Identification of Flavonoid Markers for the Botanical Origin of *Eucalyptus* Honey

Isabel Martos, Federico Ferreres, and Francisco A. Tomás-Barberán*

Laboratorio de Fitoquímica, Departamento de Ciencia y Tecnología de Alimentos, CEBAS (CSIC), P.O. Box 4195, 30080 Murcia, Spain

European *Eucalyptus* honeys showed a common and characteristic HPLC profile in which the flavonoids myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone), tricetin (5,7,3′,4′,5′-pentahydroxyflavone), quercetin (3,5,7,3′,4′-pentahydroxyflavone), luteolin (5,7,3′,4′-tetrahydroxyflavone), and kaempferol (3,5,7,4′-tetrahydroxyflavone) were identified. Their contents, and relative amounts, in the analyzed honey samples were quite constant and supported their floral origin. In addition, ellagic acid and the propolis-derived flavonoids pinobanksin, pinocembrin, and chrysin were detected in most samples. The contents of these nonfloral phenolics were much more variable as could be expected for their propolis origin. Myricetin, tricetin, and luteolin had not been identified as floral markers in any other honey sample previously analyzed in our laboratory (chestnut, citrus, rosemary, lavender, acacia, rapeseed, sunflower, heather, lime tree, etc.) or reported in the literature, suggesting that these could be useful markers. Only in some individual heather samples produced in Portugal has tricetin previously been detected in minor amounts. These samples, however, were contaminated with *Eucalyptus* as revealed by their pollen analysis and the lack of tricetin or their glycosides in heather floral nectar. It remains to be established if myricetin, tricetin, and luteolin originate from *Eucalyptus* floral nectar where the corresponding glycosides should be present.

Keywords: *Honey; Eucalyptus; flavonoids; botanical origin; quality*

INTRODUCTION

In the past few years there has been an increasing interest in finding objective analytical methods that could complement pollen analysis in the determination of the floral origin of honey. Volatile compounds (Bonaga et al., 1986), aromatic and degraded carotenoid-like substances (Tan et al., 1988, 1989a,b, 1990; Wilkins et al., 1993), amino acids (Davies, 1975; Bosi and Battaglini, 1978), degradation products of phenylalanine (Speer and Montag, 1987), aromatic aldehydes and heterocycles (Häusler and Montag, 1990), aromatic acids and their esters (Steeg and Montag, 1988), and phenolic compounds (Amiot et al., 1989; Ferreres et al., 1992, 1994a,b; Sabatier et al., 1992) have been found in honey and have been related to the floral origin. In fact, the flavonoid hesperetin proved to be a useful marker for the floral origin of citrus honey (Ferreres et al., 1993) and as a marker showed some advantages over methyl anthranilate, another biochemical marker of the floral origin of citrus honey (Ferreres et al., 1994b).

Eucalyptus honey has a dark color and characteristic flavor. Previous studies reported the norisoprenoids, monoterpenes, and other volatile constituents of *Eucalyptus leucoxylon* and *E. melliodora* honeys produced in Australia (D'Arcy et al., 1997). Some volatiles were quite characteristic of these honey samples, suggesting that Australian honeys have distinctive chemical constituents. Another study reported the flavor and free amino acid composition of eucalyptus honeys and found diketones, hydroxyketones, 3-hexanal, sulfur compounds, and alkanes that were characteristic of the eucalyptus samples, as well as a high content of proline (Bouseta et al., 1996). In addition, the presence of a small amount of dehydrovomifoliol, a characteristic compound of heather honey, was also reported in eucalyptus honey (<1 mg/kg of honey) (Häusler and Montag, 1991).

The purpose of the present work was the HPLC analysis of the phenolic compounds present in different European *Eucalyptus* honeys and the identification of possible markers for the floral origin of these samples.

MATERIALS AND METHODS

Honey Samples. Commercial eucalyptus honey samples (nine) were obtained from different markets and beekeeper's associations in Italy, Portugal, and Spain. The geographical origin and year of production of the different samples are shown in Table 1. Samples were stored at 0 °C until analyzed.

Sample Extraction for HPLC Analysis. The different honey samples (100 g each) were mixed with five parts of water (pH 2 with HCl) until completely fluid and filtered through cotton to remove solid particles. The filtrate was then passed through a column (25 × 2 cm) of Amberlite XAD-2 (Fluka Chemie; pore size = 9 nm, particle size = 0.3-1.2 mm) (Tomás-Barberán et al., 1992). The phenolic compounds remain on the column, while sugars and other polar compounds elute with the aqueous solvent, which results in flavonoid recovery >95% (Ferreres et al., 1994a; Tomás-Barberán et al., 1992). The column was washed with acid water (pH 2 with HCl, 100 mL) and subsequently with distilled water (~300 mL). The whole phenolic fraction was then eluted with methanol (~300 mL) and taken to dryness under reduced pressure (40 °C). The residue was redissolved in 5 mL of water and extracted with diethyl ether (5 mL × 3) (Ferreres et al., 1994c). The ether extracts were combined, concentrated under reduced pressure, and redissolved in 0.5 mL of methanol for HPLC analysis. Samples were stored under N_2 until analyzed. Repeatability of the HPLC analysis was 5% (RSD).

* Author to whom correspondence should be addressed (e-mail fatomas@natura.cebas.csic.es).

10.1021/jf991166q CCC: $19.00
Published on Web 04/22/2000

Table 1. Analysis of Nectar-Derived Flavonoids in *Eucalyptus* Honey Samples

sample	origin	year	1	2	3	4	5	total
A	Italy	1997	171.6	790.1	283.4	283.1	71.2	1599.4
B	Italy	1997	330.2	832.5	485.7	418.3	55.3	2122.0
C	Spain	1997	100.5	289.8	165.0	211.1	45.2	811.6
D	Portugal	1997	46.6	108.1	68.3	103.7	23.2	350.0
E	Spain (Levante)	1998	337.0	1006.2	422.0	338.3	75.2	1878.7
F	Spain (Salamanca)	1998	142.1	585.2	371.7	214.4	175.4	1488.8
G	Spain	1998	225.2	909.8	309.2	274.6	57.9	1776.8
H	Spain	1998	178.2	818.2	328.4	159.6	70.7	1415.1
I	Spain	1998	171.9	803.2	338.5	241.0	62.3	1616.9
mean			189.3	682.6	308.0	254.9	70.7	1451.0
SD			96.3	299.4	126.7	100.9	42.4	549.2
SD/mean			0.509	0.439	0.411	0.396	0.599	0.378

[a] 1, myricetin; 2, tricetin; 3, quercetin; 4, luteolin; 5, kaempferol. Values are μg/100 g of honey.

HPLC Analysis of Honey Flavonoids. All HPLC analyses were carried out using a Merck-Hitachi liquid chromatograph L-6200 with a Merck-Hitachi L-3000 diode array detector and a Merck-Hitachi AS-2000A autosampler. Data were stored and processed with DAD-Manager software (Merck). The column used was a LichroCART RP-18 (Merck) (12.5 $\times$ 0.4 cm, 5 μm particle size) eluted with water/formic acid (19:1, v/v) (solvent A) and methanol (solvent B) at 1 mL/min. The following gradient was used: 30% methanol (15 min) isocratic, then increasing to reach 40% methanol at 20 min, 45% methanol at 30 min, 60% methanol at 50 min, and 80% methanol at 52 min, and finally isocratic at 80% methanol until 60 min. The flavonoids were detected with a diode array detector (Merck-Hitachi L-3000) to obtain the UV spectra of the different phenolic compounds, and the chromatograms were recorded at 340 and 290 nm.

Flavonoid Quantitation. Wherever possible, the different honey flavonoids were identified by chromatographic comparisons with authentic compounds (commercial or previously isolated and identified from honey) (Ferreres et al., 1991, 1992) and by matching their UV spectra with those of the standards. Honey flavonoids were quantified by the absorbance of their corresponding peaks in the chromatograms as reported previously (Ferreres et al., 1994c).

Isolation of Unknown Flavonoids. This was achieved on 15 kg of eucalyptus honey from different origins in Spain. Batches of 1 kg were dissolved with water ($\sim$3 L, adjusted to pH 2 with HCl), mixed with 500 g of Amberlite XAD-2 (Supelco, mean pore diameter = 90 Å, mesh size = 20$-$60 μm, mean surface area = 300 m^2/g), and stirred for 30 min, to allow the adsorption of phenolic compounds. The Amberlite particles were packed on a glass column (80 $\times$ 4 cm), and the column was washed with 2 L of acid water to remove sugars and other polar compounds and retain flavonoids and other honey phenolics. The column was then washed with distilled water (1 L), and the adsorbed compounds were eluted with methanol (0.75 L), until the eluent became colorless. The remaining waters were submitted to an additional adsorption on Amberlite XAD-2 resin to increase flavonoid recovery and to recover those compounds that had not been adsorbed during the first treatment. All of the methanol extracts were combined and concentrated under reduced pressure (40 °C) until complete removal of methanol. The remaining water extract ($\sim$150 mL) was then extracted with diethyl ether (150 mL $\times$ 5 times), and the flavonoid markers were found in the organic phase, which was concentrated under reduced pressure and redissolved in 20 mL of methanol. This extract was then column chromatographed on a Sephadex LH-20 column (40 $\times$ 3 cm) (Pharmacia, Uppsala, Sweden) using methanol. The separation of different fractions was monitored by a 360 nm light, and the fractions obtained were analyzed by HPLC under the specific conditions for honey flavonoid analysis described above. Compounds **1**$-$**5** were isolated. All of these compounds were isolated and purified by semipreparative HPLC on a Spherisorb octadecylsilane (ODS-2) column (25 $\times$ 1 cm, 5 μm particle size), with a solvent flow of 5 mL/min using as mobile phase of different methanol/water solutions in an isocratic manner starting with 33% methanol and increasing the methanol proportion to elute the compounds. The purity of the isolated compounds was tested by analytical HPLC (see above conditions) and stored after freeze-drying.

Flavonoid Identification. The isolated unknown compounds were identified by a combination of UV spectrophotometry in methanol and, after the addition of the classical alkali and metal reagents (Mabry et al., 1970), and by EIMS (Hewlett-Packard electron impact mass spectrometer, 70 eV, direct inlet) and ^{1}H NMR in DMSO-d_6 (Brüker, 350 MHz).

RESULTS

HPLC Analysis of Flavonoid Extracts from *Eucalyptus* Honeys of Different European Origins. *Eucalyptus* spp. honey samples from different European origins (Italy, Portugal, and Spain) were diluted with water and filtered through an Amberite XAD-2 column to retain phenolic compounds and remove polar compounds. Phenolics were then eluted with methanol, and the methanol extract was analyzed by HPLC on reversed-phase columns. All of the samples showed a common chromatographic profile at 340 nm (Figure 1). The main compounds showed characteristic UV spectra for flavonols or flavones. Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) (**1**), quercetin (3,5,7,3′,4′-pentahydroxyflavone) (**3**), and kaempferol (3,5,7,4′-tetrahydroxyflavone) (**5**) were tentatively identified by their UV spectra and HPLC retention time. Flavonoids **2** and **4** were the main flavonoids present in these extracts, but they were not identified in the HPLC analysis. In addition, the characteristic propolis-derived flavonoids were detected (pinobanksin, pinocembrin, chrysin, and methylated quercetin and kaempferol derivatives) in variable amounts. The chromatographic profiles obtained for eucalyptus honey samples from Spain, Italy, or Portugal were very similar, in which myricetin, quercetin, kaempferol, and compounds **2** and **4** were present as the main constituents, with different amounts of the propolis-derived flavonoids (Figure 1).

The UV spectra of compounds **2** and **4** were very similar (Figure 2). These spectra, together with their chromatographic behavior indicated that they were either highly hydroxylated flavones or highly hydroxylated flavonols, in which the hydroxyl at the 3-position was substituted by a methyl ether. Flavonoid **2** had not been detected in previous extensive studies in any of the monofloral honey samples analyzed in our laboratory (citrus, rosemary, lavender, rape, acacia, sunflower, chestnut, heather, lime tree, etc.).

Flavonoid Identification. The isolated flavonoids were identified by UV spectrophotometry, EIMS, and ^{1}H NMR analyses.

Compound **1** showed a UV spectrum corresponding to a flavonol with a free hydroxyl at the 3-position (BI

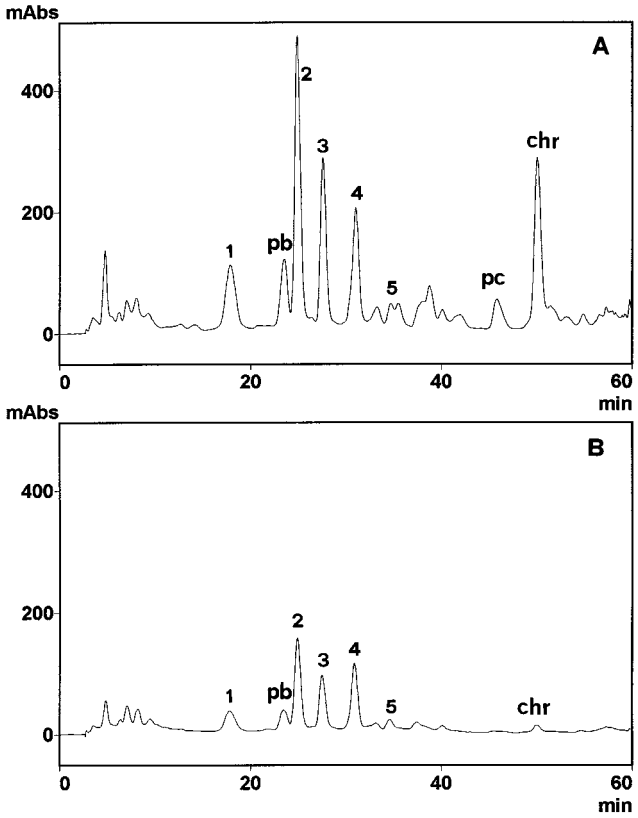

Figure 1. HPLC chromatograms of flavonoid extracts of European eucalyptus honey samples (340 nm): (A) eucalyptus honey from Italy; (B) eucalyptus honey from Spain. Peaks: 1, myricetin; 2, tricetin; 3, quercetin; 4, luteolin; 5, kaempferol; pb, pinobanksin; pc, pinocembrin; chr, chrysin.

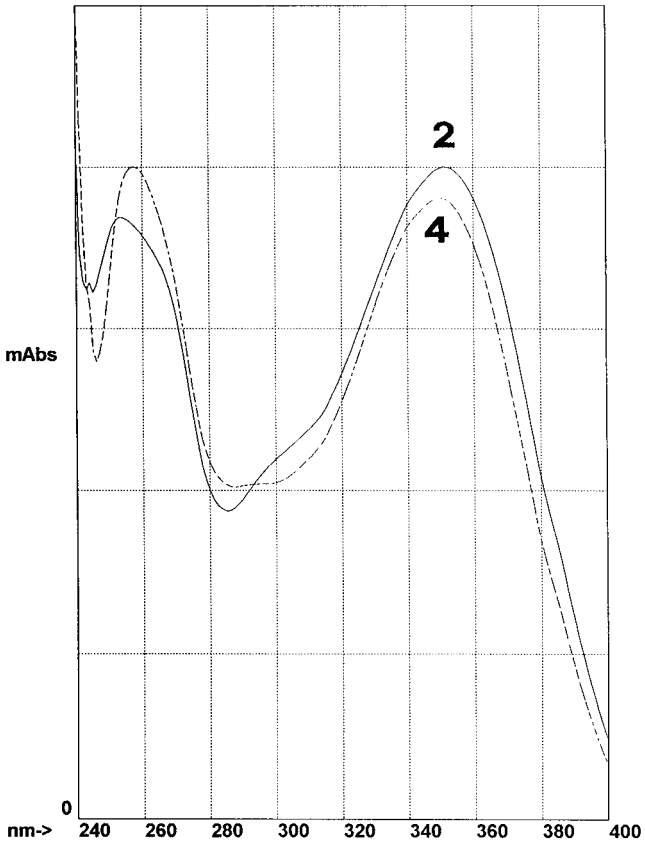

Figure 2. UV spectra of compounds **2** (tricetin) and **4** (luteolin) isolated from eucalyptus honey.

Table 2. Propolis-Derived Flavonoids and Ellagic Acid Contents of the *Eucalyptus* Honeys

sample	ellagic acid	pinobanksin	pinocembrin	chrysin	total propolis
A		1611.2	446.3	114.8	1770.3
B		549.9	1066.1	394.4	2010.4
C	0.8	34.4	46.4	11.1	91.9
D	18.6	1136.3	522.2	110.2	1768.7
E	3.3	350.2	168.2	35.2	553.6
F	8.9	695.7	296.3	82.4	1074.4
G	2.8	110.2	71.3	25.0	206.5
H		388.4	139.0	42.8	570.2
I	7.2	125.7	70.0	29.2	224.9
mean		555.8	314.0	93.9	919.0
SD		532.9	329.5	118.8	757.5
SD/mean		0.959	1.050	1.266	0.824

[a] Values are μg/100 g of honey.

in methanol at 375 nm) and with a highly oxygenated B-ring (heavy decomposition after the addition of alkaline reagents) (Table 2). The UV study (Mabry et al., 1970) showed clearly that hydroxyls at the 3-, 5-, and 7-positions were free and that three adjacent hydroxyls at the 3′-, 4′-, and 5′-positions were also present, the spectra being coincident with those of myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) (Mabry et al., 1970). The nature of this compound was confirmed by chromatographic comparison (HPLC) with an authentic standard of myricetin (Sigma, St. Louis, MO).

Compound **2** showed a UV spectrum in methanol typical of either a flavone or a 3-methylflavonol (Figure 2). Its UV study after the addition of the classical alkaline and metal reagents (Mabry et al., 1970) showed free hydroxyls at the 5- and 7-positions and a possible 3′,4′,5′-trihydroxy substitution as suggested by the decomposition observed after the addition of the alkaline reagents (Table 3). These data coincide with those reported by Voirin (1983) for tricetin (5,7,3′,4′,5′-pentahydroxyflavone). Its EIMS spectrum shows a molecular ion at *m*/*z* 302 (100% relative intensity) in accordance with a pentahydroxylated flavone. A peak at *m*/*z* 274 (M − CO) is common in highly hydroxylated flavonoids (Tomás-Barberán and Tomás-Lorente, 1986). The fragmentation of the flavonoid nucleus into A- and B-ring fragments (retro Diels−Alder fragmentation) is commensurate with this flavonoid substitution (B_2 fragment at *m*/*z* 153 for a trihydroxylated B-ring). The ^{1}H NMR spectrum in DMSO clearly shows the aromatic protons assigned at the hydrogens at the 3-, 6-, and 8-positions of the flavonoid nucleus [singlet integrating one proton at 6.53 ppm (H-3); doublets (J = 2 Hz) at 6.40 and 6.18 ppm for H-6 and H-8]. In addition, the spectrum showed a singlet corresponding to two aromatic protons (6.96 ppm), which was assigned to H-2′ and H-6′ of the flavonoid nucleus, in accordance with a 3′,4′,5′-trihydroxylated flavonoid (Markham and Geiger, 1993). Thus, this compound was identified as tricetin, 5,7,3′,4′,5′-pentahydroxyflavone.

Compound **3** was identified as quercetin, a very common compound in different honeys, and compound **5** as kaempferol, which was present in smaller amounts. These compounds were identified by their UV spectra (Table 3) and chromatographic comparisons with authentic standards.

Compound **4** showed a UV spectrum in methanol very similar to that of compound **2**. Its HPLC retention time indicated that this was less polar (less hydroxylated) than **2** (higher retention time). Its UV study indicated free hydroxyls at the 5-, 7-, 3′-, and 4′-positions and the

Table 3. UV Study of the Isolated Flavonoids[a]

compd	MeOH	+NaOMe	+$AlCl_3$	+$AlCl_3$+HCl	+NaOAc
1	255, 267sh, 301, 375	263sh, 285sh, 320, 430 (dec)	271, 314, 430	267, 275sh, 309sh, 363, 428	269sh, 355 (dec)
2	248, 267, 302sh, 351	264, 276sh, 328sh, 405 (dec)	271, 311sh, 418	274, 304, 361, 390	272, 322sh, 380
3	255, 269sh, 302sh, 370	247sh, 321 (dec)	272, 304sh, 333, 458	265, 301sh, 359, 428	257sh, 274, 329, 390 (dec)
4	254, 266sh, 292sh, 348	268, 330sh, 402	274, 300sh, 330sh, 425	273, 295sh, 355, 385sh	269, 320sh, 394
5	253sh, 266, 284sh, 322sh, 367	278, 316, 416 (dec)	260sh, 268, 303sh, 350, 424	256sh, 269, 303sh, 348, 424	274, 303, 387

[a] sh, shoulder; (dec), decomposition.

presence of a dihydroxyl grouping on the B-ring (Mabry et al., 1970) (Table 3). Its EIMS spectrum showed an M^+ at *m/z* 286 in accordance with a tetrahydroxyflavone. Its ^{1}H NMR analysis showed a singlet integrating one proton at 6.65 ppm (H-3), two doublets (J = 2 Hz) at 6.44 (H-8) and 6.18 (H-6) ppm, a doublet (J = 8 Hz) at 6.88 ppm corresponding to H-5′, a double doublet (J = 8 and 2 Hz) at 7.41 ppm (H-6′), and a doublet (J = 2 Hz) at 7.38 ppm (H-2′). All of these data are consistent with a 5,7,3′,4′-tetrahydroxyflavone (Markham and Geiger, 1993). Thus, compound **4** was identified as luteolin and its structure confirmed by chromatographic comparisons with an authentic marker.

Flavonoid Quantitation in Different Honey Samples. When commercial eucalyptus honey samples were analyzed, the same flavonoid pattern was virtually always observed (Table 1), with the presence of the characteristic flavonoids shown in the HPLC chromatograms of Figure 1. Only 1 commercial sample (of the 10 analyzed), labeled as eucalyptus honey, failed to contain these compounds, and its floral origin was questioned (data not shown).

Two different types of flavonoids were distinguished in the HPLC chromatograms. Compounds **1**−**5**, which are fully hydroxylated flavonoids, constitute the characteristic fingerprint of eucalyptus honeys along with and other flavonoids that are common to most honeys produced in temperate areas and that originate in propolis, a plant resin collected by bees for different uses in the hive. These propolis-derived flavonoids include flavanones such as pinobanksin (3,5,7-trihydroxyflavanone) and pinocembrin (5,7-dihydroxyflavanone) and flavones with an unsubstituted B-ring such as chrysin (5,7-dihydroxyflavone). These compounds are known to originate in poplar bud exudates, which is the main source of propolis in temperate regions. We have previously shown that propolis flavonoids are also present in beeswax, and from the wax matrix they can diffuse into the honey. The content of these flavonoids in honey is quite variable because it depends on the degree of propolis contamination in the hive and beeswax.

The content of nectar-derived flavonoids **1**−**5** of the analyzed samples was quite consistent (>1500 μg/100 g of honey) with the exception of samples C and D, which had a smaller flavonoid content. The main flavonoid was in all samples tricetin (**2**), whereas kaempferol (**5**) was present in the smallest amounts and the other three compunds were in intermediate content values. As could be expected, the content of propolis-derived flavonoids was much more variable. This is evident from the data shown in Table 2 and the chromatograms shown in Figure 1, in which the consistency of flavonoids **1**−**5** is clearly observed as well as the large differences in the content of chrysin, pinocembrin, and pinobanksin, although the wavelength at which these chromatograms were recorded (340 nm) was not the optimum for flavanone detection (290 nm). This large variability can be observed from the large differences observed in the propolis-derived flavonoid content of the analyzed samples, which ranges between <100 μg/100 g for sample C and >2000 μg/100 g in sample B (Table 2). Ellagic acid, a dimer of gallic acid which is a constituent of ellagitannins, was also detected in some samples in small amounts (generally <10 μg/100 g of honey). These values are much smaller than those reported previously for ellagic acid, which was detected at levels ranging between 100 and 650 μg/100 g of heather honey (Ferreres et al., 1996a).

DISCUSSION

European *Eucalyptus* honeys show a common HPLC flavonoid pattern characterized by highly hydroxylated flavonoids, namely, myricetin, tricetin, quercetin, luteolin, and kaempferol. Quercetin and kaempferol are common honey flavonoids found in many other monofloral honey samples. For instance, kaempferol was the main flavonol in the chromatograms of rosemary honey (Gil et al., 1995), and its analysis was suggested as a complementary technique in floral origin determinations, as this compound was also detected, in glycosidic form, in rosemary nectar. Quercetin was detected as one of the main flavonoids in sunflower honey, suggesting its possible use as marker of this floral origin. However, we have failed to identify quercetin glycosides in sunflower nectar, although quercetin 3-rutinoside (rhamnosyl 1−6-glucoside) was found as the main flavonoid constituent of sunflower pollen (Soler et al., 1995). The other three flavonoids, myricetin, tricetin, and luteolin, have not been associated with any specific floral origin, and in fact, they have not been described as floral flavonoids in any honey sample analyzed so far. There is only one previous paper reporting tricetin, luteolin, and myricetin in honey. These compounds were detected as minor constituents in some samples of Portuguese heather honeys (2−40 μg/100 g of honey) (Ferreres et al., 1994a). However, their use as possible floral markers was later disregarded, as they, or their corresponding glycosides, were not detected in heather floral nectar (Ferreres et al., 1996a,b). Their presence as trace compounds in these Portuguese heather honeys could be explained by the contamination with *Eucalyptus*, as revealed by the pollen analysis of these samples that showed the occurrence of *Eucalyptus* pollen in many of the analyzed heather honey samples (Andrade et al., 1999).

These results show that eucalyptus honeys have a distinctive flavonoid profile that could probably be used in objective analytical determinations of their floral origin. The study of *Eucalyptus* spp. floral nectar to confirm the nectar origin of these flavonoids will also be necessary, although nectar collection in the case of

Eucalyptus flowers is not an easy task, due to the location of flowers high in the trees and to the small amount of nectar produced. In addition, a larger number of *Eucalyptus* honey samples from different geographical origins should also be analyzed to prove the utility of these flavonoids as markers of floral origin in this type of honey.

ACKNOWLEDGMENT

We are grateful to Prof. J. Sánchez and Celestino Santos from Salamanca University and Prof. Huidobro from Santiago University for providing honey samples for flavonoid extraction and identification.

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Received for review October 25, 1999. Revised manuscript received February 23, 2000. Accepted March 2, 2000. The present work has been financially supported by MAPA-INIA (Grant API98-001).

JF991166Q