Flavonoids in Monospecific *Eucalyptus* Honeys from Australia

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The HPLC analyses of Australian unifloral *Eucalyptus* honeys have shown that 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) are present in all samples. These compounds were previously suggested as floral markers of European *Eucalyptus* honeys. The present results confirm the use of flavonoid analysis as an objective method for the botanical origin determination of eucalyptus honey. Honeys from *E. camaldulensis* (river red gum honey) contain tricetin as the main flavonoid marker, whereas in honeys from *E. pilligaensis* (mallee honey), luteolin is the main flavonoid marker, suggesting that species-specific differences can be detected with this analysis. The main difference between the flavonoid profiles of Australian and European *Eucalyptus* honeys is that in the Australian honeys, the propolis-derived flavonoids (pinobanksin (3,5,7-trihydroxyflavanone), pinocembrin (5,7-dihydroxyflavanone), and chrysin (5,7-dihydroxyflavone)) are seldom found and in much smaller amounts.

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

INTRODUCTION

The study of phytochemical constituents of honeys, as markers of their floral origin, has been the aim of different research projects during the past decade. Volatile compounds (Bonaga et al., 1986), aromatic and degraded carotenoid-like substances (Tan et al., 1989, 1990; Wilkins et al., 1993), degradation products of phenyl alanine (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., 1993, 1994a, 1994b; Sabatier et al., 1992) have been found in honey and have been related to the floral origin. As part of our research to find floral markers for the objective determination of the botanical origin of honeys, we have recently reported the occurrence of the flavonoids myricetin, tricetin, and luteolin as markers for the European *Eucalyptus* honey (Martos et al., 2000). European *Eucalyptus* honeys are produced from *Eucalyptus camaldulensis* and *E. globulus*. In the market this honey type is labeled as eucalyptus honey, without any specific mention of the plant species from which the samples were produced. In Australia, however, honeys from different *Eucalyptus* species are produced and can be found in the market. Thus, honeys from *E. melliodora* (yellow box honey), *E. camaldulensis* (river red gum honey), and *E. pilligaensis* (mallee honey), as well as many others, are available. The occurrence of volatile norisoprenoids, monoterpenes, and benzene derivatives in monospecific *Eucalyptus* Australian honeys has been previously reported, and differences between the composition of blue gum (*E. leucoxylon*) and yellow box (*E. melliodora*) honeys were detected (D'Arcy et al., 1997).

The aim of the present work was to analyze the flavonoid content of monofloral *Eucalyptus* honeys from Australia and to determine if the markers found in European *Eucalyptus* honey samples are also present in the Australian samples. This would therefore confirm their utility as floral markers that could be used in the objective determination of the floral origin of eucalyptus honeys. The presence of species-specific markers was also explored.

MATERIALS AND METHODS

Honey Samples. Seven samples of mallee (*E. pilligaensis*) honey, two samples of yellow box (*E. melliodora*) honey, and six samples of river red gum (*E. camaldulensis*) honey were the important commercial floral honeys collected for this study. During the 1995−96 flowering season, individual Australian apiarists supplied seven mallee honey samples sourced from different geographical areas of north New South Wales (N NSW) and south-east Queensland (SE QLD). During the 1997−98 flowering season, six river red gum honey samples were sourced from different areas in Victoria and South Australia (SA). Two yellow box honey samples were collected from SE QLD and north New South Wales, Australia during the 1995−96 flowering season. All the samples were stored in a freezer at temperatures of −18 to −24 °C. Details of the honey samples used in this study are given in Table 1.

Sample Extraction (Column Chromatography). Extraction was carried out as described previously (Martos et al., 2000). Briefly, honey samples (100 g each) were mixed with five parts of water (0.05M HCl) until completely fluid, and the fluid samples were 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). The column was washed with acid water (0.05M HCl, 100 mL) and subsequently rinsed with distilled water (ca 300 mL). The whole phenolic fraction was

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Table 1. Analyzed Unifloral Eucalyptus Honey Samples from Australia

sample code	common name	botanical origin	date	origin
M847(K7796)	River red gum	*E. camaldulensis*	1998	Clare, SA
M848(K8068)	River red gum	*E. camaldulensis*	1998	Barossa Valley, SA
M849(K7727)	River red gum	*E. camaldulensis*	1998	Geelong, Victoria
M850(K7617)	River red gum	*E. camaldulensis*	1998	Mildura, Victoria
M851(K8451)	River red gum	*E. camaldulensis*	1998	Bannockburn, Victoria
M852(K7631)	River red gum	*E. camaldulensis*	1998	Maryborough, Victoria
Q1837	Mallee	*E. pilligaensis*	1996	Pilliga Scrub, N NSW
Q2023	Mallee	*E. pilligaensis*	1996	Inglewood, SE QLD
Q2139	Mallee	*E. pilligaensis*	1996	Pilliga Scrub, N NSW
Q2183	Mallee	*E. pilligaensis*	1996	Pilliga Scrub, N NSW
Q2236	Mallee	*E. pilligaensis*	1996	Goondiwindi & Tara, SE QLD
Q2257	Mallee	*E. pilligaensis*	1996	Pilliga Scrub, N NSW
Q2436	Mallee	*E. pilligaensis*	1996	Inglewood, SE QLD
Y/box 3039	Yellow box	*E. melliodora*	95/96	SE QLD & N NSW
Y/box Z7643	Yellow box	*E. melliodora*	1995	Stanthorpe, SE QLD

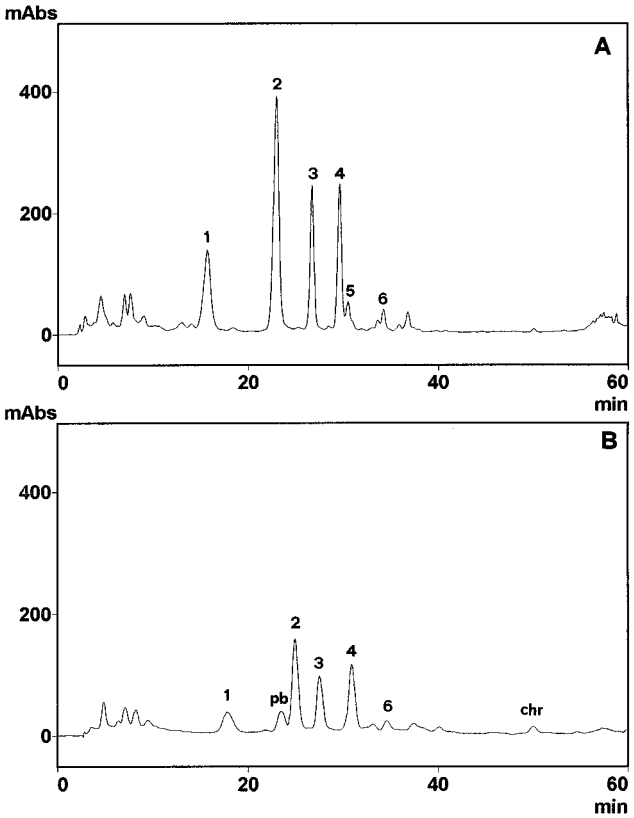

Figure 1. HPLC chromatograms of flavonoids from unifloral eucalyptus honeys (340 nm). (**A**) *Eucalyptus camaldulensis* (river red gum honey, Australia); (**B**) *E. camaldulensis* (eucalyptus honey, Spain). Flavonoids are (**1**) myricetin; (**2**) tricetin; (**3**) quercetin; (**4**) luteolin; (**5**) quercetin 3-methyl ether; (**6**) kaempferol; (**pb**) pinobanksin; and (**chr**) chrysin.

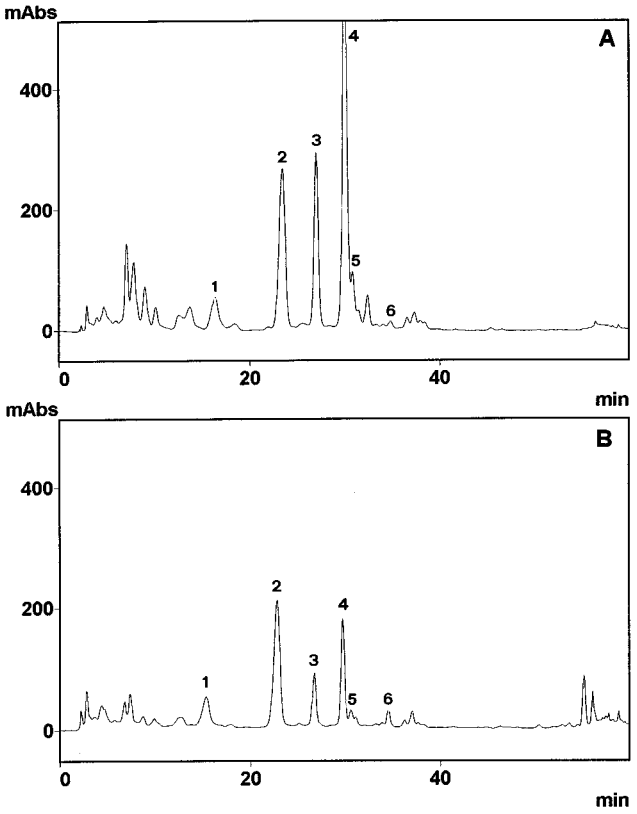

Figure 2. HPLC chromatograms of flavonoids from unifloral eucalyptus honeys (340 nm). (**A**) *Eucalyptus pilligaensis* (mallee honey, Australia); (**B**) *E. melliodora* (yellow box honey, Australia). Flavonoids are (**1**) myricetin; (**2**) tricetin; (**3**) quercetin; (**4**) luteolin; (**5**) quercetin 3-methyl ether; and (**6**) kaempferol.

then eluted with methanol (ca 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). The ether extracts were combined, concentrated under reduced pressure, and redissolved in 0.5 mL of methanol for HPLC analysis.

HPLC Analysis. HPLC analyses were carried out by using a Merck−Hitachi Liquid Chromatograph L-6200. Separations were carried out on a column (LichroCART RP-18, Merck; 12.5 × 0.4 cm, 5 μm particle size) using water−formic acid (19:1, v:v) (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min. The following gradient was used: 30% methanol flowed through the column isocratically with solvent A for 15 min, and then increased to 40% methanol at 20 min, 45% methanol at 30 min, 60% methanol at 50 min, and 80% methanol at 52 min, and, finally, flowed isocratically again with 80% methanol until 60 min. The flavonoids were detected with a photodiode-array detector (Merck-Hitachi L-3000) to obtain the UV spectra of the various phenolic compounds, and the chromatograms were monitored at 340 nm. Flavonoids were identified and quantified as reported previously (Martos et al., 2000).

RESULTS AND DISCUSSION

The HPLC analyses of the available monofloral *Eucalyptus* honeys from Australia showed that all the samples had a common flavonoid profile that was similar with that previously reported for European *Eucalyptus* honeys. The HPLC chromatograms recorded at 340 nm showed the characteristic compounds myricetin (**1**), tricetin (**2**), quercetin (**3**), luteolin (**4**), and kaempferol (**6**). In addition, a minor flavonoid, that had not been detected in any of the European *Eucalyptus*

Table 2. Flavonoid Content of Unifloral *Eucalyptus* Honeys

		Flavonoids[a,b] (μg/100 Honey)																		
botanical origin	sample	myricetin		tricetin		quercetin		luteolin		quercetin 3-Me		kaempferol		pinobanksin		pinocembrin		chrysin		total
E. camaldulensis	M847	277.7	(13.1)	913.4	(43.2)	433.9	(20.5)	390.3	(18.4)	86.5	(4.1)	15.0	(0.7)							2116.8
	M848	151.7	(16.9)	383.0	(42.6)	162.6	(18.1)	167.6	(18.6)	20.1	(2.2)	14.1	(1.6)							899.1
	M849	289.0	(13.0)	1085.9	(49.0)	334.7	(15.1)	412.1	(18.6)	77.3	(3.5)	19.3	(0.9)							2218.3
	M850	153.1	(7.2)	855.4	(40.2)	427.2	(20.1)	474.9	(22.3)	104.1	(4.9)	25.7	(1.2)	65.5	(3.1)			22.8	(1.1)	2128.7
	M851	268.4	(16.3)	648.2	(39.3)	292.1	(17.7)	332.9	(20.2)	31.3	(1.9)			24.9	(1.5)	9.5	(0.6)	6.9	(0.4)	1650.3
	M852	232.4	(14.3)	733.6	(45.1)	309.5	(19.0)	214.6	(13.2)	35.1	(2.2)	56.7	(3.5)	28.6	(1.8)	8.9	(0.5)	8.5	(0.5)	1627.9
	mean	228.7	(13.5)	769.9	(43.2)	326.7	(18.4)	332.1	(18.6)	59.1	(3.1)	26.2	(1.6)	39.7	(2.1)	9.2	(0.6)	12.7	(0.7)	
	s.d.	62.1	(3.5)	242.3	(3.5)	100.1	(2.0)	119.2	(3.0)	34.6	(1.2)	17.7	(1.1)	22.4	(0.8)	0.4	(0.0)	8.8	(0.4)	
E. pilligaensis	Q1837	97.6	(5.8)	352.8	(20.9)	400.2	(23.8)	744.7	(44.2)	t		47.3	(2.8)	41.9	(2.5)					1684.6
	Q2023	23.9	(1.8)	178.9	(13.8)	399.8	(30.8)	611.7	(47.2)	64.4	(5.0)	9.4	(0.7)					9.1	(0.7)	1297.3
	Q2139	t		30.9	(3.5)	303.3	(34.7)	435.4	(49.8)	46.3	(5.3)	34.3	(3.9)	12.9	(1.5)	10.6	(1.2)			873.8
	Q2183	44.6	(4.0)	201.1	(18.0)	279.3	(25.0)	333.1	(29.8)	37.7	(3.4)	169.3	(15.1)	36.4	(3.3)	17.8	(1.6)			1119.3
	Q2236	88.8	(5.0)	418.1	(23.6)	306.3	(17.3)	822.4	(46.4)	85.5	(4.8)	50.5	(2.9)							1771.5
	Q2257	38.2	(2.6)	135.1	(9.2)	410.9	(27.8)	814.9	(55.2)			39.9	(2.7)	36.4	(2.5)					1475.5
	Q2436	57.3	(3.2)	97.1	(5.5)	519.9	(29.4)	888.9	(50.3)	101.5	(5.7)	101.5	(5.7)							1766.2
	mean	58.4	(3.7)	202.0	(13.5)	374.2	(27.0)	664.4	(46.1)	67.1	(4.8)	64.6	(4.8)	31.9	(2.4)	14.2	(1.4)	4.6	(0.4)	
	s.d.	29.2	(1.5)	138.3	(7.7)	84.2	(5.6)	211.9	(8.0)	26.6	(0.9)	53.8	(4.8)	12.9	(0.7)	5.1	(0.3)	6.4	(0.5)	
E. melliodora	YB3039	t		117.2	(34.6)	124.5	(36.8)	96.8	(28.6)											338.4
	YB7643	126.2	(12.9)	463.8	(47.6)	114.4	(23.3)	227.5	(23.3)	14.4	(1.5)	28.3	(2.9)							974.7
	mean	63.1	(6.5)	290.5	(41.1)	119.5	(24.3)	162.2	(26.0)	7.2	(0.7)	14.2	(1.5)							
	s.d.	89.2	(9.2)	245.1	(9.2)	7.1	(17.7)	92.4	(3.7)	10.2	(1.0)	20.0	(2.1)							
E. camaldulensis	Europe mean	189.3	(8.0)	682.6	(28.8)	308.0	(13.0)	254.9	(10.8)			70.7	(3.0)	555.8	(23.5)	314.0	(13.2)	93.9	(4.0)	2370

[a] 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; quercetin 3-methyl ether, 5,7,3',4'-tetrahydroxy-3-methoxyflavone; kaempferol, 3,5,7,4'-tetrahydroxyflavone; pinobanksin, 3,5,7-trihydroxyflavanone; pinocembrin, 5,7-dihydroxyflavanone; chrysin, 5,7-dihydroxyflavone. [b] Values in parentheses are % of each individual flavonoid in samples.

samples analyzed previously, was detected in Australian samples and identified as quercetin 3-methyl ether (**5**). This compound was identified by its UV spectrum and chromatographic comparisons with an authentic standard previously isolated and identified from different honey samples (Ferreres et al., 1991). The characteristic HPLC chromatograms of unifloral *Eucalyptus* honeys from Australia are shown in Figures 1 and 2. It is clear that all the analyzed samples (river red gum, mallee, and yellow box honeys) have a common, and genus-specific, flavonoid profile. This profile is similar to that of the European *Eucalyptus* honeys previously reported. In Figure 1 the characteristic HPLC chromatograms of river red gum honey (*E. camaldulensis* from Australia) (**A**) and European *Eucalyptus* honey (*E. camaldulensis*) (**B**) are shown. It is clear from these chromatograms that in honeys produced from *E. camaldulensis* tricetin (**2**) is the main flavonoid and the relative percentage of the other flavonoids is very constant (Table 2). In Figure 2, the characteristic chromatograms of mallee honey (*E. pilligaensis*) (**A**) and yellow box honey (*E. melliodora*) (**B**) are shown. In the case of mallee honey luteolin (**4**) is the main flavonoid detected in the chromatograms, whereas yellow box honey has a flavonoid profile similar to that of river red gum honey. These characteristics were generally found in all the analyzed samples from the same floral origin (Table 2).

The content of the individual flavonoids in the analyzed honey samples is shown in Table 2, where the data are compared with the mean flavonoid content of the European samples (*E. camaldulensis*) analyzed previously (Martos et al., 2000). These quantitative data confirm the qualitative differences observed when the HPLC profiles were analyzed. In this table, the content and relative percentage of each individual flavonoid are shown. In Australian *E. camaldulensis* honeys the main flavonoid in the chromatograms is tricetin (**2**), which represents 43.2% of the total flavonoids, with quercetin (**3**) and luteolin (**4**) as secondary flavonoids (18.4 and 18.6%, respectively), and myricetin in smaller percentage (13.5%). These values are in good agreement with the values found for European *E. camaldulensis* honeys (Martos et al., 2000), in which tricetin is the main flavonoid, and quercetin and luteolin are in a secondary position. The mean content of the individual flavonoid markers in European and Australian *E. camaldulensis* honeys is very similar (Table 2) and confirms their use as floral markers. In *E. pilligaensis* honey the main flavonoid is luteolin (46.1%), with quercetin and tricetin in smaller amounts (27.0 and 13.5%, respectively), and the myricetin content being much smaller (3.7%). The two samples available of *E. melliodora* honey had marked differences in their total flavonoid contents, as one of the samples had only 338.8 μg/100 g honey and the other had 974.7 (which is within the range of the eucalyptus honeys from other floral origins). The mean flavonoid profile was similar, in percentage of the individual flavonoids, to that of *E. camaldulensis*. A larger number of *E. melliodora* samples should be studied to confirm its similarity with *E. camaldulensis* honeys.

The main difference observed between the Australian and the European honeys , was the content of propolis-derived flavonoids (pinobanksin (**7**), pinocembrin (**8**), and chrysin (**9**)). These compounds were present in significant amounts in most European honey samples with an average close to 1000 μg/100 g of honey (Martos et al., 2000), but these compounds were present only in very small amounts, or were not detected, in Australian samples (Table 2). This difference could be explained by the origin of propolis flavonoids. In temperate regions of the Northern Hemisphere, poplars (the main source for propolis in this region) are the preferred source selected by bees for propolis production. In temperate regions of the Southern Hemisphere, where poplars are not native, the bees seek different plant sources to produce propolis, and the characteristic poplar phenolics are seldom found. Poplars are, however, imported in these regions, and sometimes the poplar constituents are detected in honeys produced in the Southern Hemisphere (Tomás-Barberán et al., 1993). This can be the reason the analyzed *Eucalyptus* honeys produced in Australia contain only small amounts of these compounds (Table 2).

As a conclusion we can confirm that the flavonoids previously reported as floral markers of eucalyptus honey, are also valid as markers for monofloral eucalyptus honeys from different geographic regions, and from different *Eucalyptus* species.

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