Flavonoid Composition of Tunisian Honeys and Propolis

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Flavonoids and other phenolics of Tunisian honey samples and propolis were analyzed to find correlations between botanical and geographical origin and chemical composition. Flavonoid content of honey was very variable (20−2400 μg/100 g). The richest samples contained the characteristic propolis flavonoids, while those containing fewer flavonoids were devoid of these substances. This is a difference with honeys from temperate areas, in which the poplar-derived flavonoids are present in all honey samples. In Tunisian honeys, and propolis, a new flavonoid, myricetin 3,7,4′,5′-tetramethyl ether, was detected. This is characteristic of *Cistus* spp. leaf exudates. Another minor compound was identified as quercetin 3,7,3′-trimethyl ether. They were present in high amounts in propolis but were only detected in small amounts in honey. These results show that in border areas, such as Tunisia, where poplars are not always available for propolis collection, other plant sources can be used and their constituents detected in honey.

Keywords: *Phenolics; honey; propolis; flavonoids; characterization; geographical origin; botanical origin*

INTRODUCTION

The search for objective analytical methods that could complement pollen analysis in the determination of the floral and geographical origin of honey has been the purpose of researchers dealing with honey chemical composition during the past decade. Volatile compounds (Bonaga and Giumanini, 1986), aromatic and degraded carotenoid-like substances (Tan et al., 1988, 1989a,b, 1990; Wilkins et al., 1993), amino acids (Davies, 1975), degradation products of phenylalanine (Speer and Montag, 1987), aromatic aldehydes and heterocycles (Häusler and Montag, 1990), aromatic acids and their esters (Speer and Montag, 1984; Steeg and Montag, 1988), and plant phenolic metabolites (Amiot et al., 1989; Ferreres et al., 1992, 1994; Sabatier et al., 1992) have been found in honey and have been related to its floral origin. In fact, the flavanone hesperetin proved to be a useful marker for the floral origin of citrus honey (Ferreres et al., 1993), the flavonol kaempferol of rosemary honey (Gil et al., 1995), and ellagic and abscisic acids of *Erica*-type-heather honey (Ferreres et al., 1994, 1996b).

In addition, the chemical composition of honey has been found to be related to the geographical origin of honey (Tomás-Barberán et al., 1993a). In temperate areas, were *Populus* species are well represented, the phenolic compounds that accumulate in poplar bud exudates and propolis (namely, pinocembrin, chrysin, pinobanksin, galangin, and tectochrysin) (Wollenweber et al., 1987) are the main constituents of the phenolic extracts of honey (Tomás-Barberán et al., 1993a). On the other hand, in places where *Populus* species are not common trees (as in the case of the tropics and very arid areas), bees seek a different resin source to be incorporated in propolis and indirectly in honey. Thus, in tropical Venezuela, compounds from the resinous exudates of *Clusia* spp. are incorporated in propolis and honey from the same geographical region (Tomás-Barberán et al., 1993b), and in the Arizona desert, highly methylated flavones (i.e. xanthomicrol) were detected in propolis in addition to the classical poplar flavonoids (Wollenweber et al., 1987). In addition, it seems that in cases where no suitable resinous plant material is available for the bees at a reasonable distance from the hive, then they may select other alternative sources such as road tar or car workshop glue, which is incorporated in propolis and beeswax (personal observation).

The objective of this work was to study the plant phenolic metabolite composition of honey samples from northern Africa (Tunisia), with different floral origins, and propolis from the same region and compare them with those produced in Europe. No North African honey or propolis has been studied for phenolic constituents so far. The study of these substances is of interest due to their possible role in the determination of the geographical and floral origin of honey.

MATERIALS AND METHODS

Honey Samples. These were produced in Tunisia in different localities and from different floral origins (Table 1). The samples were directly collected from beekeepers and were without commercial manipulation to avoid possible changes during handling, storage, and processing. Samples were stored at 5 °C in the dark until used. For comparative purposes, rosemary and orange honeys produced in Spain, which have been extensively studied in previous works (Ferreres et al., 1993; Gil et al., 1995), were also analyzed.

Propolis Samples. Propolis collected in Menzel mhiri (Tunisia) and in La Alcarria (Spain) were used for this study.

Flavonoid Extraction. Honey samples (100 g) were thoroughly mixed with 500 mL of distilled water (adjusted to pH 2 with concentrated HCl), until completely fluid, by stirring with a magnetic stirrer at room temperature. The solution was then filtered through cotton to remove solid particles. The filtrate was mixed with Amberlite XAD-2 (100 g) (Fluka

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Table 1. Tunisian Honey Samples Studied in the Present Work

code	floral origin[a]	place of production	date of harvest
eucalyptus 1	*Eucalyptus* spp.	Sejnane	July 1994
eucalyptus 2	*Eucalyptus* spp.	Grombalia	Aug 1994
eucalyptus 3	*Eucalyptus* spp.	Sejnane	July 1995
thyme 1	*Thymus algeriensis*	Mateur	May 1994
thyme 2	*Thymus algeriensis*	Biserte	July 1994
rosemary 1	*Rosmarinus officinalis*	Kasserine	March 1994
rosemary 2	*Rosmarinus officinalis*	Kairouan	March 1995
orange 1	*Citrus sinensis*	Borj Touil	May 1991
orange 2	*Citrus sinensis*	Beni Khalled	May 1995
orange 3	*Citrus sinensis*	Nabeul	May 1995
rape	*Brassica campestris*	Mateur	May 1995
sunflower	*Helianthus annus*	Korba	Sept 1995
multifloral	multifloral	Menzel Temime	May 1994

[a] Samples were monofloral (obtained from one species) with the exception of *Eucalyptus* samples, which originated from different *Eucalyptus* species (spp.), and the multifloral sample, which originated from different genera.

Chemie, Bucks, Switzerland; pore size 9 nm, particle size 0.3−1.2 mm) and stirred with a magnetic stirrer for 10 min, which was considered enough time to adsorb honey phenolics with a recovery >80% (Tomás-Barberán et al., 1992). The Amberlite particles were then packed in a glass column (25 × 2 cm). The column was washed with acidified water (200 mL) and distilled water (300 mL) successively to remove all sugars and other polar constituents of honey. The phenolic compounds remained adsorbed in the column (Ferreres et al., 1991) and were then eluted with methanol (400 mL); this extract was then concentrated under reduced pressure (40 °C). The residue was resuspended in distilled water (5 mL) and extracted with diethyl ether (5 mL × 3). The extracts were combined, and the diethyl ether was removed by flushing with nitrogen. The dried residue was then redissolved in 0.5 mL of methanol (HPLC grade), filtered through a 0.45 μm mesh, and analyzed by HPLC.

The propolis samples (ca. 1 g) were extracted with methanol (25 mL) at room temperature for 24 h, and the extract was filtered through filter paper, concentrated to dryness under reduced pressure (40 °C), and redissolved in methanol (1 mL). The sample was then filtered through a 0.45 μm mesh before HPLC analyses.

HPLC Analysis of Honey and Propolis Phenolics. This was achieved on a reversed-phase column LiCrochart RP-18 (Merck, Darmstadt, Germany) (12.5 × 0.4 cm, 5 μm particle size), using as mobile phase water/formic acid (19:1, v:v) (A) and methanol (B), with a constant solvent flow rate of 1 mL min^{-1}, starting isocratically with 30% B in A until 15 min, then installing a gradient to reach 40% B at 20 min, 45% B at 30 min, 60% B at 50 min, 80% B at 52 min, and 90% B at 60 min, then becoming isocratic until 65 min. Honey extracts were analyzed using an L-6200 Merck-Hitachi chromatograph, with a multichannel photodetector L-3000, and samples were injected with an Merck-Hitachi AS-2000A autosampler. The column was maintained at room temperature, and the chromatograms were processed with DAD-Manager software (Merck-Hitachi). The retention times of the different phenolics detected in Tunisian honey samples, when analyzed under these conditions with this equipment, are shown in Table 2. They were quantified by their absorbance in the HPLC chromatograms against external standards, the flavanones as pinocembrin (at 290 nm), the flavones with unsubstituted ring B (chrysin, tectochrysin, and galangin) as chrysin (at 340 nm), and the rest of flavonoids as quercetin (at 340 nm). Ellagic acid and the caffeic acid derivatives phenylethyl caffeate and dimethylallyl caffeate were quantified as ellagic acid (290 nm) and dimethylallyl caffeate (340 nm), respectively.

Propolis extracts were analyzed under the same column, HPLC pump, and solvent conditions, but in this case the column was thermostated at 40 °C, the detector was a Shimadzu SPD-M6A, and the samples were injected manually with a Rheodyne injector with a 20 μL loop. The data were processed with Class M10-A software (Shimadzu).

Identification of Phenolics in Honey. Honey phenolics were identified by their characteristic UV spectra recorded with a diode array detector and by chromatographic comparisons with authentic markers (commercial or previously isolated and identified from honey or propolis samples) (Ferreres et al., 1991, 1992). The caffeic acid esters were kindly provided by Prof. E. Wollenweber (Darmstadt, Germany). The different phenolics identified in Tunisian honeys are shown in Table 2.

Isolation of Flavonoids from Tunisian Propolis. Propolis (10 g) was cut in small pieces and extracted in an Soxhlet apparatus with methanol, until the extract had no color. When the methanol extract reached room temperature, there was a precipitate (mainly beeswax), which was separated by centrifugation at 6000*g*. The supernatant was concentrated under reduced pressure until reaching a syrup-like consistency and redissolved in 3 N NaOH. This was extracted with diethyl ether. The aqueous fraction was taken to pH 3 by addition of concentrated HCl and extracted again with diethyl ether. The flavonoids were located by HPLC in this second diethyl ether extract. This fraction was taken to dryness, redissolved in methanol, and chromatographed on a Sephadex LH-20 colunm (40 × 3 cm) with methanol. The different fractions were followed under UV light (365 nm), and their composition was tested by HPLC (0 min, 30% methanol; 30 min, 80% methanol in water + 5% formic acid). The fraction containing flavonoid **T** was then purified by semipreparative HPLC [Spherisorb ODS-2, 5 μm, 25 × 0.7 cm, room temperature, 2 mL min^{-1}, isocratic separation methanol/water (58:42, v:v)]. During the purification of compound **T**, a second flavonoid (**T′**) was detected in much smaller amount. This compound eluted in HPLC very close to compound **T** but with higher R_t (**T** = 25.0 min; **T′** = 25.3 min) under these conditions. Compound **T′** was also isolated for structural identification.

Identification of Flavonoids from Tunisian Propolis. The structures were determined by UV spectrophotometry in methanol and after the addition of the classical alkaline and metal reagents (Mabry et al., 1970), by EIMS (electron impact mass spectrometry), and by ^{1}H NMR (nuclear magentic resonance).

*Compound **T**:* UV (λ_{max} nm) MeOH 267, 305 sh, 346; + NaOMe 266, 300 sh, 364 (decrease in absorbance); + $AlCl_3$ 276, 307, 355, 402 sh; + $AlCl_3$ + HCl 276, 307, 352, 401 sh; + NaOAc 266, 297, 350; + NaOAc + H_3BO_3 267, 302 sh, 347. EIMS (70 eV, direct inlet) *m*/*z* (rel intensity) 374 (M, 100), 373 (M − H, 46), 359 (M − Me, 67), 331 (M − 43, 34), 271 (22), 313 (M − 43 − 18, 15), 173 (44), 167 (A_1 + H, 43), 150 (B_1 − 28, 21), 138 (A_1 − 28, 21), 135 (B_1 − 43, 21). ^{1}H NMR (Varian, 300 MHz, in DMSO-*d*) δ 7.28 (H-2′, d, $J_{2'-6'}$ = 2Hz), 7.20 (H-6′, d, $J_{6'-2'}$ = 2 Hz), 6.75 (H-8, d, J_{6-8} = 2 Hz), 6.39 (H-6, d, J_{6-8} = 2 Hz); methoxyl singlets at 3.87, 3.86, 3.82, and 3.77.

*Compound **T′**:* UV (λ_{max} nm) MeOH 256, 294 sh, 355; + NaOMe 265, 298 sh, 406 (increase in absorbance); + $AlCl_3$ 275, 313 sh, 363, 405 sh; + $AlCl_3$ + HCl 275, 312 sh, 361, 404 sh; + NaOAc 263, 298 sh, 366, 412; + NaOAc + H_3BO_3 257, 268, 293 sh, 357. EIMS (70 eV, direct inlet) *m*/*z* (rel intensity) 344 (M, 12.5), 343 (M − H, 8), 329 (M − 15, 11), 301 (M − 43, 20), 283 (M − 43 − 18, 4), 167 (A_1 + H, 37), 158 (56), 151 (B_2, 50), 138 (A_1 − 28, 18), 135 (B_2 − 16, 49), 123 (B_2 − 28, 56), 108 (B_2 − 43, 38). ^{1}H NMR (Varian, 300 MHz, in DMSO-*d*) δ 7.67 (H-2′- d, $J_{2'-6'}$ = 2 Hz), 7.63 (H-6′, dd, $J_{2'-6'}$ = 2 Hz, $J_{5'-6'}$ = 8 Hz), 6.96 (H-5′, d, $J_{5'-6'}$ = 8 Hz), 6.79 (H-8, d, J_{6-8} = 2 Hz), 6.38 (H-6, d, J_{6-8} = 2 Hz), 3.87 (2 × OMe, s), 3.82 (1 × OMe, s).

RESULTS

Phenolic Compounds Content in Tunisian Honeys. Thirteen honey samples collected in different Tunisian localities and with diffferent floral origin (as indicated by the beekeepers and checked by the places where the hives were located and the flora available for foraging) were extracted and the phenolic compounds analyzed by HPLC. The results are shown in Table 3. The total phenolic content was very variable and ranged

Table 2. Phenolics Metabolites Detected in Tunisian Honey Samples

common name	structure	R_t HPLC (min)	no.
ellagic acid	gallic acid dimer	10.01	A
pinobanksin	3,5,7-trihydroxyflavanone	12.78	B
unknown flavanone	?	14.80	C
hesperetin	5,7,3′-trihydroxy-4′-methoxyflavanone	18.44	D
quercetin	3,5,7,3′,4′-pentahydroxyflavone	21.95	E
luteolin	5,7,3′,4′-tetrahydroxyflavone	23.85	F
3-methylquercetin	5,7,3′,4′-tetrahydroxy-3-methoxyflavone	24.02	G
8-methoxykaempferol	3,5,7,4′-tetrahydroxy-8-methoxyflavone	25.64	H
kaempferol	3,5,7,4′-tetrahydroxyflavone	26.51	I
apigenin	5,7,4′-trihydroxyflavone	28.82	J
isorhamnetin	3,5,7,4′-tetrahydroxy-3′-methoxyflavone	30.52	K
pinocembrin	5,7-dihydroxyflavanone	32.34	L
phenylethyl caffeate	caffeic acid ester	35.45	M
pinobanksin 3-acetate	3,5,7-trihydroxy-3-acetylflavanone	37.25	N
dimethylallyl caffeate	caffeic acid ester	38.12	O
quercetin 3,7-dimethyl ether	5,3′,4′-trihydroxy-3,7-dimethoxyflavone	39.58	P
chrysin	5,7-dihydroxyflavone	41.91	Q
galangin	3,5,7-trihydroxyflavone	42.99	R
galangin 3-methyl ether	5,7-dihydroxy-3-methoxyflavone	45.01	S
myricetin 3,7,4′,5′-methylether	5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone	47.17	T
pinocembrin 7-methyl ether	5-hydroxy-7-methoxyflavanone	50.34	U
tecthochrysin	5-hydroxy-7-methoxyflavone	57.20	V

Table 3. Phenolic Metabolites Content of Tunisian Honeys[a]

		eucalyptus			thyme		rosemary		orange					
	no.	1	2	3	1	2	1	2	1	2	3	rape	sun-flower	multi-floral
ellagic acid	A	86.59	48.06	848.89		36.80								2.81
pinobanksin	B					59.15				56.74	105.38	132.49	107.59	
unidentified flavanone	C					40.72								
hesperetin	D								7.42	8.68	18.08			3.68
quercetin	E	123.17	76.19	29.22		13.57								
luteolin + 3-mequerc	F+G	11.48	8.75	8.52	2.18	4.01								
8-methoxykaempferol	H	30.21	47.13	22.40	21.57	40.40	9.27	94.64	25.52	28.01	19.70		81.67	
kaempferol	I	63.07	70.59	28.30	19.51	136.57	9.84	29.24	36.27	65.33	111.68		24.25	248.19
apigenin	J		1.99	202.10	2.16	2.53				11.73	78.72	579.13		
isorhamnetin	K	20.86	32.29	13.55	1.36	67.22		2.74	4.15	7.03	27.78	52.46		122.44
pinocembrin	L			33.95		20.02		13.95	2.46	30.57	74.85	129.13	128.67	
phenylethyl caffeate	M					19.80				1.18	2.86		1.96	
pinobanksin 3 acetate	N										17.94	10.29	8.69	
dimethylallyl caffeate	O					8.45								
quercetin 3,7-dimethyl ether	P											37.26		
chrysin	Q			156.68		2.53		27.93	tr	177.21	434.55	846.65	1258.05	2.23
galangin	R					1.30				45.50	17.85	303.02	383.89	
galangin 3 Me	S										29.90	50.00	93.47	
myricetin 3,7,4′,5′-Me	T	44.22	8.62	17.77		61.79		8.31	19.63	4.74	21.73	13.76	tr	54.42
pinocembrin 7-Me	U											11.80	29.22	
tectochrysin	V			47.84				8.46			35.37	68.71	32.44	
other flavonols	W	481.39	190.71	54.12										
total		860.99	484.33	614.50	46.78	514.86	19.11	185.27	95.45	436.71	996.37	2377.47	2150.21	433.77

[a] Values are μg/100 g of honey.

between 20 and 2400 μg/100 g of honey. The richest honeys in phenolics were those from rape and sunflower. Some samples, as rosemary 1, were very poor in phenolic constituents (ca. 20 μg/100 g).

The phenolic profiles detected in the different samples analyzed were quite different (Table 3), which is a remarkable difference from the honey samples of Europe, which in spite of having different floral origins have a quite common phenolic profile, characterized by the presence of propolis-derived flavonoids as main constituents (Soler et al., 1995). In the present study, only 5 of the 13 samples analyzed had a flavonoid profile similar to those of European honeys (samples thyme 2, orange 2 and 3, rape and sunflower) and showed the characteristic poplar-derived compounds pinobanksin, pinocembrin, chrysin, galangin, and related compounds. Interestingly, other Tunisian samples from the same floral origins (thyme 1 and orange1) were devoid of the poplar (propolis)-derived flavonoids, supporting the assumption that these phenolics originate in propolis. It is important to remark that for those honey samples in which poplar-derived phenolics are not observed, the phenolic content is very small (sample thyme 1 and orange 1), illustrating the importantance of the contribution of propolis-derived compounds to the phenolic composition of honey (>90% in many instances).

Differences were also found between honey samples from different floral origins. Thus, *Eucalyptus* honeys were characterized by the presence of ellagic acid and a number (two or three) of relatively polar flavonols (eluting in the chromatogram earlier than quercetin) included in Table 3 as compound **W** for quantitation purposes. In addition, these honey samples contained other flavonols such as quercetin, kaempferol, and isorhamnetin, and only sample eucalyptus 3 contained a small amount of poplar-derived flavonoids. Rosemary samples contained kaempferol and 8-methoxykaempferol as floral-derived flavonoids, and orange honey samples contained the characteristic hesperetin, in amounts similar to those reported in Spanish *Citrus* honeys (Ferreres et al., 1993). The other three monofloral

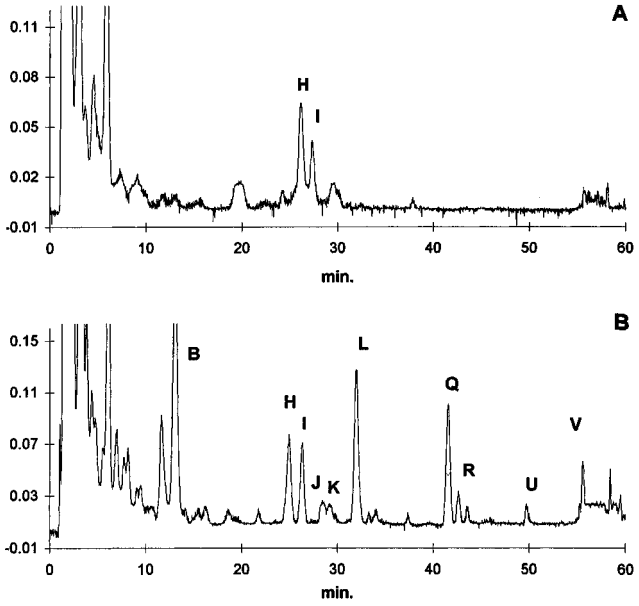

Figure 1. HPLC phenolic profiles of Tunisian (A) and Spanish (B) rosemary honeys. Chromatograms were recorded at 290 nm. HPLC conditions are given under Materials and Methods. For compound identification, see Table 2.

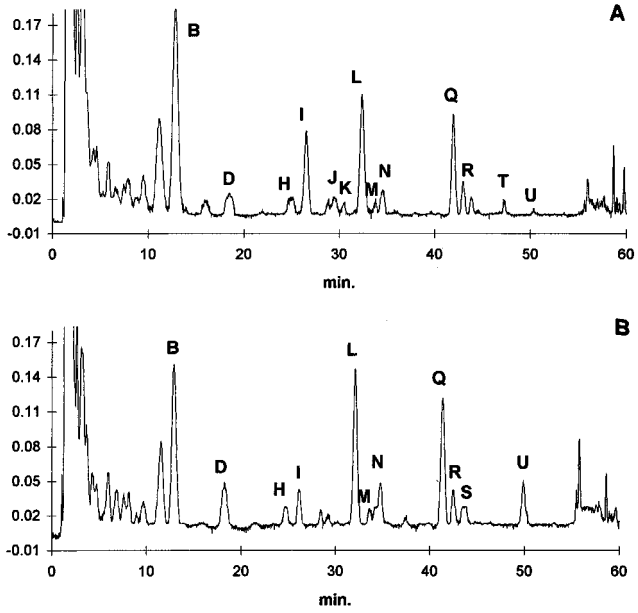

Figure 2. HPLC phenolic profiles of Tunisian (A) and Spanish (B) citrus honeys. Chromatograms were recorded at 290 nm. HPLC conditions are given under Materials and Methods. For compound identification, see Table 2.

honey samples analyzed, rape, sunflower, and thyme, did not show specific phenolic constituents.

To illustrate the differences in honey phenolics due to the geographical origin (propolis-derived phenolics) and the similarities between floral-derived phenolics, monofloral samples (*Citrus* and rosemary) from the same floral origins from Tunisia and Spain were studied. In Figure 1, the HPLC phenolic profiles of rosemary honeys from Tunisia (rosemary 1) and Spain are shown. It is clear that the profiles are quite different. The sample from Tunisia is completely lacking the poplar-derived flavonoids, while these are the main constituents in the Spanish sample (data not shown). In the North African sample, only the floral-derived metabolites, kaempferol (**I**) and 8-methoxykaempferol (**H**), are present, these compounds appearing in amounts and proportions similar to those in the Spanish sample.

In Figure 2, the phenolic profiles of citrus honeys from Tunisia and Spain are shown. In this case, hesperetin (**D**), the floral marker of *Citrus* honey, was detected in both samples in similar amounts, and in both cases, the major flavonoids were those originating from poplar-derived propolis including pinobanksin, pinocembrin, chrysin, and galangin. In addition, a new compound (**T**) was detected in the Tunisian sample. This compound was detected in small amounts in 11 of the samples analyzed (Table 3), and it had not been previously detected in any of the European samples analyzed so far in our laboratory. This lipophilic flavonoid had a UV spectrum similar to that of a kaempferol 3-methyl ether (Mabry et al., 1970) and did not cochromatograph with any of the standards of kaempferol mono-, di-, or trimethyl ethers available in our laboratory. Its UV spectrum clearly indicated that the hydroxyl at the 5-position was free.

Due to the wide distribution of this unusual compound in Tunisian honeys, its propolis origin was envisaged, and this prompted us to study propolis collected in this geographical area. When a propolis sample collected in Tunisia was extracted and analyzed by HPLC, the characteristic compounds of European or North American propolis (i.e. chrysin, galangin, tectochrysin, pinocembrin, pinobanksin, dimethylallyl caffeate, phenylethyl caffeate, etc.) were also detected. In addition, compound **T** was present as a majority compound (Figure 3).

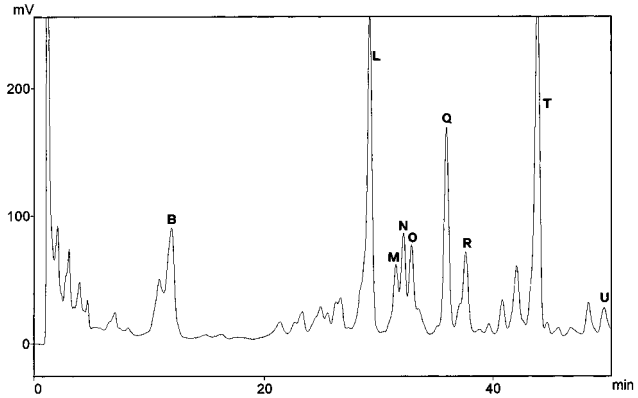

Figure 3. HPLC phenolic profile of Tunisian propolis. Chromatogram was recorded at 290 nm. HPLC conditions are given under Materials and Methods. For compound identification, see Table 2.

In Figure 3, the phenolic profile of extracts from Tunisian propolis is shown. This differs from the European propolis in the presence of compound **T**. On the other hand, Spanish propolis contains the characteristic compounds of poplar bud exudates, namely pinobanksin, pinocembrin, pinobanksin 3-acetate, chrysin, galangin, galangin 3-methyl ether, pinocembrin 7-methyl ether, and tectochrysin, which are characteristic of most temperate areas including Europe, North America, and Asia (Tomás-Barberán et al., 1993a). Compound **T** was then isolated from Tunisian propolis and a second minor compound (**T′**), which eluted with the same retention time as **T** under the conditions used for honey flavonoids analyses, was also isolated.

Identification of the Characteristic Flavonoids of Tunisian Propolis. For compound **T**, the UV−vis data in methanol and after the addition of the classical alkaline and metal reagents (Mabry et al., 1970) readily showed that the natural compound had a free hydroxyl

at the 5-position and that the hydroxyls at 3, 7, and 4′ were blocked. The molecular ion in EIMS indicated a molecular weight of 374, consistent with a dihydroxytetramethoxyflavone. The ^{1}H NMR spectrum clearly demonstrated that the substitution in the A ring of the flavonoid is a 5-hydroxy-7-methoxy, since there are two doublets at 6.38 and 6.75 ppm for the protons at the 6- and 8-positions, respectively, these being 6.20 and 6.38 ppm, respectively, for 5,7-dihydroxyflavonoids (Markham and Geiger, 1994). The signals for protons in ring B show two doublets at 7.20 and 7.28 ppm, which should be assigned to those protons at positions 2′ and 6′. This means that ring B is not symmetrical and that the hydroxyl at 3′ should be free and those at 4′and 5′ should be blocked. Four methoxyl signals were observed in the ^{1}H NMR spectrum, confirming the data obtained in the EIMS. The structure of compound **T** is therefore 5,3′-dihydroxy-3,7,4′,5′-tetramethoxyflavone. This compound has been previously reported from bud exudates of *Betula nigra*, in the leaf exudates of *Cistus* spp., and in the leaf exudates of *Geranium* (Wollenweber, 1994).

The UV spectrum of compound **T′** in methanol and after addition of the classical shift reagents showed that this had free hydroxyls at positions 5 and 4′ and a possible substitution at 3, 7, and 3′. The EIMS spectrum is consistent with a dihydroxytrimethoxyflavone. The retro-Diels−Alder fragments obtained in the EIMS spectrum for the A and B rings clearly indicate that the A ring is monohydroxylated and monomethoxylated and that ring B also has one hydroxyl and one methoxyl (Mabry and Markham, 1975). The ^{1}H NMR spectrum confirms that the substitution of the A ring is 5-hydroxy-7-methoxy- (same signals as compound **T**) and that ring B has a 3′,4′-dioxygenated substitution (H-5′ doublet, 6.9 ppm; H-2′ doublet, 7.6; and H-6′ double doublet). Therefore, the structure of compound **T′** is consistent with 5,4-dihydroxy-3,7,4′-trimethoxyflavone (pachypodol). This has been previously reported from *Cistus* leaf resin and *Geranium* leaf exudate (Wollenweber, 1994).

DISCUSSION

The phenolic contents observed in most of the Tunisian honey samples analyzed were considerably smaller than those previously reported in Spanish honey samples from different floral origins (some of them of the same floral origin as the samples from Tunisia). For instance, in Spanish rosemary honey, the total phenolic content ranged between 700 and 2000 μg/100 g (Gil et al., 1995), and multifloral honey samples from La Alcarria (Spain) had contents between 500 and 2000 μg/100 g (Ferreres et al., 1992).

The Tunisian honeys analyzed showed very high variability in their phenolic constituents. Ellagic acid (**A**), a dimer of gallic acid produced from ellagitannins, had only been detected to date in Portuguese heather honey samples (Ferreres et al., 1996a) and in French *Calluna* honey (Soler et al., 1995), and it is detected here in 5 (eucalyptus 1, 2, and 3; thyme 2; and the only multifloral honey analyzed) of 13 samples analyzed, although its content is smaller than those detected in heather honey samples. It is also interesting to mention that the caffeic acid esters phenylethyl caffeate (**M**) and dimethylallyl caffeate (**O**), which are characteristic compounds of propolis from temperate areas, as well as of poplar bud exudates and of some honeys produced in these regions, were also detected, although in very different amounts, in some Tunisian honey samples.

At least three unidentified flavonols were also detected in significant amounts in the two *Eucalyptus* samples, suggesting that these compounds could be related to the floral origin of this honey sample.

These results confirm the possible use of flavonoids as floral origin markers of honey, along with the previously reported data on other honey samples from Europe with the same floral origin, since *Citrus* honey is the only one containing the flavanone hesperetin and kaempferol is present in rosemary honey (although it is also present in many other species). These results suggest that *Eucalyptus* honey samples should be studied in much more detail, to evaluate if the flavonols detected in samples of the origin analyzed here are a common feature in the *Eucalyptus* honey and if is there any relationship with the constituents of *Eucalyptus* nectar.

The flavonols quercetin, kaempferol, 8-methoxy-kaempferol, and isorhamnetin and the flavones luteolin and apigenin have been previously identified in many honey samples from different geographical and botanical origins (Soler et al., 1995); the flavones (chrysin and galangin and their methyl ethers) and the flavanones (pinocembrin and pinobanksin and derivatives) with an unsubstituted ring B have been reported as the main flavonoid constituents of poplar bud exudates (Wollenweber et al., 1987) and propolis produced in temperate areas (Tomás-Barberán et al., 1993a), from which they diffuse to beeswax and honey in the hives (Tomás-Barberán et al., 1993c). The caffeic acid esters dimethylallyl caffeate and phenylethyl caffeate are also common constituents of poplar bud exudates and propolis (Greenaway et al., 1988) and have also been detected in some European honey samples (Soler et al., 1995). Other phenolics are more related to the floral origin and can be specific and, therefore, used as floral markers. Thus, the flavanone hesperetin is characteristic of Citrus honey (Ferreres et al., 1993), and the gallic acid dimer ellagic acid, which is produced as a degradation product of ellagitannins, seems to be especially abundant in Ericaceae honeys (Ferreres et al., 1994).

The characteristic flavonoid of Tunisian propolis, myricentin 3,7,4′,5′-tetramethyl ether (**T**), was detected in the majority of the analyzed samples, although in rather small amounts. This contrasts with the high amount of this substance in the analyzed propolis. The explanation for the small amount detected in honey could be related to the high lipophilicity of this substance and, therefore, by the preference of this propolis substance to be dissolved in the beeswax rather than in honey, in which the solubility of more hydrophilic flavonoids is more favored. This would be similar to what happens with the lipophilic poplar flavonoid tectochrysin, which is present in relevant amounts in poplar bud exudate and propolis, but its presence in honeys in which poplar flavonoids are the main constituents is not very significant. This compound is, however, concentrated in the beeswax due to its high lipophilicity (Tomás-Barberán et al., 1993c).

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