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Quantitative High-Performance Liquid Chromatography Analyses of Flavonoids in Australian *Eucalyptus* Honeys

LIHU YAO,[†,§] YUEMING JIANG,[†] BRUCE D'ARCY,*[,§] RIANTONG SINGANUSONG,[#] NIVEDITA DATTA,[§] NOLA CAFFIN,[§] AND KATHERINE RAYMONT[⊥]

South China Institute of Botany, The Chinese Academy of Sciences, Guangzhou ReYiJu 510650, People's Republic of China; School of Land and Food Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia; Department of Agro-Industry, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Muang, Phitsanulok 65000, Thailand; and School of Agriculture and Horticulture, The University of Queensland, Gatton, Queensland 4343, Australia

Flavonoids of nine Australian monofloral *Eucalyptus* honeys have been analyzed and related to their botanical origins. The mean content of total flavonoids varied from 1.90 mg/100 g of honey for stringybark (*E. globoidia*) honey to 8.15 mg/100 g of honey for narrow-leaved ironbark (*E. crebra*) honey, suggesting that species-specific differences occur quantitatively among these *Eucalyptus* honeys. All of the honey samples analyzed in this study have a common flavonoid profile comprising tricetin (5,7,3′,4′,5′-pentahydroxyflavone), quercetin (3,5,7,3′,4′-pentahydroxyflavone), and luteolin (5,7,3′,4′-tetrahydroxyflavone), which, together with myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) and kaempferol (3,5,7,4′-tetrahydroxyflavone), were previously suggested as floral markers for European *Eucalyptus* honeys. Thus, flavonoid analysis could be used as an objective method for the authentication of the botanical origin of *Eucalyptus* honeys. Moreover, species-specific differences can also be found in the composition of honey flavonoid profiles. Among these honeys, bloodwood (*E. intermedia*) honey contains myricetin and tricetin as the main flavonoid compounds, whereas there is no myricetin detected in yapunyah (*E. ochrophloia*), narrow-leaved ironbark (*E. crebra*), and black box (*E. largiflorens*) honeys. Instead, these types of *Eucalyptus* honeys may contain tricetin, quercetin, and/or luteolin as their main flavonoid compounds. Compared to honeys from other geographical origins, the absence or minor presence of propolis-derived flavonoids such as pinobanksin, pinocembrin, and chrysin in Australian honeys is significant. In conclusion, these results demonstrate that a common flavonoid profile exists for all of the *Eucalyptus* honeys, regardless of their geographical origins; the individual species-specific floral types of *Eucalyptus* honey so common in Australia could be possibly differentiated by their flavonoid profile differences, either qualitatively or quantitatively or both.

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

INTRODUCTION

Eucalypts occur naturally only in Australia. The trees found in other places, such as red gums in the Mediterranean region or blue gums in California, are the result of introductions from Australia (*1*). As such, the eucalypts are the most conspicuous element of the Australian vegetation, constituting ∼95% of the trees, and dominate the woodlands, with the species and varieties consisting of 550−600 more or less distinct forms, plus many hybrids (*1*). Therefore, the phytochemical profiles, such as profiles of flavonoids and phenolic acids, in the *Eucalyptus* floral honeys of Australia could be distinctive from the other types (botanical origins) of honeys. They may also vary in the *Eucalyptus* honeys originating from different *Eucalyptus* floral varieties (*2*, *3*) and/or from different geographical origins (*4*−*6*).

In the assessment of honey flavors and organoleptic quality, hydrocarbons (*7*), phenylalanine decomposition products (*8*), aromatic aldehydes (*9*), aromatic carboxylic acids and their esters (*10*), and degraded carotenoids have been analyzed and correlated to the flavor quality of floral honeys. For example, the volatile compounds of unifloral honeys produced in New Zealand (*11*, *12*) and Australia (*13*, *14*) were found to be characteristic only of the corresponding honeys of specific botanical origins.

With HPLC becoming more available in many laboratories, nonvolatile compounds such as flavonoids (*15*−*19*) and other

* Author to whom correspondence should be addressed (telephone +61 7 3346 9190; fax +61 7 3365 1177; e-mail B.DArcy@uq.edu.au).
[†] The Chinese Academy of Sciences.
[§] The University of Queensland, Brisbane.
[#] Naresuan University.
[⊥] The University of Queensland, Gatton.

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Table 1. Australian Unifloral *Eucalyptus* Honeys Used in This Experiment

sample code	common name	floral origin	year	origin
T/T LT1054	bloodwood	*E. intermedia*	1999	Kempsey, NSW
T/T LT286	bloodwood	*E. intermedia*	1999	Wauchope, NSW
B/wood A4131	bloodwood	*E. intermedia*	1998	Maryborough, QLD
Yap Z8325	yapunyah	*E. ochrophloia*	1995	Channel Country, QLD
Yap Z8174	yapunyah	*E. ochrophloia*	1995	Channel Country, QLD
Yap A3911	yapunyah	*E. ochrophloia*	1998	Quilpie, QLD
NL/IB A3988	narrow-leaved ironbark	*E. crebra*	1998	Millmerran, QLD
BT/IB A3942	blue top ironbark	*E. nubila*	1998	Western Creek, DLD
G/T A3895	gum top	*E. moluccana*	1998	Barakula, QLD
ST/Bark A4110	stringybark	*E. globoidia*	1997	New England, NSW
Black/B A5629	black box	*E. largiflorens*	1999	Goondiwindi, QLD

phenolic compounds (*2*, *20*−*24*) as well as abscisic acid (*25*−*27*) have been analyzed and used as indicators for the botanical origin and, hence, the quality of honey. In the research of floral markers for the objective determination of the botanical origin of honeys, the flavonoids myricetin, tricetin, luteolin, quercetin, and kaempferol have been suggested as markers for the European *Eucalyptus* honey (*5*); a preliminary study on three Australian *Eucalyptus* honeys, river red gum, mallee box, and yellow box honeys, has further confirmed the above flavonoid profiles for *Eucalyptus* honeys (*6*). However, prior to this study, no other data were available on the flavonoids in many other types of Australian *Eucalyptus* honey. Thus, data on the phytochemical constituents could provide a database for authentication of the botanical origins of Australian *Eucalyptus* honeys.

The present work represents a characterization of the flavonoids of unifloral *Eucalyptus* honeys from Australia and a determination of the potential of these compounds to serve as floral markers for Australian *Eucalyptus* honeys.

MATERIALS AND METHODS

Honey Samples. In Australia, floral sources of honeys are identified using a standard method for honey sourcing. That is, the individual apiarists and the honey packers who supplied the honey samples use the following procedures to identify the floral sources of Australian honey: sensory properties such as the aroma, taste, and color of the honey; the location of the beehives; seasons of the honey collected; and the availability of the floral sources nearby. This method is considered to be accurate and has been extensively used and accepted by the Australian honey industry (*28*). For this study, individual Australian apiarists supplied the *Eucalyptus* honey samples, sourced from the main honey production regions New South Wales (NSW) and Queensland (QLD), during the corresponding flowering season. The geographical locations, botanical origins, and sourcing dates of these honeys are detailed in **Table 1**. All of the honey samples were stored at a temperature of −18 to −24 °C prior to analysis.

Sample Extraction (Column Chromatography). Extraction was carried out as described previously (*5*, *6*, *29*, *30*). Namely, liquefied honey samples (100 g) were thoroughly mixed with five parts (500 mL) of distilled water, and adjusted to pH 2 with concentrated HCl until completely fluid. The fluid samples were then filtered through cotton wool to remove the solid particles. The filtrate was mixed with 150 g of Amberlite XAD-2 (Supelco, Bellefonte, PA; pore size = 9 nm, particle size = 0.3−1.2 mm) and stirred in a magnetic stirrer for 10 min, which was considered enough to absorb honey flavonoids with a recovery rate of >80% (*29*, *30*). The Amberlite particles were then packed in a glass column (42 × 3.2 cm), and the column was washed with acidified water (pH 2 with HCl, 250 mL) and subsequently rinsed with distilled water (300 mL) to remove all sugars and other polar constituents of honey. The flavonoids remain absorbed on the column (*15*) and can be eluted with methanol. The whole flavonoid fraction was then eluted with methanol (400 mL). This extract was concentrated to dryness under reduced pressure in a rotary evaporator at 40 °C. The residue was redissolved in distilled water (5 mL) and extracted with diethyl ether (5 mL × 3). The ether extracts were combined, and the diethyl ether was removed by flushing with nitrogen. The dried residue was then redissolved in 1 mL of methanol (HPLC grade) and filtered through a 0.45 μm membrane filter, ready for HPLC analysis.

HPLC Analysis. Analyses of the extracts from unifloral Australian honeys were carried out using a Shimadzu Class-VP HPLC system with a computer-controlled system containing upgraded Class-VP 5.03 software. Separations were carried out on a reversed phase column LiChroCART RP-18 (Merck, Darmstadt, Germany; 12.5 cm × 0.4 cm, particle size = 5 μm), using a mobile phase of water/formic acid (19:1, v/v) (solvent A) and methanol (solvent B) at a constant solvent flow rate of 1 mL/min. The following gradient was used, according to the method of Martos et al. (*29*) and Yao et al. (*24*): 30% methanol (B) flowed through the column isocratically with solvent A for 15 min and then was increased to 40% methanol at 20 min, 45% methanol at 30 min, 60% methanol at 50 min, 80% methanol at 52 min, and 90% methanol at 60 min. Finally, isocratic elution with 90% methanol was done until 65 min. The honey extracts were injected with a SIL-10A XL autoinjector, and the flavonoids were detected using a multichannel photodiode array detector (SPD-M10A VP) to obtain the UV spectra of flavonoids. In addition, the chromatograms were monitored at 290 and 340 nm, because the majority of the honey flavonoids show their UV absorption maxima around these two wavelengths (*29*). The flavonoids were identified and quantified according to the method reported previously (*5*, *6*, *24*, *29*). When the authentic compounds for some honey flavonoids were unavailable, the stored UV spectra extracted using the same HPLC method as for the honey analysis and their corresponding retention times were used for identification. In this study, the flavonoids were quantified using the external standard method and authentic compounds (*5*, *6*, *24*, *29*). The flavanones (such as pinobanksin) were quantified as pinocembrin at 290 nm; the flavones with an unsubstituted ring B (such as chrysin) were quantified as chrysin at 340 nm; the flavone kaempferol and its methyl ether were quantified as kaempferol at 340 nm; and the remaining flavones were quantified as quercetin at 340 nm.

The phenolic acids that elute at the beginning of each chromatogram were also analyzed but will be reported elsewhere.

RESULTS AND DISCUSSION

Flavonoids in Bloodwood (*E. intermedia*) Honey. The HPLC chromatogram for bloodwood honey recorded at 340 nm (**Figure 1A**) shows that myricetin (35.6%) and tricetin (24.6%) together represent 60.2% of total flavonoids, with luteolin and quercetin as the secondary flavonoids (**Table 2**). Such relative levels of these four compounds as part of a flavonoid profile may be characteristic for Australian bloodwood honey; there have been no similar flavonoid profiles reported in this study or other studies (*5*, *6*). The main difference between bloodwood honey and other *Eucalyptus* honeys analyzed so far (*5*, *6*, *26*) is that in the former, myricetin is a dominant flavonoid, whereas in the latter, myricetin is only the second or third or a minor component or is not detected. Thus, high levels (>1−5 mg/100 g) of myricetin may be characteristic of bloodwood honey.

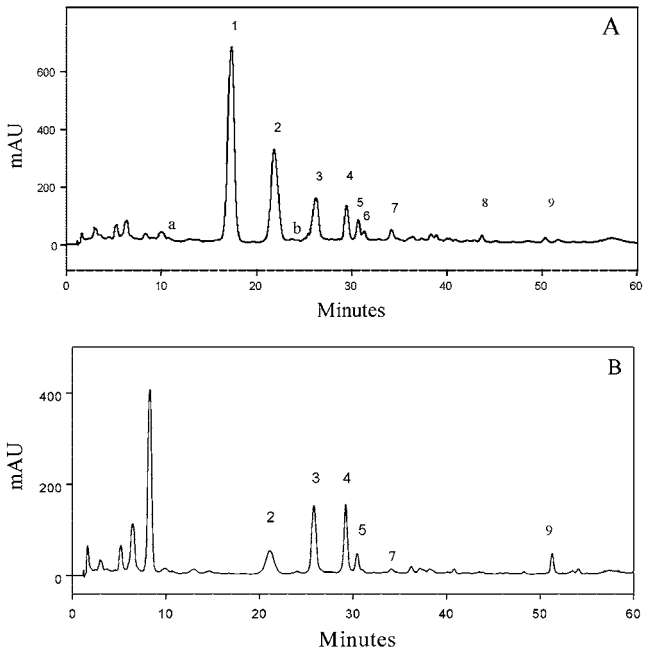

Figure 1. HPLC chromatograms of flavonoids in Australian *Eucalyptus* honeys at 340 nm: (A) bloodwood (*E. intermedia*) honey; (B) yapunyah (*E. ochrophloia*) honey. Flavonoids peaks: (1) myricetin, (2) tricetin, (3) quercetin, (4) luteolin, (5) quercetin 3-methyl ether, (6) kaempferol, (7) kaempferol 8-methyl ether, (8) pinocembrin, (9) chrysin; (a) unknown flavonoid F01; (b) unknown flavonoid F02 (peaks of phenolic acids are not numbered).

There are two minor compounds that could not be finally identified from bloodwood honey because no authentic compounds were available for comparison, as described previously (*24*). These compounds were temporarily identified as F01 and F02. In Australian bloodwood honey, the content of F01 represents only 2.2% of total flavonoids (**Table 2**), whereas F02 was detected in only one of the three bloodwood honeys.

Flavonoids in Yapunyah (*E. ochrophloia*) Honey. The content of total flavonoids in yapunyah honey is 2.23 mg/100 g of honey (**Table 2**), much smaller than that of bloodwood honey. The main flavonoids in yapunyah honey are tricetin (27.4%), luteolin (27.3%), and quercetin (20.6%), together representing 75.3% of total flavonoids (**Figure 1B**; **Table 2**). Unlike bloodwood honey and some other *Eucalyptus* honeys (*6*), yapunyah honey does not contain myricetin. However, the absence of myricetin is not a characteristic of yapunyah honey alone, because it was found in this study that narrow-leaved ironbark and black box honeys also do not contain myricetin. These results showed that Australian yapunyah honey has a common flavonoid profile comprising tricetin, quercetin, and luteolin, which was proposed as the floral marker for European *Eucalyptus* honeys (*5*) and for Australian river red gum (*E. camaldulensis*) and mallee box (*E. pilligaensis*) honeys (*6*). In addition, the flavanones F01 and pinocembrin are present in a higher percentage of the total flavonoid content in yapunyah honey than in most of the other Australian *Eucalyptus* honeys (**Table 2**), although they are present in much smaller amounts than in European *Eucalyptus* honeys (*5*, *6*).

Flavonoids in Narrow-Leaved Ironbark (*E. crebra*) Honey. The flavonoid profiles of narrow-leaved ironbark honey were found to be similar to those of European *Eucalyptus* honeys reported previously (*5*, *6*), which are dominated by luteolin, tricetin, and quercetin (**Figure 2**). Furthermore, this Australian honey contains an unknown F02, as discussed earlier, which

Table 2. Flavonoid Content of Unifloral Australian *Eucalyptus* Honeys

	content of flavonoids (mg/100 g of honey)[a,b]															
sample	Myr	Tri	F01	Que	Lut	3MQ	Kae	8MK	PC	DMQ	IRM	Chr	F02	PB	F03	total
T/T LT1054	3.57 (45.4)	1.89 (24.1)	0.16 (2.0)	0.85 (18.8)	0.49 (6.3)	0.27 (3.5)	0.16 (2.1)	0.19 (2.4)	0.26 (3.3)							7.83
T/T LT286	2.62 (44.3)	1.43 (24.1)	0.20 (3.3)	0.65 (11.0)	0.34 (5.8)	0.21 (3.5)	0.13 (2.2)		0.07 (1.1)	0.11 (1.8)	0.17 (2.9)					5.91
B/wood A4131	1.74 (16.8)	2.65 (25.6)	0.14 (1.4)	1.25 (12.0)	3.00 (29.0)	0.23 (2.2)	0.13 (1.3)				0.13 (1.2)	0.31 (3.0)	0.77 (7.5)			10.35
mean	2.64 (35.6)	1.99 (24.6)	0.17 (2.2)	0.91 (11.3)	1.28 (13.7)	0.24 (3.1)	0.14 (1.8)	0.10	0.16 (2.2)	0.05	0.15 (2.1)	0.15	0.39			
SD[c]	0.91 (16.2)	0.62 (0.9)	0.03 (1.0)	0.30 (0.7)	1.49 (13.3)	0.03 (0.7)	0.02 (0.5)		0.14 (1.6)		0.03 (1.2)					
Yap Z8325		1.06 (38.9)	0.15 (5.3)	0.36 (13.3)	0.73 (26.6)	0.17 (6.2)	0.08 (2.9)		0.18 (6.8)							2.74
Yap Z8174		0.43 (21.6)	0.15 (7.8)	0.61 (30.9)	0.47 (23.7)	0.19 (9.6)						0.13 (6.5)				1.97
Yap A3911		0.36 (21.5)	0.12 (7.5)	0.29 (17.7)	0.52 (31.5)	0.15 (8.8)	0.05 (2.8)	0.07 (4.2)	0.10 (6.0)							1.66
mean		0.62 (27.4)	0.14 (6.9)	0.42 (20.6)	0.57 (27.3)	0.17 (8.2)	0.06 (2.9)	0.04 (1.6)	0.14 (6.4)			0.06 (2.9)				
SD		0.39 (10.0)	0.02 (1.3)	0.17 (9.1)	0.14 (3.9)	0.02 (1.8)	0.02 (0.1)		0.06 (0.5)							
NL/IB A3988		2.13 (26.2)	0.08 (1.0)	1.30 (16.0)	3.19 (39.2)	0.22 (2.7)	0.11 (1.4)		0.06 (0.7)	0.27 (3.3)			0.78 (9.6)		0.23 (2.8)	8.15
BT/IB A3942	0.38 (11.8)	0.80 (25.0)	0.07 (2.2)	0.48 (15.1)	0.81 (25.2)	0.14 (4.3)	0.07 (2.1)	0.06 (1.9)	0.07 (2.1)				0.33 (10.3)			3.20
G/T A3895	0.11 (2.4)	1.26 (28.0)	0.06 (1.4)	0.84 (18.5)	1.43 (31.7)	0.17 (3.7)	0.08 (1.9)				0.11 (2.4)		0.45 (10.1)			4.52
ST/Bark A4110	0.17 (8.8)	0.55 (28.7)	0.11 (5.6)	0.19 (9.8)	0.29 (15.0)	0.12 (6.4)	0.05 (2.5)	0.04 (2.1)	0.06 (3.0)		0.24 (12.7)	0.10 (5.4)		0.10 (5.0)		1.90
Black/B A5629		0.54 (21.9)	0.08 (3.1)	0.44 (17.6)	0.63 (25.6)	0.19 (7.8)	0.06 (2.5)		0.05 (2.1)		0.40 (16.2)	0.08 (3.3)				2.48

[a] Myr, myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone); Tri, tricetin (5,7,3′,4′,5′-pentahydroxyflavone); Que, quercetin (3,5,7,3′,4′-pentahydroxyflavone); Lut, luteolin (5,7,3′,4′-tetrahydroxyflavone); 3MQ, quercetin 3-methyl ether (5,7,3′,4′-tetrahydroxy-3-methoxyflavone); Kae, kaempferol (3,5,7,4′-tetrahydroxyflavone); 8MK, kaempferol 8-methyl ether (3,5,7,4′-tetrahydroxy-8-methoxyflavone); PC, pinocembrin (5,7-dihydroxyflavanone); DMQ, quercetin 3,3′-dimethyl ether (5,7,4′-trihydroxy-3,3′-dimethoxyflavone); IRM, isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone); Chr, chrysin (5,7-dihydroxyflavone); PB, pinobanksin (3,5,7-trihydroxyflavanone); F01, unknown flavonoid 1; F02, unknown flavonoid 2; F03, unknown flavonoid 3. [b] Values in parentheses are percent of each individual flavonoid in the total flavonoids detected in the samples. [c] Standard deviation.

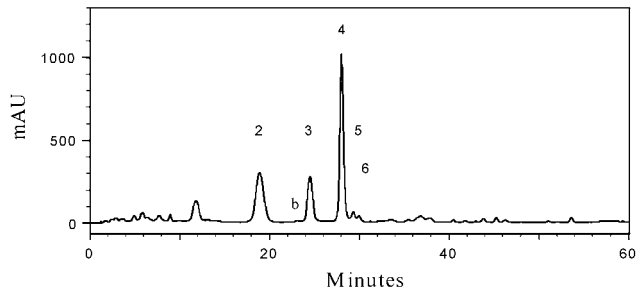

Figure 2. HPLC chromatogram of flavonoids in Australian narrow-leaved ironbark (*E. crebra*) honey at 340 nm. Flavonoid peaks: (2) tricetin, (3) quercetin, (4) luteolin, (5) quercetin 3-methyl ether, (6) kaempferol; (b) unknown flavonoid F02 (peaks of phenolic acids are not numbered).

was not detected in the European *Eucalyptus* honeys. The flavonoid profile of these three compounds for narrow-leaved ironbark honey is quite similar to the flavonoid profiles found in the other floral types of Australian *Eucalyptus* honey, such as bloodwood and yapunyah honeys (**Figures 1** and **2**). In the narrow-leaved ironbark honey, the content of total flavonoids is the second largest amount among the Australian *Eucalyptus* honeys analyzed here, being 8.15 mg/100 g of honey, with the main flavonoids being luteolin, tricetin, and quercetin (**Table 2**). In this floral type of honey, luteolin represents 39.2% of total flavonoids, with tricetin (26.2%) as the second and quercetin (16.0%) as the third flavonoid (**Table 2**). The level of luteolin in the narrow-leaved ironbark honey was high compared to that of the other *Eucalyptus* honeys.

Flavonoids in Blue Top Ironbark (*E. nubila*) Honey. The content of total flavonoids in Australian blue top ironbark honey is 3.20 mg/100 g of honey, with luteolin and tricetin being the main flavonoids (**Table 2**), representing 25.2 and 25.0% of total flavonoids, respectively, and quercetin, myricetin, and F03 being the secondary flavonoids. These results demonstrate that blue top ironbark honey has a common flavonoid profile that has been detected in many other *Eucalyptus* honeys analyzed (*5*, *6*). The main difference between this honey type and other *Eucalyptus* honeys may be its very even distribution of the main flavonoids in the profile (**Table 2**); most other honeys have either myricetin, tricetin, or luteolin dominating their flavonoid profiles.

Flavonoids in Gum Top (*E. moluccana*) Honey. The flavonoid profile of Australian gum top honey is very similar to that of Australian blue top ironbark honey, with the main flavonoids luteolin, tricetin, and quercetin representing 78.2% of total flavonoids (4.52 mg/100 g of honey) in this honey (**Table 2**). The main difference between these two types of honeys may be the difference in the contribution of luteolin and myricetin to their flavonoid profiles. In blue top ironbark honey, these two compounds represent 25.2 and 11.8% of total flavonoids, respectively, whereas in gum top honey they represent 31.7 and only 2.4% of total flavonoids.

Flavonoids in Stringybark (*E. globoidia*) and Black Box (*E. largiflorens*) Honeys. In Australian stringybark honey, the main flavonoid, tricetin, represents 28.7% of total flavonoids, with luteolin and isorhamnetin being the secondary flavonoids (15.0 and 12.7%, respectively) and myricetin being in a smaller amount (8.8%) (**Table 2**). The content of total flavonoids in Australian black box honey is 2.48 mg/100 g of honey, with luteolin, tricetin, quercetin, and isorhamnetin being the main flavonoids (**Table 2**). Luteolin and tricetin together represent 47.5% of total flavonoids in black box honey, whereas quercetin and isorhamnetin are the secondary flavonoids (**Table 2**).

Main Difference in Flavonoid Occurrence between Australian and European *Eucalyptus* Honeys. The main difference between the Australian and European *Eucalyptus* honeys is the content of propolis-derived flavonoids such as pinobanksin, pinocembrin, and chrysin. These compounds are present in significant amounts in most European honey samples (*5*, *6*), including *Eucalyptus* honeys. For example, pinobanksin alone has been found to be 1.61 mg/100 g of honey in European river red gum honey, whereas it reaches 2.02 mg/100 g of honey in heather (*Erica* spp.) honey, 2.31 mg/100 g of honey in acacia (*Robinia pseudoacacia*) honey, and 3.12 mg/100 g of honey in sunflower (*Helianthus annuus*) honey (*26*). However, in Australian *Eucalyptus* honey samples (**Table 2**), these propolis-derived compounds were either present in very small amounts or not detected. This difference could be due to the origin of propolis flavonoids (*30*, *31*). That is, poplars are the main source of propolis in temperate regions of the northern hemisphere and are the preferred source for bees to produce propolis (*30*). In contrast, poplars are not native in Australia. Thus, the bees have to find different plant sources to produce propolis, and hence the characteristic poplar flavonoids are rarely found in the Australian *Eucalyptus* honeys. Furthermore, beekeepers in Australia attempt to maintain high standards of hive construction that reduce the need for bees to seal gaps, thus reducing the production of propolis (*28*). Honeybees in Australia have a different foraging behavior toward more honey production (*28*). In this way, the behavior of honeybees has been modified to collect more nectar for honey production rather than the glues for propolis. Thus, the source of propolis-derived flavonoids is limited in Australian honeys.

Floral Markers for *Eucalyptus* Honeys Studied. The HPLC analyses of Australian unifloral *Eucalyptus* honeys show that all of the samples have a common and genus-specific flavonoid profile. The characteristic flavonoids tricetin, quercetin, luteolin, and quercetin 3-methyl ether were found in all of the honey samples analyzed. This result further confirms previous findings (*5*, *6*) that tricetin, quercetin, and luteolin represent a flavonoid profile that could be used as a marker for *Eucalyptus* honeys. However, myricetin was not found in some of the *Eucalyptus* honeys such as yapunyah honey, narrow-leaved ironbark honey, and black box honey, in contrast to previous findings for other *Eucalyptus* honeys (*5*, *6*), confirming some species-specific distribution of the flavonoids occurs in Australian *Eucalyptus* honeys. However, because there were only limited numbers of Australian honey samples available for this study, a larger number of samples of each floral honey type should be studied to confirm their similarities and differences and to assign individual marker compounds that distinguish honeys sourced from different species of eucalypt trees, so common in Australia.

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