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ARTICLE

Use of Quinoline Alkaloids as Markers of the Floral Origin of Chestnut Honey

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To identify potential floral markers of chestnut honey, the phytochemicals present in chestnut floral nectar collected by bees were analyzed. Two nitrogen-containing compounds were detected, isolated, and identified as 4-hydroxyquinaldic acid (kynurenic acid) and 4-quinolone-2-carboxylic acid by ^{1}H NMR and ^{13}C NMR. In addition, chestnut nectar contained the monoterpene 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid, its gentiobioside ester, and the flavonol quercetin 3-pentosylhexoside. These nectar markers were found in different chestnut unifloral honey samples, although the flavonol was not detected in all samples analyzed. The terpenoid derivatives had previously been found in linden and tilia honeys. These results show that quinoline alkaloids are potentially good markers of chestnut honey, as they were not detected in any other unifloral honey analyzed so far. They are present at concentrations ranging from 34 to 65 mg/100 g of honey in the samples analyzed. In addition, the terpenoid and flavonoid derivatives present in nectar, although not exclusively characteristic of this floral origin, are good complementary markers for the determination of the floral origin of chestnut honey.

KEYWORDS: Floral markers; botanical origin; nectar; chestnut; kynurenic acid; HPLC-MS-MS

INTRODUCTION

HPLC analysis of floral nectar phytochemicals is potentially an effective and objective method to help in honey floral origin determinations. This technique can complement the well-established pollen and sensory analyses. Different studies have explored the application of the analysis of honey phytochemical constituents for this purpose. Thus, flavonoids and other phenolics (*1*−*4*), volatile compounds (*5*), aromatic and degraded carotenoid-like substances (*6*, *7*), amino acids (*8*), and aromatic aldehydes and heterocycles (*9*) have been evaluated to establish the floral origin of honey. The following phytochemicals have been proposed for the determination of honey floral origins: kaempferol for rosemary honey (*10*); myricetin, tricetin, and luteolin for *Eucalyptus* honey (*3*); abscisic acid for heather honey (*11*); homogentisic acid for strawberry tree (*Arbutus unedo*) honey (*12*); methyl syringate for manuka honey (*13*); hydroxyquinaldic acid for chestnut honey (*14*, *19*); carboxylic monoterpene glycosidic esters for linden honey (*15*); hesperetin for *Citrus* honey (*16*); and kaempferol rhamnosides for acacia (*Robinia*) honey (*4*).

Chestnut blossom honey production is important in many European countries. Verification of its floral origin is based essentially on sensory analysis, because there are no specific chemicophysical parameters for this kind of honey and palynological analysis is often inconclusive because chestnut pollen is hyper-represented. Therefore, the identification of floral markers for chestnut honey is of great practical interest.

Chestnut honeys are dark in color and have a strong and characteristic flavor. Previous studies have suggested that volatile compounds, such as 3-aminoacetophenone and 1-phenylethanol, could be used as markers of the floral origin of chestnut honey (*17*). The occurrence of potential markers of chestnut honey was reported in a study of European unifloral honeys in which five unidentified markers were detected (**CH1**−**CH5**) and showed characteristic UV spectra (*18*). These markers were not detected in any of the other unifloral honeys analyzed. One of these compounds was identified as kynurenic acid (*14*, *19*, *20*). However, in these previous studies, the occurrence of these markers, or their precursors, in chestnut nectar had not been explored due to the small size of chestnut flowers, and therefore the status of the floral origin markers was not confirmed.

The aim of the present study was the analysis of chestnut flower nectar phytochemicals by HPLC-DAD-MS-MS, using nectar collected by bees, and the evaluation of the possibility of using these compounds as markers of the botanical origin of chestnut honey.

MATERIALS AND METHODS

Reagents. Chlorogenic acid (5-*O*-caffeoylquinic acid), quercetin, and hesperetin were purchased from Sigma (St. Louis, MO), and chrysin (5,7-dihydroxyflavone) was from Carl Roth OGH (Karlsruhe, Germany). Formic and acetic acids were of analytical grade and methanol was of HPLC grade and supplied by Merck (Darmstadt, Germany).

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Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this study.

Collection of Chestnut (*Castanea sativa*) Nectar. Due to the small size of chestnut flowers and the relatively small amount of nectar that they produce, the collection of nectar directly from the flowers was not possible. Chestnut nectar was collected by placing the hives in a chestnut wood during the blooming period. After it was verified from the combs that bees were collecting chestnut nectar (the fresh "uncapped" honey was collected and identified by sensorial methods at the CRA-API), bees returning to the hive were captured and dissected directly in the field and only the honey stomach full of nectar was collected; the content was extracted and immediately frozen at −20 °C. About 1 mL of nectar was collected from nearly 50 honey bee stomachs.

Honey Samples. Seven chestnut honey samples from different regions in Italy were analyzed. These samples were provided and certified by the Agricultural Research Council—Bee and Silkworm Research Unit (CRA-API, Bologna, Italy). Origin of samples was mainly central northern Italy: three samples from Emilia Romagna (C-00, C-1390, C-1473), one from Siena (C-1453), and two from Arezzo (C-1454, C-1456); one sample was from the Piedmont region (C-1757). All honey samples included in this study were stored in the dark at 4 °C until analysis. Pollen analysis revealed that the percentage of *C. sativa* pollen was >90% in all cases.

Extraction of Markers from Nectar. Chestnut nectar collected from the bee stomach was diluted with ultrapure water and centrifuged at 7000 rpm for 10 min, in a Centromix centrifuge (Selecta, Barcelona). The supernatant was filtered through a reversed phase Sep-Pak solid phase extraction (SPE) cartridge (Waters Millipore, USA). This cartridge was previously activated with 10 mL of methanol and then with 10 mL of water. The supernatant was filtered through the cartridge and washed with 10 mL of water. The phytochemical compounds absorbed in the cartridge were then eluted with 1 mL of methanol. The methanol fraction was filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp., USA) and stored at −20 °C until further analysis by HPLC-DAD-MS-MS.

Sample Preparation for the Analysis of Chestnut Floral Markers in Honey. Honey samples (10 g each) were diluted 5-fold with ultrapure water until completely fluid. This solution was filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp., USA) before HPLC analyses.

Extraction of Floral and Propolis-Derived Phytochemicals from Honey. In addition of floral nectar phytochemicals, honey contains propolis-derived phenolics, and their analysis needs extraction and concentration. Honey samples (10 g each) were mixed with 5 parts of water acidified to pH 2 with HCl until completely fluid and filtered through cotton to remove solid particles. Then the filtrate was passed through a C_{18} cartridge (Sep-Pak) previously activated with methanol (10 mL) and water (10 mL). The phenolic compounds and markers remained in the cartridge, whereas sugars and other polar compounds were eluted with water. The cartridge was washed with distilled water (10 mL), and the phenolic fraction was then eluted with methanol (2 mL). The methanol extracts were stored at −20 °C until further analysis by HPLC-DAD-MS-MS. This extraction process was necessary for the analysis of propolis-derived phytochemicals that were present in small amounts in honey but recovered only 70−80% of the alkaloids present in nectar (results not shown).

HPLC-DAD-MS-MS Analysis of Nectar and Honey Metabolites. All HPLC-MS analyses were achieved using an Agilent 1100 series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (G1312 A), an autosampler (G1313 A), a degasser (G1322 A), and a photodiode array detector (G1315 B) controlled by software (v. A08.03). The mass detector was an ion trap spectrometer (G2445A) equipped with an electrospray ionization (ESI) system and controlled by software (v. 4.1). The nebulizer gas was nitrogen; the pressure and the flow rate of the dryer gas were set at 65 psi and 11 L/min, respectively. The full-scan mass covered the range of m/z 100−1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. The capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass spectrometry data were acquired in the negative mode for the identification of phenolic compounds and in the positive mode for the identification of nitrogen-containing markers of chestnut honey. The column used was a 250 mm × 4 mm i.d., 5 μm, C_{18} LiChroCART RP-18 column protected with a 4 mm × 4 mm i.d. LiChroCART guard column (Merck). Elution was with water/formic acid (19:1 v/v) (solvent A) and methanol (solvent B). The elution was accomplished with a solvent flow rate of 1 mL/min, starting with 10% B to reach 30% B at 20 min, 45% B at 30 min, 60% B at 40 min, 70% B at 45 min, 90% B at 60 min, and then became isocratic for 5 min. Chromatograms were recorded at 290, 320, and 340 nm.

The potential markers of chestnut honey and the propolis-derived flavonoids were identified according to their UV spectra, molecular weights, retention times, and MS-MS fragments, and, whenever possible, chromatographic comparisons with authentic markers (commercial or previouly isolated and identified from honey or propolis) were performed. The markers (**CH1**−**CH3**; **CH5**) were quantified as compound **CH1** at 290 nm. Calibration curves were prepared for the UV detector. The calibration curves were linear in the range of 10−530 μM for kynurenic acid and were characterized by correlation coefficients of >0.99. The limit of detection was 2 μM, and the limit of quantification was 5 μM. The flavanones were quantified as hesperetin at 290 nm, the flavonols as quercetin and the flavones as chrysin at 340 nm. Compounds **L1** and **L2** were not quantified as no commercial marker was available and the amount isolated was not enough to run calibration curves.

Isolation of Compounds CH1, CH2, and L1. To isolate and purify the chestnut honey markers 12 kg of chestnut honey was used. The extraction of the markers was achieved in 1 kg batches. One kilogram of honey was dissolved in water acidified to pH 2 with HCl and mixed with 500 g of Amberlite XAD-2 (Supelco, Bellefonte, PA; mean pore diameter = 90 Å, mesh size = 20−60 μm, mean surface area = 300 m^2/g) to ca. 3 L and stirred with a magnetic stirrer for 30 min to allow the adsorption of phenolic compounds. Then it was packed in a glass column (84×4 cm) and washed with acid water (ca. 2 L) to remove sugars and polar compounds and retain alkaloids, flavonoids, and other honey phenolics. The column was washed with distilled water (ca. 1 L). Phenolic compounds were then eluted with MeOH (ca. 0.75 L) until a colorless eluent was obtained. The remaining water was submitted to additional adsorption on Amberlite XAD-2 to complete the recovery of phytochemicals from honey.

The whole sample (12 kg) was processed in this way. The methanol extracts from each extraction were combined and concentrated under reduced pressure to 20 mL of methanol. This extract was then chromatographed on a Sephadex LH-20 column (40×3 cm) (Pharmacia, Uppsala, Sweden) using methanol. The separation of different fractions was monitored using a 360 nm light, and the fractions obtained were analyzed by HPLC under the specific conditions for honey metabolites described above. The fractions containing the markers were joined, and **CH1**, **CH2**, and **L1** were isolated and purified by semipreparative HPLC on a Spherisorb octadecylsilane (ODS-2) column (25×1 cm, 5 μm particle size) with a solvent flow rate of 5 mL/min using as mobile phase methanol/water solutions in an isocratic manner starting with 10% methanol and increasing the methanol proportion to elute compounds (30%). The purity of isolated compounds was tested by analytical HPLC and stored at room temperature after freeze-drying.

Phytochemicals Identification. The isolated compounds were identified by a combination of UV spectrophotometry in methanol and, after the addition of the classical shift reagents (*21*), EIMS (Hewlett-Packard electron impact mass spectrometer, 70 eV, direct inlet), ^{1}H NMR and ^{13}C NMR in DMSO- d_6 (Brüker, 350 mHz).

RESULTS AND DISCUSSION

Analysis of Compounds from *C. sativa* Nectar. The chestnut nectar obtained from the honey stomach of bees was prepared using a SPE cartridge and analyzed by HPLC, and its chromatographic profile is shown in **Figure 1A**. A total of six different compounds were identified as the main constituents of chestnut nectar. The chromatographic peaks CH1, CH2, CH5, L1, and L2 showed UV spectra with characteristic maxima and shoulders (**Figure 1**). These potential markers were previously detected in chestnut honey by our group in 2001, but they were not identified (*18*). In addition, compound **F** showed the characteristic UV spectrum of a flavonol.

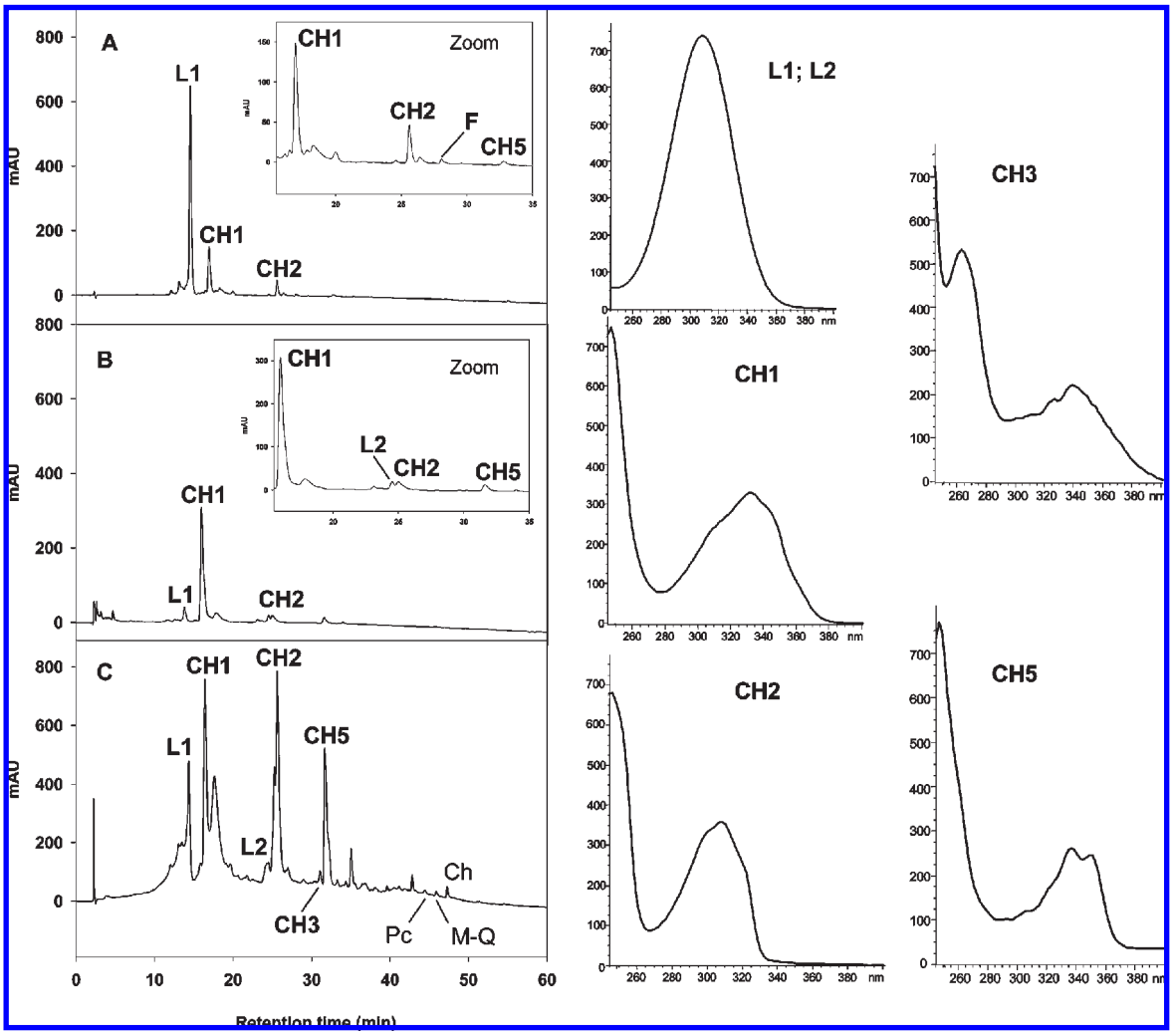

Figure 1. HPLC chromatograms of chestnut nectar and honey phytochemicals (290 nm) and their UV spectra: (**A**) chestnut nectar extracted with C_{18} SPE cartridge; (**B**) chestnut honey diluted with ultrapure water; (**C**) chestnut honey acidified and extracted with C_{18} SPE cartridge. Peaks: CH1, 4-hydroxyquinaldic acid (kynurenic acid); CH2, 4-quinolone 2-carboxylic acid; CH3 and CH5, unidentified markers of chestnut honey; F, quercetin 3-*O*-pentosylhesoxide; L1, 6-*O*-(*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl ester 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylate; L2; 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid; Pc, pinocembrin; M-Q, quercetin methyl ether; Ch, chrysin.

The structures of **CH1**, **CH2**, and **L1** were studied by means of a combination of their UV and mass spectra registered by HPLC-DAD-MS-MS/ESI in positive and negative modes. The MS study of compound **CH1** showed an ion at *m*/*z* 190 $[M + H]^+$, and its MS/MS analysis showed that the most abundant fragment ion was that at *m*/*z* 162 that indicated the loss of C═O (**Figure 2A**).The MS analysis of **CH2** showed a molecular weight identical to that of **CH1** (*m*/*z* 190 $[M + H]^+$). MS^2 of this compound produced as the main fragment an ion at *m*/*z* 172 that indicated the loss of one molecule of H_2O (**Figure 2B**). Its MS study, in the negative ion mode, showed that the pseudomolecular ion was at *m*/*z* 188 for **CH1** and **CH2**. Their MS^2 showed for both compounds that the most abundant ion was that corresponding to the loss of HCOOH (*m*/*z* 144), confirming the presence of a carboxyl residue, very probably bound to a substituted indole or hydroxylated (iso)quinaldic acid in both structures (**Figure 3**) (*14*, *19*). The fragmentation of **CH1** (**Figure 2A**) is in agreement with the results reported by other authors which indicated that this compound could be 4-hydroxyquinaldic acid or kynurenic acid (*14*, *22*). However, the molecular structure of **CH2** could not be identified by its MS studies. To confirm the definitive structures it was necessary to isolate and analyze these markers by ^1H NMR and ^{13}C NMR. Thus, compounds **CH1**, **CH2**, and **L1** were isolated by preparative column chromatography.

Compound **CH1** showed a UV spectrum of 340sh, 335sh, 332, 308, 282sh (**Figure 1**) consistent with that recorded with HPLC-DAD. Its EIMS showed a molecular ion at *m*/*z* 189, confirming that this was a nitrogen-containing compound. Its MS spectrum is shown in **Table 1**, and a MS spectra library search showed that this compound could be tentatively identified as 4-hydroxy-2-quinolinecarboxylic acid (4-hydroxyquinaldic acid). ^1H NMR analysis shows the presence of five aromatic protons in the molecule. The chemical shifts and coupling constants were in agreement with those reported for kynurenic acid (*19*) (**Table 1**), supporting the structure suggested above. The ^{13}C NMR spectrum showed the response of a carboxylic carbon (163.8 ppm), and the rest of the assignments were consistent with previously published data for this compound (**Table 1**) (*19*).

The isolated compound **CH2** showed a UV spectrum of 330sh, 309, 283 sh. This spectrum was similar to that of **CH1**, but the maxima had shorter wavelengths (**Figure 1**), indicating a molecule with less resonance. Its MS spectrum showed a molecular ion at *m*/*z* 189, showing that this was an isomer of compound **CH1**. Its ^1H NMR spectrum showed five protons, the same as that of compound **CH1**, but the chemical shifts were quite different (**Table 1**). This was particularly relevant for the hydrogens at H-3 and H-6. The ^{13}C NMR spectrum was very close to that of **CH1**,

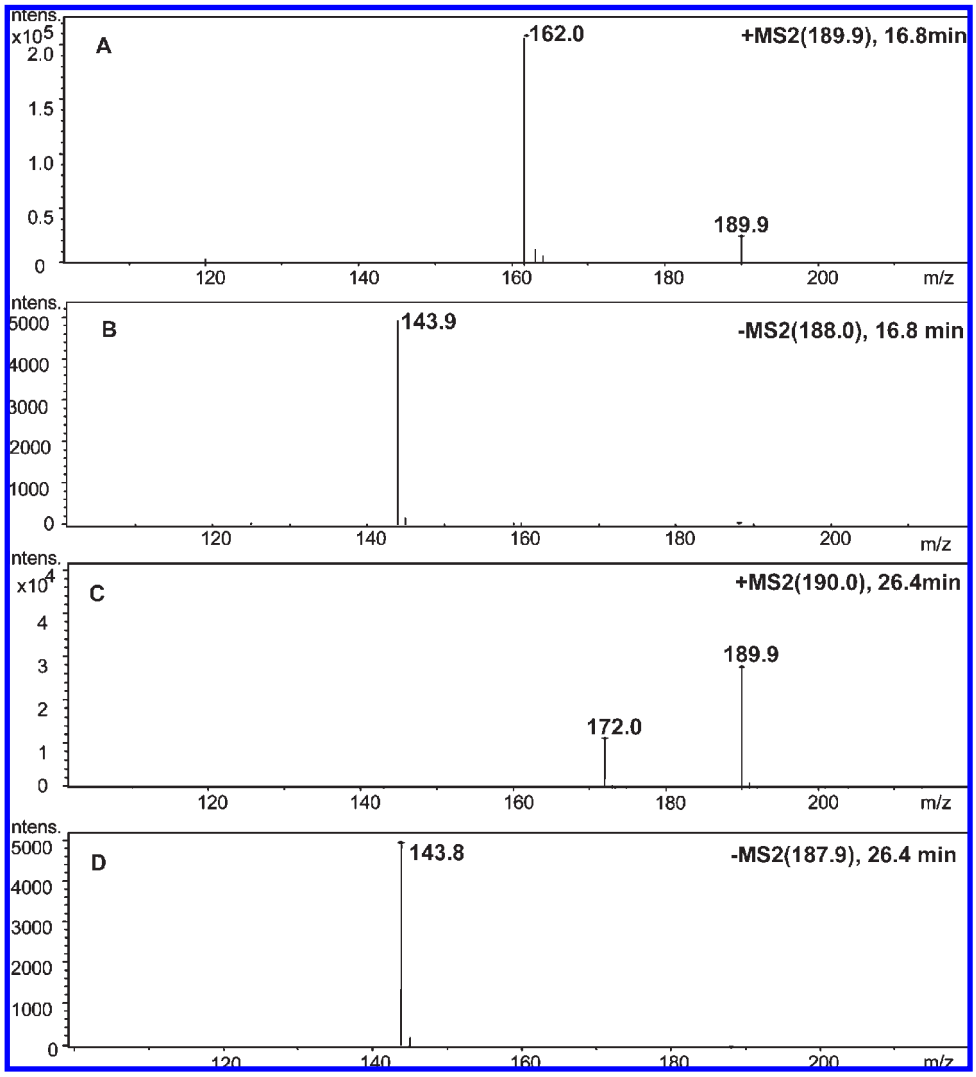

Figure 2. HPLC-MS-MS analyses (MS^2) of compounds **CH1** and **CH2**: (**A**) **CH1** in positive mode; (**B**) **CH1** in negative mode; (**C**) **CH2** in positive mode; (**D**) **CH2** in negative mode.

suggesting a very similar molecule. Differences were observed for the carbons at C-2 and C-10. As this is a nitrogen-containing compound the possibility of protonation of the nitrogen to give a quinaldinium cation exists (**Figure 4**). This can produce the quinolone tautomer that could explain the differences observed in the UV spectrum and the differences in the NMR data. The presence of a proton in a nitrogen atom affects considerably the electronic environment of the protons in the quinaldic acid molecule. The interconversion of compounds **CH2** and **CH1** in acid solution supports the hypothesis that **CH2** is the tautomer form of compound **CH1** (**Figure 4**). Its ^{13}C NMR spectrum (**Table 1**) is consistent with a compound with this molecular structure.

Compound **CH5** was detected as a minor peak in the UV chromatogram at 290 nm (**Figure 1A**). HPLC-MS study in positive mode showed that its $[M - H]^-$ ion was at *m*/*z* 241. MS^2 analysis of this ion showed a fragment at *m*/*z* 213 corresponding to the loss of C=O. **CH5** could not be identified due to the small amount present that prevented its isolation for ^1H NMR and ^{13}C NMR analyses. The MS study, UV spectra, and HPLC retention time suggest that this compound could be an intermediate of the tryptophan metabolism pathway, similar to **CH1** and **CH2**. **CH5** did not correspond with any of those nitrogen-containing compounds previously reported in chestnut honey, including 2-oxaylaminobenzoic acid, *N*-formylanthranilic acid, and anthranilic acid (*20*).

The UV spectra of compounds **L1** and **L2** with a maximum at 305 nm suggest that these could coincide with the markers of tilia and linden unifloral honeys, which have been previously described in other studies (*15*, *18*). The first one was the most abundant compound detected in nectar, and the second one was detected in smaller proportions (**Figure 1A**). The MS analysis of **L1** showed a deprotonated ion at *m*/*z* 505. The MS^2 scan of the $[M - H]^-$ ion yielded two fragment ions at *m*/*z* 323 and 181. The first fragment indicated the loss of the terpenoid aglycone (*m*/*z* 181), and the second one was obtained as result of the loss of two sugar moieties (*m*/*z* 181). This fragmentation tentatively confirmed that **L1** was the 1-*O*-*β*-gentiobioside ester of the monoterpene. NMR analysis supported that this was 4-(1-hydroxy-1-methylethyl) cyclohexa-1,3-diene-1-carboxylic acid (*15*) (**Figure 3**). MS study of **L2** showed that this had an ion at *m*/*z* 181, but its ionization was poor under the analytical conditions used. MS^2 analysis showed a main fragment at *m*/*z* 163, due to the loss of water, *m*/*z* 152, due to the loss C=O, and *m*/*z* 135, due to the loss of HCOOH. The molecular ion and fragments supported that compound **L2** was tentatively identified as the monoterpene 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid,

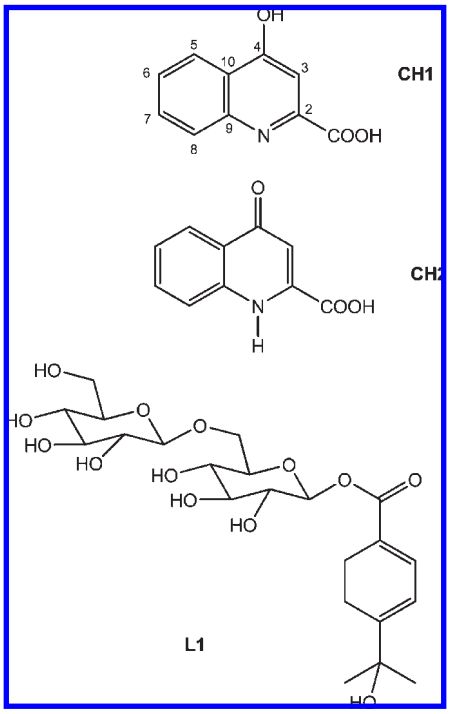

Figure 3. Compounds **CH1** (4-hydroxyquinaldic acid), **CH2** (4-hydroxyquinaldinium cation), and **L1**, 6-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl ester 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylate.

Table 1. ^{1}H NMR and ^{13}C NMR Analyses of Chestnut Markers

	CH1	CH2	kynurenic acid[a]
protons			
H-3	6.68 s	8.88 s	6.63 s
H-5	8.09 brd *J* = Hz	8.28 brd *J* = Hz	8.08 d
H-6	7.32 brt *J* = Hz	7.85 brt *J* = Hz	7.36 t
H-7	7.66 brt *J* = Hz	7.59 brt *J* = Hz	7.69 t
H-8	7.99 brd *J* = Hz	7.86 brd *J* = Hz	7.96 d
carbons			
C-2	140.0	145.3	140.0
C-3	109.0	107.5	109.5
C-4	178.0	178.1	177.7
C-5	123.5	124.4	123.7
C-6	119.6	119.9	119.6
C-7	132.0	133.8	132.2
C-8	124.6	125.0	124.6
C-9	125.6	126.0	125.7
C-10	142.8	139.8	140.3
COOH	163.8	166.5	163.6

[a] Reference (*19*).

and this was in agreement with other authors (*15*, *23*). **L2** was released after saponification of **L1**.

Compound **F** was detected in trace amounts (**Figure 1A**). MS analysis showed a deprotonated molecular ion at *m*/*z* 595 consistent with a quercetin pentosylhexoside. Both sugars are linked to one phenolic hydroxyl as the disaccharide moiety is released in a single fragment. The UV spectra indicated that the hydroxyl in the 3-position is blocked (*21*).

Quantification of Markers in Chestnut Honey. HPLC analyses of chestnut honey extracts showed that the different compounds (**CH1**, **CH2**, **CH5**, **L1**, and **L2**) present in chestnut nectar were the main peaks in the chromatogram of chestnut honey samples (**Figure 1B**) and were characterized by HPLC-MS-MS. All of

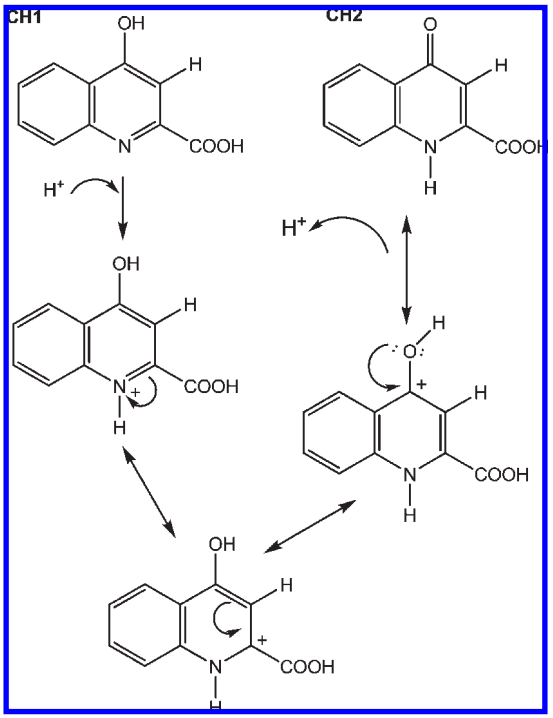

Figure 4. **CH1** and **CH2** interconversion.

Table 2. Alkaloids Content in Chestnut Honey[a]

sample	CH1	CH2	CH3	CH5	total
C-00	40.6 ± 0.8	3.0 ± 0.3	0.1 ± 0.00	2.0 ± 0.0	45.7
C-1390	30.7 ± 0.3	2.4 ± 0.0	0.1 ± 0.0	0.7 ± 0.1	33.9
C-1453	43.8 ± 0.8	2.7 ± 0.1	0.1 ± 0.0	0.6 ± 0.1	47.2
C-1454	35.1 ± 0.2	2.7 ± 0.1	0.2 ± 0.0	1.1 ± 0.02	39.1
C-1456	43.1 ± 0.2	3.3 ± 0.9	0.1 ± 0.0	1.0 ± 0.0	47.5
C-1473	58.0 ± 1.9	4.8 ± 0.4	0.1 ± 0.0	1.9 ± 0.1	64.8
C-1757	49.6 ± 0.5	4.3 ± 0.2	0.2 ± 0.1	0.8 ± 0.03	54.8

[a] Values are mg/100 g of honey.

these compounds were previously reported (although not identified) in a study of European honey in which potential floral origin markers were detected (**CH1**−**CH5**) (*18*). However, in the present study compound **CH4** was not detected, and **CH3** was present only as a minor constituent in honey. Neither compound **CH3** nor **CH4** was observed in nectar. Compounds **CH1**, **CH2**, **CH3**, and **CH5** were detected in all honeys (**Table 2**). For **CH1** the concentration in the samples analyzed ranged from 30.7 to 58.0 mg/100 g of honey; for **CH2** the range was 2.4−4.8 mg/100 g of honey, and for **CH5** the range was 0.6−2.0 mg/100 g. **CH3** was present as traces. This alkaloid content was in the range previously reported for other chestnut honey samples (10−30 mg/100 g of honey) (*20*). In the HPLC chromatogram of nectar phytochemicals (**Figure 1A**) compound **CH2** was present as a minor constituent. This was also a minor constituent in the chromatogram of honey direcly diluted, filtered, and analyzed (**Figure 1B**), whereas this was a relevant constituent in the honey extract obtained after concentration using a solid phase extraction cartridge after acidification with acid water (**Figure 1C**). This could be explained by the relatively easy interconversion of both tautomers (**CH1** and **CH2**), particularly under acidic conditions (**Figure 4**).

Compounds **L1** and **L2** were detected in chestnut nectar and in all chestnut honey samples analyzed except C-1454. **L1** was the main constituent in chestnut nectar, but it was observed in only

Table 3. Propolis-Derived Flavonoid Contents in Chestnut Honeys [a]

	sample						
flavonoid	C-00	C-1390	C-1453	C-1454	C-1456	C-1473	C-1757
pinobanksin	nd	0.3 ± 0.0	0.6 ± 0.0	nd	nd	nd	nd
pinocembrin	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	nd	0.3 ± 0.1	1.0 ± 0.0	nd
methylquercetin	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.03	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
chrysin	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
galangin	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
tectochrysin	nd	0.1 ± 0.01	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

[a] Values are mg/100 g of honey. nd, not detected.

minor proportions in honey. In a previous study, these compounds were isolated from linden nectar and honey and were suggested as markers for this floral origin (*15*). Moreover, these compounds **L1** and **L2** have been detected in studies on chestnut honey markers (*18*, *20*, *23*).

The flavonol pentosylhesoxide **F**, which was a derivative of quercetin analyzed in chestnut nectar, was detected in only some honey samples (C-1473; C-1390; C-00) but in trace amounts.

The content of markers in the different chestnut honey samples was rather variable as during honey elaboration/ripening these compounds could be degraded or transformed. Climatic conditions and geographical origin could modify the concentration of these markers in honey.

Propolis-Derived Flavonoid Quantification in Chestnut Honeys. The propolis-derived flavonoid aglycones were also extracted and analyzed by HPLC on reverse-phase columns (**Figure 1C**). All samples showed a similar and characteristic HPLC profile. The content of propolis-derived compounds was in general very small in all samples (0.1−1.0 mg/100 g), as shown in **Table 3**. These results are in accordance with those previously reported for chestnut honey (*18*). The content of these compounds in the different chestnut honey samples is quite variable because it depends on the degree of propolis contamination in the hive and beeswax (*18*, *24*).

In conclusion, these results show that nitrogen-containing phytochemicals, such as 4-hydroxyquinaldic acid, and the terpenoid and flavonoid derivatives detected in chestnut floral nectar collected by bees are detected in chestnut honey as well. These compounds have been identified and quantified in different amounts in all of the honey samples analyzed, and they can be suggested as suitable markers to be used for the determination of the floral origin of chestnut honey.

These results confirm that the identification of floral markers from nectar can be a useful tool to avoid fraud in honey authenticity as they help determine the botanical origin of honey, representing an added value to honey quality.

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