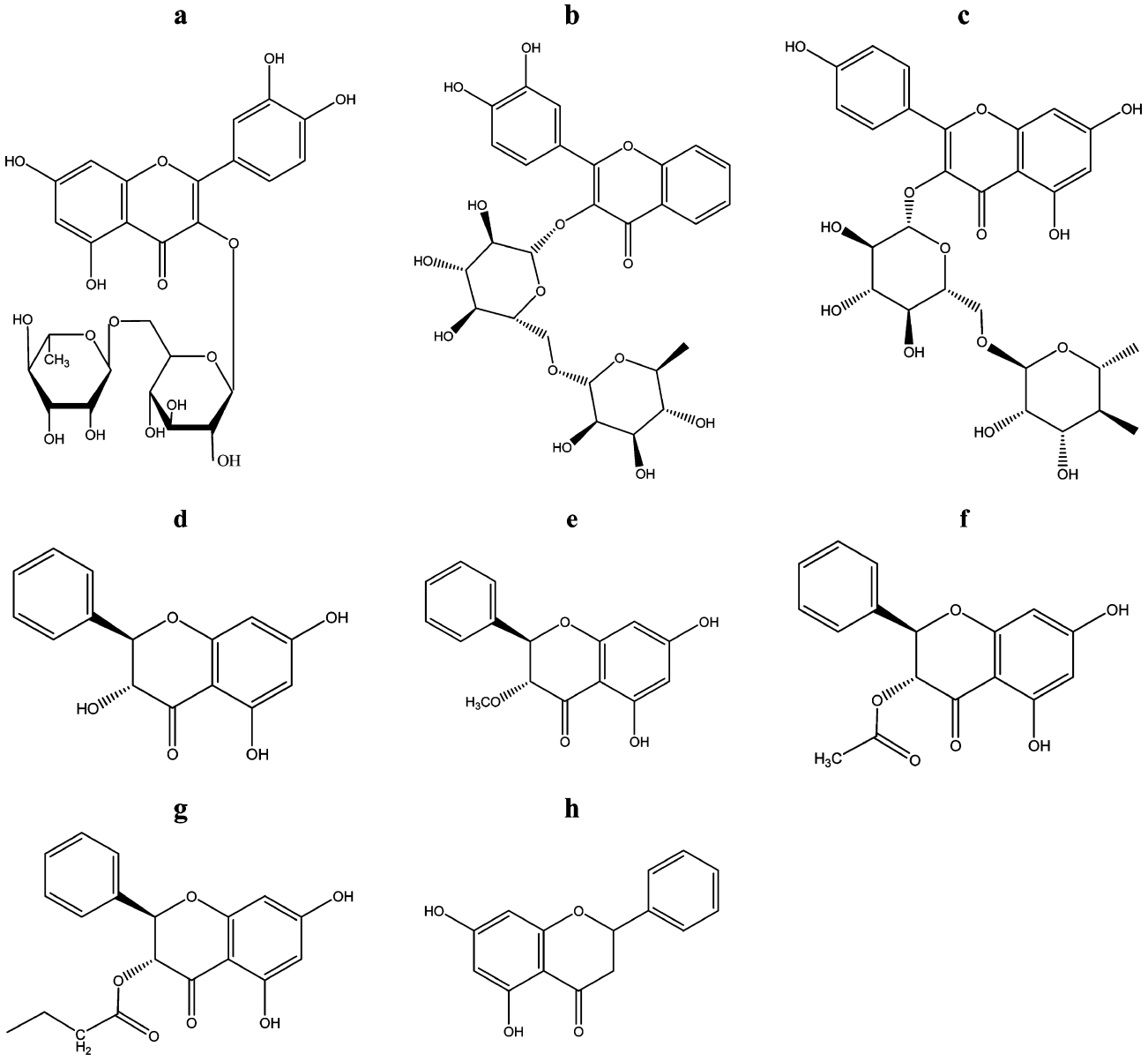

Figure 2. Chemical structures of flavonoids detected in sulla and dill honeys.

With regard to organic acids, the analysis showed the presence of four compounds belonging to this chemical class. In more detail, peak 2, detected in both sulla and dill honeys, was identified as citric acid (MW 192) because the parent ion $[M - H]^-$ at m/z 191 produced a fragment ion at m/z 129, corresponding to $[M - H - H_2O - CO_2]^-$, and another ion at m/z 111, due to the consequent loss of H_2O. Peaks 1 and 5 were identified as 4-methoxyphenyllactic acid (MW 196) and phenyllactic acid (MW 164), respectively. They were found in both sulla and dill honeys. In more detail, peak 1 was assigned to 4-methoxyphenyllactic acid because in the negative ionization mode the pseudomolecular ion at m/z 195 produced a MS/MS spectrum as previously reported in the literature by Oelschlaegel et al.[7] Peak 5 was identified as phenyllactic acid (MW 166) because its parent ion $[M - H]^-$ at m/z 165 produced fragment ions at m/z 147 and 119, corresponding to the neutral loss of H_2O and HCO_2H, respectively.[7] Peak 9, detected only in dill honey, was assigned to kynurenic acid (MW 189). Its parent ion $[M + H]^+$ at m/z 190 produced fragment ions at m/z 173 and 162, due to the loss of OH and a carbonyl group, respectively.

Phenyllactic acid is a common compound found in honeys. Its concentration ranges from several hundreds of millgrams per kilogram to <1 mg/kg and is strongly correlated to the typical honey sensorial properties.[5] Kynurenic acid derives from the tryptophan metabolic pathway and is the precursor of kynurenine and xanthurenic acid. Its presence in honey could be of interest due its biological and biochemical properties. Soto et al. showed the presence of kynurenic acid and derivatives in different botanical origin honeys. The highest kynurenic acid amounts were found in chestnut, blackberry, and multifloral honeys, with concentrations ranging from 103 to 141 to 3 mg/kg. Other kinds of honeys, such as oak, heather, orange, eucalyptus, thyme, sunflower, and acacia, were shown to contain lesser amounts (concentration <1 mg/kg) of this quinolone alkaloid. Nevertheless, no data are available on the presence of kynurenic acid in dill honey to date.[26]

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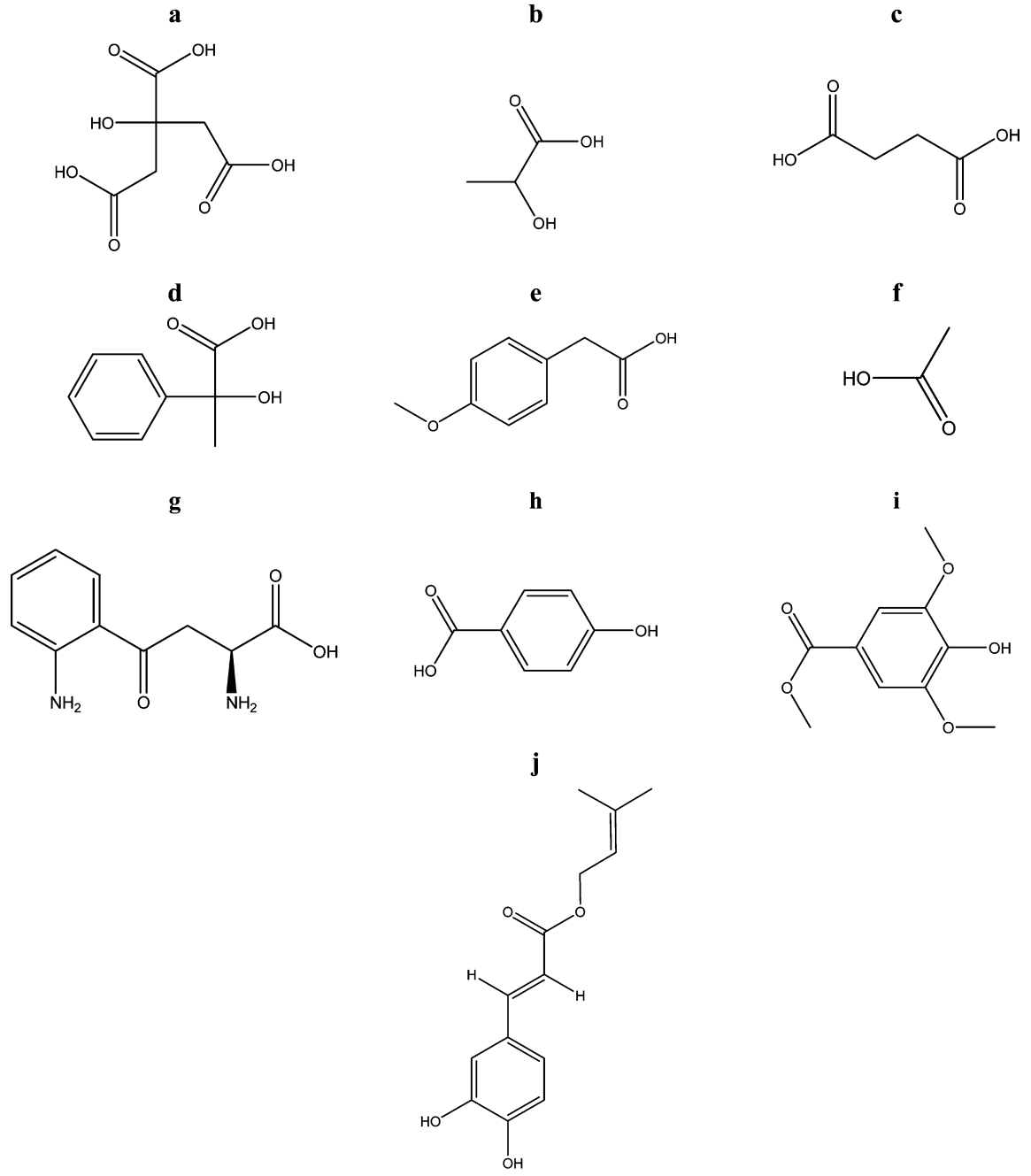

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Figure 3. Chemical structures of organic acids, phenolic acids, and their derivatives detected in sulla and dill honeys.

As far as phenolic acids and derivatives are concerned, three substances were determined. Peak 7, detected only in dill honey, was identified as *p*-hydroxybenzoic acid (MW 138) because it presented a pseudomolecular ion $[M - H]^-$ at m/z 137, which produced a fragment ion at m/z 93, obtained by the loss of the carboxylic moiety. Peak 10, present only in dill honey, was assigned to methyl syringate (MW 212) because its parent ion $[M + H]^+$ at m/z 213 produced fragment ions at m/z 181, due to the loss of a carboxymethyl group, at m/z 154, corresponding to $[M - OCH_3CO]^+$, and at m/z 121, corresponding to $[M - (OCH_3)_2CO]^+$. Peak 20, identified only in sulla honey, was assigned to caffeic acid isoprenyl ester (MW 248). Its MS spectrum showed the presence of a parent ion $[M - H]^-$ at m/z 247, whereas its MS/MS spectrum presented ions at m/z 179, due to the loss of the isoprenyl group, and at m/z 135, obtained by the consequent loss of the carboxylic moiety.[27]

The chemical structures of organic acids, phenolic acids, and their derivatives detected in sulla and dill honeys are reported in Figure 3. Methyl syringate is a common phenolic derivative that was already detected in many honeys of different botanical origins such as robinia, rape, chestnut, clover, linden blossom, daendelion, sunflower, thyme, and manuka honeys.[28] The presence of this compound in sulla honey is in agreement with Jerković et al., who determined this compound by GC-MS analysis.[28] With regard to the other phenolic and hydroxycin-

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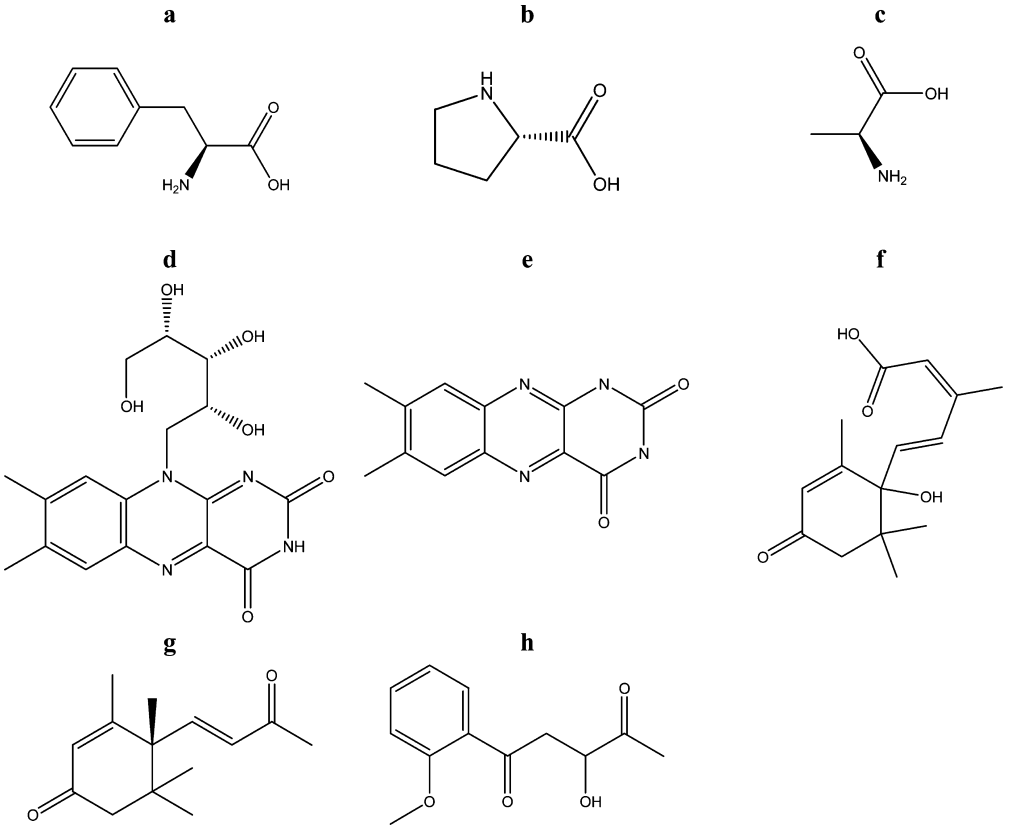

Figure 4. Chemical structures of other metabolites detected in sulla and dill honeys.

namic acids found in sulla and dill honeys, these compounds are typical secondary metabolites of plants,[29] widely distributed in many parts of the plants including flowers and nectars, and are identified in many different floral origin honeys.[10,15,30]

With regard to the other identified compounds, peak 3, found only in dill honey, was assigned to phenylalanine (MW 165) because its pseudomolecular ion at *m*/*z* 166 produced a fragment ion at *m*/*z* 120, due to the neutral loss of 46 Da, corresponding to HCOOH.

It is known that amino acids in honey are attributable to both animal and plant origin. The most abundant honey amino acid is proline, followed by phenylalanine, alanine, and glutamic acid. Phenylalanine content seems to be correlated to geographical origin, even if its presence is not always confirmed in honey, as reported by Kelly et al.[31]

In dill honey LC-MS/MS analysis showed also the presence of a riboflavin and lumichrome (7,8-dimethylalloxazin). The positive ionization mode allowed the identification of riboflavin (MW 376) as peak 8 because its MS/MS spectrum presented an intense fragment ion at *m*/*z* 243, corresponding to lumichrome, obtained from the partial loss of the ribitol moiety. Moreover, peak 13 was assigned to lumichrome (MW 242), because its MS^2 spectrum showed the presence of the same fragment ions as previously reported by Tuberoso et al.[5]

Lumichrome is a degradation product of riboflavin formed via a photochemical-induced cleavage of the ribityl group under neutral and acidic conditions. The presence of lumichrome was reported for the first time in 2011 by Tuberoso et al.[5] in thistle (*Galactites tomentosa* Moench) honey samples collected in different areas of Sardinia (Italy). More recently, the same authors reported that lumichrome occurs also in Dalmatian sage (*Salvia officinalis* L.) honey samples.[32] Our results show the occurrence of lumichrome in dill honey for the first time.

The present analysis allowed us to identify abscisic acid (MW 264), a norisoprenoid compound, derived from carotenoid degradation.[30] Abscisic acid was identified in dill honey as peak 15 because it showed fragment ions, derived from a parent ion $[M - H]^-$, at *m*/*z* 263, at *m*/*z* 219, due to the loss of the carboxylic group, and at *m*/*z* 201, corresponding to $[M - CO_3H]^-$.[33]

Abscisic acid acts as a plant hormone, inhibiting growth and helping plants to control stressful conditions. The presence of this compound in dill honey is not surprising as it is widely distributed in many honey varieties. With regard to sulla honey, the absence of abscisic acid confirms the results obtained by Jerković et al., who did not find this compound but revealed the presence of vomifoliol, a degradation product of abscisic acid.[28] On the other hand, we determined dehydrovomifoliol in both sulla and dill honeys.[7] In fact, LC-MS/MS analysis showed the presence of dehydrovomifoliol (detected in both sulla and dill honeys) and 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione (found only in dill honey), which were previously identified in manuka honey (*Leptospermum scoparium* R.Forst. & G.Forst.) and were considered specific markers of this kind of honey.[7] The assignment was possible due to the presence in the literature of MS/MS spectra of these compounds that allowed a comparison.[7] Both compounds were identified in positive ionization mode, giving fragment ions at *m*/*z* 205 and 121 for dehydrovomifoliol (MW 222, peak 5) and at *m*/*z* 205 and 135 for 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione (MW 222, peak 6), respectively.

The chemical structures of the other metabolites detected in sulla and dill honeys are reported in Figure 4.

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NMR Analysis. In accordance with literature data,[34] the ^{1}H NMR spectrum of honey in an aqueous solution is dominated by the intense signals of glucose and fructose anomeric forms (Figure 5 is an example) where total ^{1}H NMR spectra of dill and sulla honeys are compared.

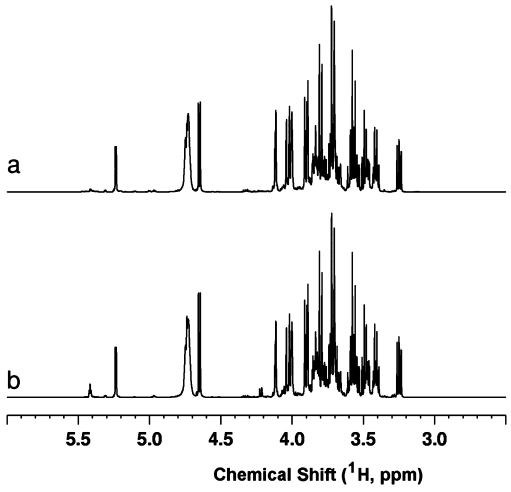

Figure 5. ^{1}H NMR spectra of dill (a) and sulla (b) honeys solutions in D_2O.

Apart from the signals of the principal components of the saccharide fraction, glucose and fructose, the signals of minor components are also observed in ^{1}H spectra. In Figure 6 the spectral region of anomeric protons (5.5−4.4 ppm) is reported not only for dill and sulla honeys but also for lemon, orange, and medlar ones. The complete assignment of these signals to individual compounds requires the application of ^{1}H−^{13}C HSQC-NMR with enhanced resolution in the ^{13}C dimension,[35] to overcome the overlapping of their ^{1}H and ^{13}C resonances,[34] the use of literature databases, and, in some cases, addition of standard compounds. The partial assignment obtained using 2D NMR experiments and literature data[34−36] was focused on ^{1}H signals not overlapped with other resonances. In this case the direct quantification by integration of corresponding ^{1}H signals was possible. Raffinose, kojiobiose, turanose, and nigerose were identified by virtue of their characteristic anomeric signals (Figure 6). The multiplet signal at 5.42 ppm showed a marked variation in the intensity among the five honeys. The structure of this multiplet (two doublets at 5.419 and 5.412 ppm partially overlapped) together with the corresponding ^{13}C correlations (a double spot in ^{1}H−^{13}C HSQC spectrum at 92.7 and 100.3 ppm) proves that it belongs to at least two different components. A detailed analysis of the literature data[34−36] revealed that at least seven di- and oligosaccharides have the anomeric CH group giving the signal with very similar ^{1}H (i.e., 5.42 ppm) and ^{13}C chemical shifts and usually present in honey: erlose, kestose, raffinose, sucrose, maltose, maltotriose, and maltotetraose. As verified by the direct addition of raffinose in our solution, the anomeric proton from the α-D-glucopyranose ring of raffinose gave a doublet signal at 5.435 ppm, and so raffinose can be excluded. The analysis of other ^{1}H and ^{13}C resonances including those outside the anomeric region indicated that kestose did not contribute to the signals at 5.419 and 5.412 ppm. Among the remaining sugars, sucrose and erlose were responsible for the doublet at

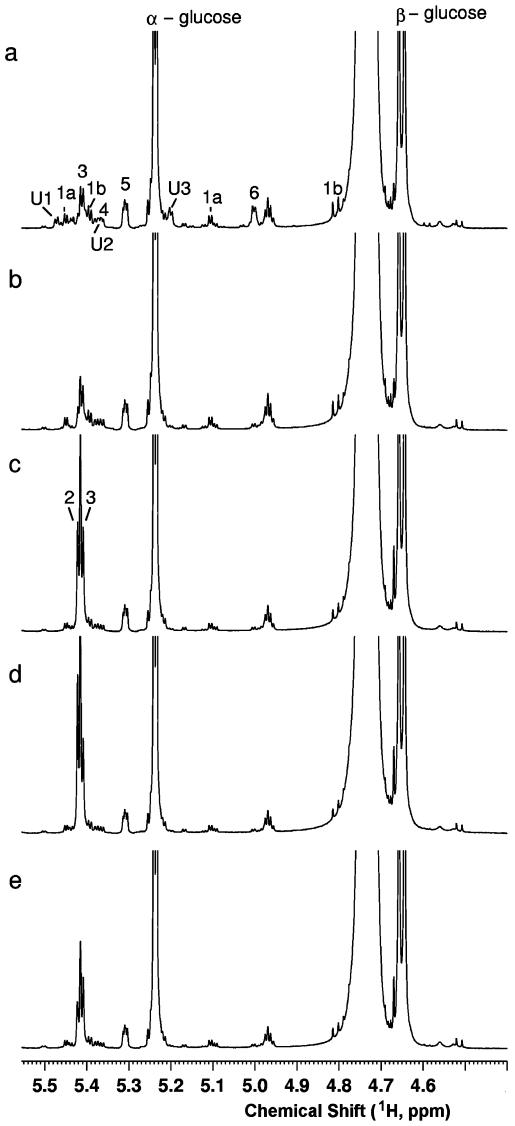

Figure 6. 5.5−4.4 ppm ^{1}H NMR spectral region of dill (a), medlar (b), sulla (c), orange (d), and lemon (e) honey solutions in D_2O. Assignments: 1a and 1b, kojibiose; 2, sucrose; 3, maltooligosaccharides; 4, nigerose; 5, turanose; 6, raffinose; U1, U2, U3, unassigned signals.

5.419 ppm, whereas erlose and maltose (according to ^{1}H−^{13}C HSQC experiment) contributed to the doublet at 5.412 ppm.

The quantification of raffinose, kojibiose, turanose, and nigerose in the five honeys has been performed by the integration of selected signals and normalization of integrals with respect to an internal standard (TSP) (Table 3). In the case of kojibiose and turanose, one of the anomeric forms (*O*-α-D-glucopyranosyl-(1→2)-β-D-glucopyranose and 3-*O*-α-D-glucopyranosyl-α-D-fructofuranose, respectively) was not quantified due to the absence of specific resonances not overlapped with the signals of the other sugars. As a consequence, the absolute content of kojibiose and turanose in honeys was underestimated. Nevertheless, taking into account that all of the anomeric forms were in equilibrium under the experimental conditions, the contents of kojibiose and turanose reported in Table 3 can be considered as relative values that can be compared between different samples.

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Table 3. Sugar Concentrations in Sulla, Dill, Lemon, Orange, and Medlar Honeys According to NMR Analysis

	concentration (mg/g)				
sugar	sulla	dill	lemon	orange	medlar
raffinose	2.8 ± 0.1	25.7 ± 0.8	2.6 ± 0.1	2.2 ± 0.1	4.1 ± 0.1
kojibiose[a]	4.2 ± 0.1	6.7 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	6.6 ± 0.1
turanose[b]	20.6 ± 0.4	22.6 ± 0.5	17.9 ± 0.2	18.0 ± 0.3	22.9 ± 0.4
nigerose	7.4 ± 0.1	nd	6.7 ± 0.2	6.8 ± 0.1	10.2 ± 0.3

[a]Only *O*-*α*-D-glucopyranosyl-(1→2)-*α*-D-glucolpyranose anomer. [b]Only 3-*O*-*α*-D-glucopyranosyl-*β*-D-fructofuranose and 3-*O*-*α*-D-glucopyranosyl-*β*-D-fructopyranose.

The values reported in Table 3 evidence the marked differences in the content of minor components of the saccharide fraction in the honeys of different botanical origins. Raffinose content showed a noticeable variation, whereas the contents of turanose were quite similar in all five honeys as well as to some extent the contents of kojibiose. The content of raffinose was particularly high in dill honey with respect to other honeys. The content of nigerose in dill honey was not determined due to overlapping of its signals with those of an unassigned compound (U2). It is noteworthy that dill honey contains still unidentified saccharides (denoted U1, U2, and U3 in Figure 6a) absent in the other four honeys, as suggested by ^{1}H NMR signals.

Especially in dill honey, the occurrence of carbohydrates such as kojibiose, turanose, and nigerose (Figure 7), which were shown to possess prebiotic effects in experimental animal studies, suggests that these honeys may exert prebiotic effects. These compounds have been found to show a large number of physiologic effects, such as increasing bifidobacteria numbers in the colon. This effect has been connected to other positive effects for human health such as the production of compounds able to inhibit potential pathogen growth, reduce blood ammonia levels, and produce vitamins and digestive enzymes. Moreover, the carbohydrates reported above showed also the capacity to increase calcium absorption and fecal weight and to decrease gastrointestinal transit time and blood lipid levels.[37,38]

Finally, in the region between 3 and 0.5 ppm (Figure 8a,b) characteristic signals of organic acids and amino acids are present. The signals of proline and lactic, acetic, and succinic acids were identified in this region in all honeys, but the intensity of proline and lactic and succinic acids resonances was much higher in dill compared to sulla honey. Another amino acid, phenylalanine, was identified only in dill (Figure 6c) and lemon honeys, whereas its content was too low to be detected in sulla (Figure 6d), medlar, and orange honeys. According to the literature, proline is the most abundant amino acid in honey followed by phenylalanine.[39] Moreover, alanine traces were observed only in the dill honey spectrum (Figure 8).

In conclusion, this investigation represents the first study of the chemical composition of sulla and dill honeys obtained in purity by Sicilian black honeybees performed with a combined method that includes HPLC-PAD-ESI-MS^n and NMR spectroscopy analyses. LC-MS/MS analysis allowed the identification of 22 compounds. In sulla honey, 14 compounds were identified, among which were 3 organic acids, 1 derivative of a phenolic acid, 4 flavonols, 1 flavanon, 4 flavanonols, and dehydrovomifoliol. In dill honey, 14 compounds were detected, among which were 4 organic acids, 2 phenolic acids and derivatives, 1 flavanonol, 1 flavanon, 1 B2 vitamin and its metabolite, 1 amino acid, 1 norisoprenoid compound, dehydrovomifoliol, and 3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione. Some compounds were previously detected in other

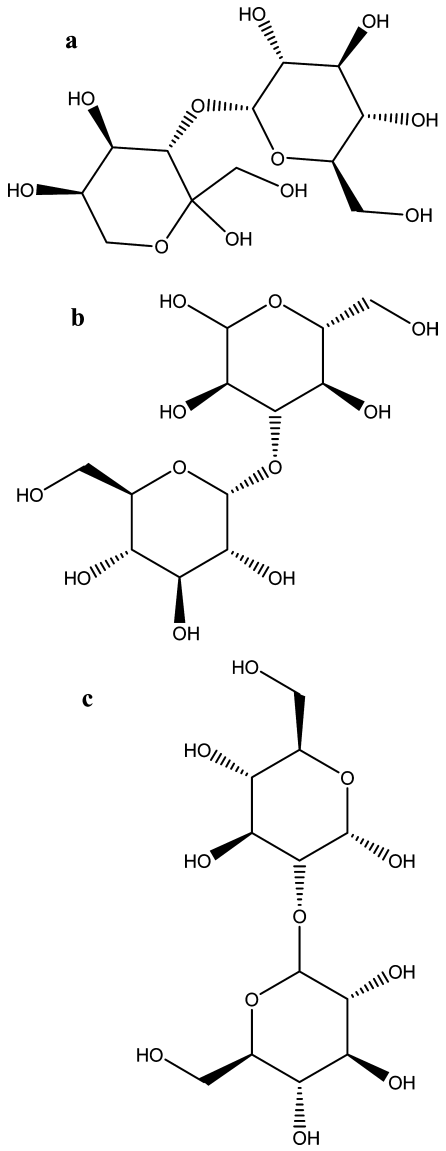

Figure 7. Chemical structures of (a) turanose (3-*O*-*α*-D-glucopyranosyl-*β*-D-fructopyranose), (b) nigerose (3-*O*-*α*-D-glucopyranosyl-D-glucose), and (c) kojibiose (2-*O*-*α*-D-glucopyranosyl-D-glucopyranose).

A. mellifera ssp. *sicula* honey samples of different botanical origins,[10] but for many other substances, this research represents the first report on their identification in these particular kinds of honey. NMR spectroscopy allowed the identification and quantification of several carbohydrates such as raffinose, kojibiose, turanose, and nigerose, the beneficial effects of which on nonpathogenic gut microorganism growth

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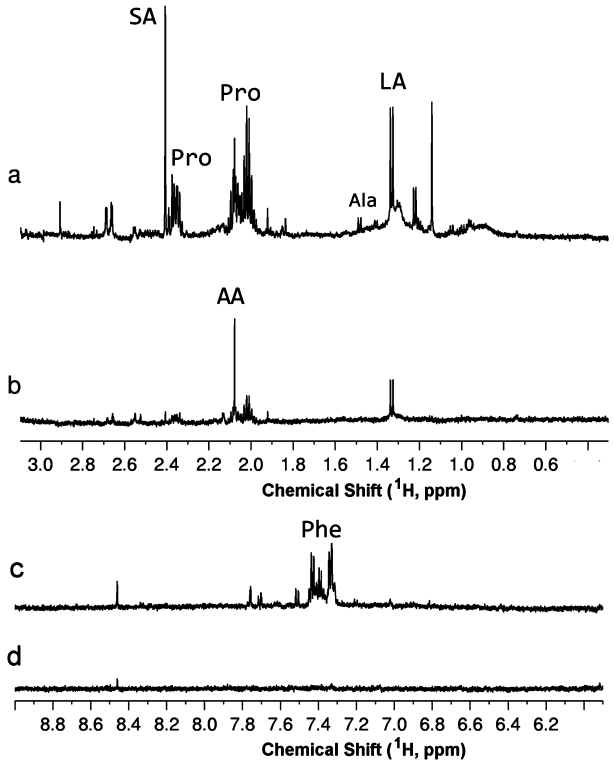

Figure 8. Two selected regions of ^{1}H NMR spectra of dill (a, c) and sulla (b, d) honey solutions in D_2O. Assignments: AA, acetic acid; Ala, alanine; LA, lactic acid; Phe, phenylalanine; Pro, proline; SA, succinic acid.

are well documented. Especially, the finding of the high content of turanose in all tested honeys and raffinose in dill honey justifies the prebiotic effect ascribed to honey.

The combination of these two methodologies allowed us to describe the phytochemical composition of each type of honey and identify many compounds that were found in these types of honey for the first time. In addition, we could compare the metabolic profiles obtained by our analysis with those already reported in the literature and provide the first phytochemical composition for that kind of honey for which no chemical data are available in the literature. On the basis of the obtained results, this combined approach can be applied conveniently for future honey analyses.

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Notes

The authors declare no competing financial interest.

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