Homogentisic Acid: A Phenolic Acid as a Marker of Strawberry-Tree (*Arbutus unedo*) Honey

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Analysis of organic acids in strawberry-tree (*Arbutus unedo*) honey showed the presence of an unknown acid as the main constituent. This compound was isolated and identified as homogentisic acid (2,5-dihydroxyphenylacetic acid) by MS and NMR techniques. Its average content in honey was 378 ± 92 mg/kg. Analysis of nectar confirmed the floral origin of the compound found in honey. Since this acid was not detected in any of the different monofloral honeys, it could be used as a marker of strawberry-tree (*A. unedo*) honey.

Keywords: *Homogentisic acid; strawberry-tree (Arbutus unedo); honey; marker*

INTRODUCTION

The floral origin of honey is mainly determined by melissopalynological analysis. (Louveaux et al., 1978). This determination shows various limitations (Ricciardelli D'Albore, 1997) depending on the beekeeping techniques (extraction, filtering, etc.), size of the pollens, the flower morphology, which may affect nectar contamination in the flowers, and consequently the pollen grains in the honey sediment compared to its respective nectar. In some flowers (i.e., *Castanea*, *Eucalyptus*), pollens are over-represented, and their percentage in the sediment is greater than the percentages of the corresponding nectar in honey. On the contrary, in other cases, such as *Arbutus*, *Citrus*, or *Rosmarinus*, pollens are under-represented. In the case of *Arbutus*, in particular, the low pollen content in the honey sediment is chiefly due to the fact that *Arbutus* flowers are in an upside-down position, so that the probability of direct nectar contamination is reduced. In this case, the performance and interpretation of melissopalynological analysis require particular care, and for a correct classification of the floral origin the sensorial and physicochemical characteristics must be related. In view of the above, it would be interesting to find specific chemical markers for a more precise diagnosis of the botanical origin (Anklam, 1998). Bonaga and Giumanini (1986) showed that was possible to correlate the chemical information of honey with the floral source. The chemical determination of honey could offer greater reliability in tracing the floral origin. Many researchers, particularly Tomás-Barberán and co-workers in Spain, have tried to identify chemical compounds that can be used as markers of floral origin in nectar or in some biochemical modifications of nectar. Ferreres et al. (1993, 1994) found flavanone hesperetin and methyl anthranilate in citrus honey. Since these compounds have been considered markers because they are present in large amounts, though not specific of a given species, as in the case of abscisic acid in heather honey (*Erica* spp.) (Ferreres et al., 1996) and kaempferol in rosemary honey (Gil et al., 1995). Tomás-Barberán suggested the use of HPLC chromatograms as chemical "fingerprints", where, though not specific, the different compounds were present in combination and in specific relative amounts. In a previous paper (Cherchi et al., 1994), the chromatograms of organic acids in different honeys showed that only strawberry-tree honey showed the presence of large amounts of a specific unknown peak. Furthermore, prevalent amounts of phenolic compounds were proven in strawberry-tree honey (Floris et al., 1994). The aim of this work was the identification of a possible marker for this typical honey, which is well-known in Sardinia, Italy, as "bitter honey", and has already been studied from melissopalynogical and physicochemical points of view (Persano Oddo et al., 1995).

MATERIALS AND METHODS

Honey and Nectar Samples. Strawberry-tree (*Arbutus unedo*) honey samples were collected in different Sardinian modern apiaries during the autumn (20 Nov−10 Dec) of 1996. They were stored at 4 °C until analyzed. Strawberry-tree nectar was collected from the flowers using Demianowicz (1963) pipets with two bulbs during the main nectar flow (Floris et al., 1991) in a shrubland in the south of the island.

Melissopalynological Analyses. Qualitative and quantitative melissopalynological analyses were carried out following the method of the International Commission of Bee Botany (Louveaux et al., 1978).

In regard to the absolute presence of pollen grains in the sediment, the samples were classified as poor when they had a content of less than 20 000 in 10 g of honey (PK/10 g) (group I); "normal" when PK/10 g was between 20 000 and 100 000 (group II); and rich when the PK/10 g was between 100 000 and 500 000 (group III). In regard to the frequency of *Arbutus*

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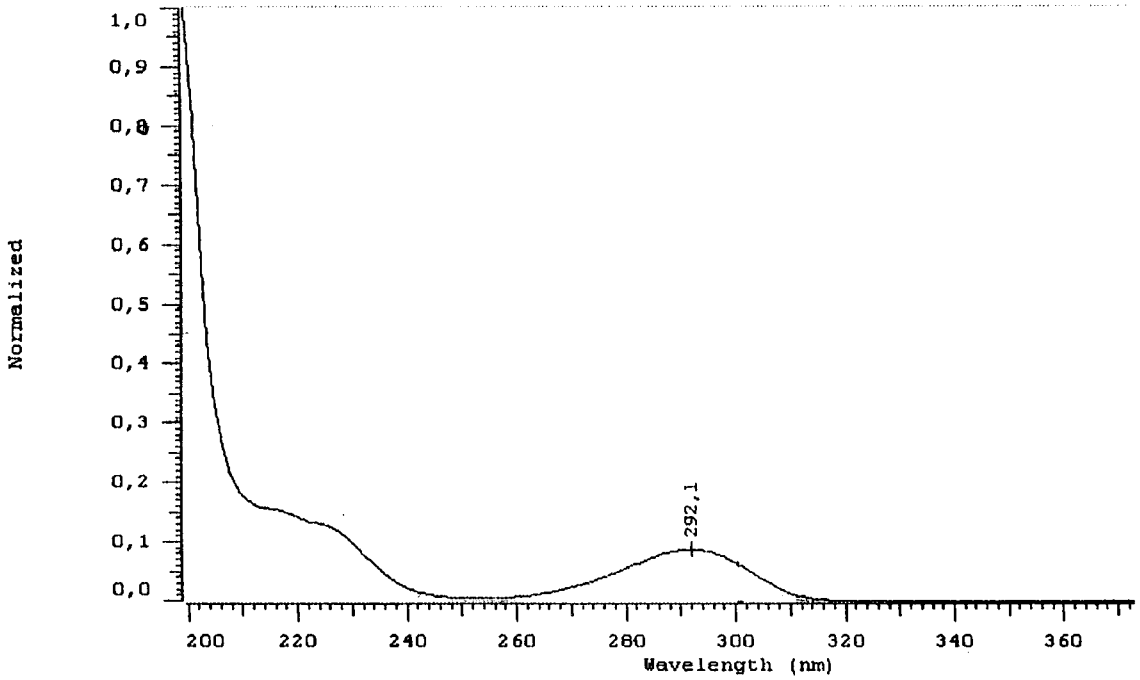

Figure 1. UV spectrum of homogentisic acid.

pollen grains, the following terms are adopted: "very frequent" when the grains were more than 45% of the total; "frequent" when they were between 16 and 45%; "rare" between 3 and 16%; "sporadic" when less than 3%.

Isolation of Homogentisic Acid from Honey. A 10 g aliquot of homogenized honey was solubilized in 10 mL of water in screw-capped 40 mL tubes. Ethyl acetate (20 mL) was added to each tube, and the mixtures were agitated in a rotary shaker for 20 min. The phases were allowed to separate, and the organic extracts were centrifuged. After centrifugation, anhydrous sodium sulfate was added to the combined extracts and evaporated to dryness by a rotary evaporator. The residue was taken up with acetone and the unknown acid isolated by preparative TLC using H_2SO_4 10^{-2} N ($R_f = 0.7$). The purity of compound was tested by analytical HPLC with diode array detection.

Identification. The pure isolated compounds were identified by MS and NMR analysis.

Apparatus and Chromatography. **HPLC**. A LaChrom 7100 HPLC pump (Merck Hitachi, Tokyo, Japan) fitted with an L 7450A diode array detector was employed. The sample (50 μL) was injected in a Spherisorb ODS2 (250 × 4.6 mm, i.d. 5 $\mu\mu$, Waddinxveen, The Netherlands). The operating conditions were the following: eluent mixture, methanol/H_2SO_4 10^{-2} N (10:90, v/v); flow, 0.7 mL/min; λ = 292 nm. The homogentisic acid concentration was calculated by the external standard method.

MS. A Hewlett-Packard electron impact mass spectrometer was used. The sample was introduced by direct insertion solid probe. The mass spectrometer operating conditions were as follows: electron ionization, 70 eV; ion source 180 °C; scan mass, range 50−550; scan interval, 1.5 s.

NMR. A Bruker AMX 500, equipped with a 5 mm reverse H/X probe head, operating at 500.13 MHz for ^{1}H spectra and at 125.77 MHz for ^{13}C spectra, was used. All the spectra were collected at 300 K in aceton-d_6. Chemical shifts (δ) are quoted relative to tetramethylsilane (TMS) and locked to the solvent signal (2.04 for ^{1}H and 29.8 for ^{13}C, respectively).

Chemicals. Acetone, ethyl acetate, and methanol were analytical-grade solvents (Carlo Erba, Milan, Italy). Homogentisic acid (2,5-dihydroxyphenylacetic acid) 97% was purchased from Aldrich (Milan, Italy). TLC plates RP-18 F_{254s} 20 × 20 cm (Merck, Darmstadt, Germany) were used. A stock standard solution of homogentisic acid (~2 g/L) was prepared in methanol. Working standard solutions was obtained by dilution with the mobile phase.

Extraction Procedure for Analytical Determination. A 10 g aliquot of homogenized honey was solubilized in 10 mL of water. Two grams of solution was weighed in a 40 mL screw-capped tube, and 10 mL of ethyl acetate was added. After agitation in a Vortex for 1 min, the phases were allowed to separate, and 5 mL of the organic extracts was dried under a nitrogen stream. The residue was taken up with 1 mL of mobile phase and injected into HPLC for analysis.

RESULTS AND DISCUSSION

Identification of Homogentisic Acid. The unknown compound isolated from strawberry-tree honey was analyzed by MS and NMR techniques. MS analysis showed that its molecular weight was 168 mu and relevant fragments were 66, 94, 122, and 150 mu. Monodimensional (^{1}H, ^{13}C) and DEPT 135 experiments showed the following data. ^{1}H NMR: 3.71 (2H, s, H-2); 6.72 (1H, dd, 8.55, 2.55, H-6); 6.83 (1H, d, 8.55, H-5); 6.85 (1H, d, 2.85, H-8); 7.8 (2H, bs, exchangeable proton); 10.6 (1H, bs, exchangeable proton). Signals at δ 7.8 and 10.6 disappear after addition of a drop of D_2O. ^{13}C NMR: 36.5 (CH_2); 115.6 (CH); 117.0 (CH); 118 (CH); 123.7 (C quat); 149.4 (C quat); 151.5 (C quat); 173.5 (C quat). (Symbols used: bs = broad signal; d = double; dd = doublet of doublets; s = singlet: C quat = quaternary carbon.)

On the basis of MS and NMR data, the structure of 2,5-dihydroxyphenylacetic acid was proposed. The identity of the compound was confirmed with a commercial authentic sample by comparison of MS, NMR, and of UV spectra (Figure 1) and the retention time obtained by HPLC.

Homogentisic Acid from Strawberry-Tree Honey and Nectar. The HPLC determination of homogentisic acid was possible using H_2SO_4 10^{-2} N as mobile phase. However, this condition gives a retention time (t_R) of >30 min. Therefore, the mobile phase was modified with a mixture containing methanol/H_2SO_4 10^{-2} N (10:90, v/v) to obtain a t_R (12.75 min) that reduced analysis time by half. The recovery assays were carried out using citrus honey (by checking for the absence of the inter-

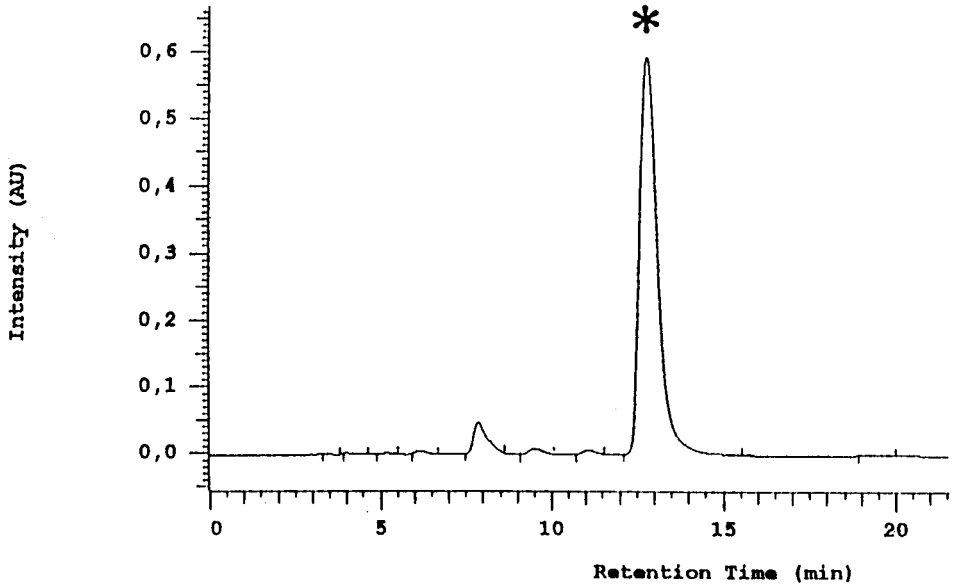

Figure 2. Chromatogram of strawberry-tree honey. *Homogentisic acid (chromatographic conditions are reported in the text).

Table 1. Homogentisic Acid in the Strawberry-Tree Honey Samples and Nectar and Absolute Content of Pollen and Frequencies of *Arbutus* Grains in Sediment

honey sample no.	homogentisic acid (mg/kg)	absolute content of pollen (PK/10 g)	frequencies of *Arbutus* pollen (%)
1	283	<10 000	<3
2	400	<10 000	16−45
3	197	<10 000	16−45
4	382	20 000−100 000	<3
5	447	<10 000	3−16
6	423	<10 000	>45
7	358	<10 000	3−16
8	340	<10 000	>45
9	342	<10 000	16−45
10	347	<10 000	16−45
11	454	<20 000	3−16
12	317	>100 000	<3
13	306	20 000−100 000	<3
14	452	<10 000	16−45
15	240	20 000−100 000	<3
16	451	<10 000	>45
17	512	<10 000	16−45
18	250	20 000−100 000	<3
19	529	<10 000	16−45
20	540	<10 000	>45
21	377	<10 000	16−45
22	329	20 000−100 000	<3
23	429	<20 000	16−45
	378 ± 92		
nectar	165		

fering peak) fortified with 50 and 500 mg/kg of homogentisic acid. Each recovery assay was replicated four times. The recoveries were >95% with a maximum coefficient of variance of 7%. Calibration graphs were constructed using the external standard method by plotting peak area versus concentration. Good linearity was achieved in the range 50−550 mg/kg with a correlation coefficient of 0.9998. In these operative conditions, the analysis was rapid and without interfering peaks (purity 1000; Figure 2).

Twenty-three samples of strawberry-tree honey were analyzed, and the homogentisic acid amount was determined (Table 1). The values ranged between 197 and 540 mg/kg with an average of 378 ± 92 mg/kg. To check the origin of this acid, a strawberry-tree nectar was analyzed. In this sample, 165 mg/kg of homogentisic acid were found. This showed that the acid present in the honey came from nectar. Since the water content of the honey samples was 18.9 ± 1.9% and that of the nectar 64.0%, the homogentisic acid should have increased by a factor 2.25 (from 165 to 371 mg/kg) while transforming the nectar into honey. Several honey samples showed higher values than the nectar sample, corrected for the concentration factor. It is therefore expected that the values of homogentisic acid content in nectar should be higher than those found in this nectar sample. Honey samples of different floral origin (*Castanea, Citrus, Eucalyptus, Thymus, Tarassacum, and Tilia*) and some of the same botanic family, the Ericaceae (*Calluna, Erica,* and *Rhododendron*), were analyzed, but no homogentisic acid was found. Therefore, this acid could be used as a marker of strawberry-tree honey. Under melissopalynological analysis, a strawberry-tree honey sample with 80 mg/kg of homogentisic acid showed to a "frequent" presence of *Rosmarinus* pollen, suggesting it was a mixed honey.

Melissopalynological Characteristics. The results of this analysis confirmed a sediment poor in pollen (PK/10 g <20 000) in 17 (74%) honey samples. More precisely, the absolute number of pollen grains was less than 10 000 in 16 samples; between 20 000 and 100 000 in another five samples, and more than 100 000 only in sample 12 (Table 1).

The *Arbutus* pollen was "very frequent" or "frequent" in 14 samples; "rare" in another three samples, and "sporadic" in the remaining six samples (Table 1). The wide range of frequencies recorded for *Arbutus* is mainly due to the diverse incidence of the accompanying pollen. In some cases, these belong to polliniferous species (i.e., *Cistus* spp.) or to species with an over-representation of pollens. In "rare" or "sporadic" samples, we recorded considerable presence of Myrtaceae, particularly *Eucalyptus* and *Cistus,* typical component of the Sardinian honey spectra; these honeys are probably derived from secondary or tertiary contamination of the pollen spectra.

CONCLUSIONS

Since strawberry-tree honey derives from flowers (*A. unedo* L.) with an under-represented amount of pollen, melissopalynological analysis alone does not normally

allow a precise diagnosis of the botanical origin. For this reason, some chemical or physical properties must to be used to confirm the results of pollen analysis.

In this paper, homogentisic acid (2,5-dihydroxyphenylacetic acid) has been isolated from strawberry-tree honey and identified. Since this acid has not been detected so far in others honeys of different botanical origin (*Castanea, Calluna, Citrus, Erica, Eucalyptus, Rhododendron, Thymus, Tarassacum,* and *Tilia*,), it could be used as a marker of this unifloral honey.

The amount of homogentisic acid in the examined samples of strawberry-tree honeys varied from 197 to 540 mg/kg. In regard to microscopical analysis, the samples may be considered strawberry-tree unifloral through a correct interpretation of the pollen spectra. In fact, in the cases where very low frequencies ("sporadic" or "rare") of *Arbutus* pollen was observed, accompanying pollen (*Cistus, Eucalyptus*) from flowers of polliniferous or over-represented species was also recorded.

On the basis of these preliminary results, a 200 mg/kg content may be temporarily indicated as the minimum quantity of homogentisic acid that should define a honey produced mainly from strawberry-tree flowers.

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LITERATURE CITED

Anklam, E. A review of analytical methods to determine the geographical and botanical origin of honey. *Food Chem*. **1988**, *63*, 549−562.

Bonaga, G.; Giumanini, A. G. The volatile fraction of chestnut honey. *J. Apic. Res*. **1986**, *25*, 113−120.

Demianowicz, Z. La secretion du nectar et le rendement en miel des plus importantes plantes melliferéres en Pologne. *Apimondia* **1963**, *XIX*, 180−183.

Cherchi, A.; Spanedda, L.; Tubweroso, C.; Cabras, P. Solid-phase extraction and HPLC determination of organic acid in honey. *J. Chromatogr. A* **1994**, *669*, 59−64.

Ferreres, F.; García-Viguera, C.; Tomás-Lorente, F.; Tomás-Barberán, F. A. Hesperetin, a marker of the floral origin of citrus honey. *J. Sci. Food Agric*. **1993**, *61*, 121−123.

Ferreres, F.; Giner, J. M.; Tomás-Barberán, F. A. A comparative study of hesperetin andmethyl anthranilate as markers of the floral origin of citrus honey. *J. Sci. Food Agric*. **1994**, *65*, 371−372.

Ferreres, F.; Andrade, P.; Tomás-Barberán, F. A. Natural occurrence of abscisic acid in heather honey and floral nectar. *J. Agric. Food Chem*. **1996**, *44*, 2053−56.

Floris, I.; Lentini, A.; Prota, R. Flora di interesse apistico della Sardegna (I. Indagine sul potenziale mellifero di *Arbutus unedo* L. in Sardegna settentrionale). *Atti Convegno MURST 40%, Sassari* **1991**, 189−200.

Floris, I.; Vacca, V.; Franco, M. A.; Del Caro, A.; Marras, P. M.; Reniero, F. Fenoli totali e rapporto 13C/12C di mieli uniflorali della Sardegna. *Apicoltur,* **1994**, *9*, 119−133.

Gil, M. I.; Ferreres, F.; Ortiz, A.; Subra, E.; Tomás-Barberán, F. A. Plant phenolic metabolites and floral origin of rosemary honey. *J. Agric. Food Chem*. **1995**, *43*, 2833−38.

Louveaux, J.; Maurizio, A.; Vorwohl, G. Methods of melissopalynology. *Bee World* **1978**, *59*, 139−157.

Persano Oddo, L.; Piazza, M. G.; Sabatini, A. G.; Accorti, M. Characterization of unifloral honeys. *Apidologie* **1995**, *26*, 453−465.

Ricciardelli D'Albore, G. C. Textbook of melissopalynology. *Apimondia* **1997**, 308.

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