Chapter 33
Flavonoids in Stingless-Bee and Honey-Bee Honeys

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33.1 Introduction

Honey produced in cerumen pots by stingless bees is a tropical ingredient for medicinal preparations since the Mayans (see Ocampo Rosales Chap. 15 in this book), widely relished before Columbus (Schwarz 1948). The Neotropical diversity of stingless bees, some 400 species reported by Camargo and Pedro (2007), is a challenge for any phytochemical investigation considering bee–plant interaction.

The sugar and water acidic matrix of honey has a set of minor components used as quality indicators, such as hydroxymethylfurfural and diastase activity (Bogdanov 1999). All the natural products and minerals of nectar and plant exudates used for honey-making are concentrated in honey as such or transformed by the bees and associated microflora.

Flavonoids are plant secondary metabolites that are associated with different physiological and ecological functions, such as protection of plant epithelial cells from ultraviolet rays, defense against biotic and abiotic stress, plant pigmentation, and signaling for interaction with animals, including bees, microbes, and other plants (Harborne 1982).

Flavonoids from floral nectar, pollen (Tomás-Barberán et al. 1989), and different plant exudates (Tomás-Barberán et al. 1993a) are incorporated into honey by the bees, and the metabolites present in plants can be modified during the honey elaboration process, mainly by the action of bee enzymes, bee microbiota metabolism, and chemical transformations during honey maturation.

Honey flavonoid profiles help to determine botanical (Ferreres et al. 1992, 1993, 1994, 1996b; Soler et al. 1995; Martos et al. 2000) and geographical

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(Tomás-Barberán et al. 1993b) origins of honey. It seems clear that honey contains complex phenolic and flavonoid profiles that could be associated with floral and geographical origin, although it is rather difficult to establish valid floral origin biomarkers, specific for a given plant. The study of potential phenolic fingerprints would be a very appropriate methodology for this purpose (Tomás-Barberán et al. 2001). Changes occur in the flavonoid profile with honey maturation in the bee nest and provide a method for evaluation of the degree of honey ripening (Truchado et al. 2010).

The flavonoid content in *Apis mellifera* honey has been extensively studied (Frankel et al. 1998; Ferreres et al. 1996a; Martos et al. 1997). The content in stingless-bee honey, however, has only been recently reported for samples from Australia (Persano Oddo et al. 2008) and Venezuela (Truchado et al. 2011), although previous qualitative studies exist (Vit et al. 1997; Vit and Tomás-Barberán 1998).

Recent research correlates flavonoid content (measured by a spectrophotometric method) to the antioxidant activity of honey produced by several species of stingless bees (Rodríguez-Malaver et al. 2007, 2009; Persano Oddo et al. 2008; Duarte et al. 2012). In this chapter the flavonoids of stingless-bee honey are reviewed, including new data presented here, from several countries.

33.2 Methods of Extraction and Analysis of Flavonoids in Honey

In the analysis of flavonoids from honey, a major problem is the extraction of these minor compounds from a matrix very rich in polar compounds (sugars). This problem is successfully solved by filtration of the diluted honey in acidified water, through nonionic polymeric resins such as Amberlite XAD (Ferreres et al. 1991). This methodology is combined with a final liquid–liquid extraction in which the flavonoids are extracted from water with dyethyl ether. The extraction renders flavonoid extracts that contain most flavonoid aglycones present in *Apis mellifera* honey—the main flavonoids present. Recent studies reveal that some unifloral honey, e.g., *Robinia pseudoacacia* (Fabaceae, Papilionoideae), contains mainly flavonoid glycosides, considered an uncommon honey trait (Truchado et al. 2008). For its analysis, extraction using solid phase extraction cartridges, in combination with HPLC-MS analyses, is considered very useful. In fact, in a more recent paper, the widespread occurrence of flavonoid glycosides in *A. mellifera* honey from different floral origins is demonstrated (Truchado et al. 2009b) although in most cases, flavonoid aglycones are the main metabolites. For stingless-bee honey, since this type of honey contains glycosides in a higher proportion than aglycones (Vit et al. 1997), the same extraction methodology was applied to a number of samples collected in South America and Australia.

The methodology used was the following. Flavonoid compounds from honey samples (5 g) were isolated with a Sep-Pak solid phase extraction cartridge (reversed phase C18 cartridge). The samples were diluted with ultrapure water and centrifuged at 9,000 × *g* for 10 min. The supernatants were filtered through a cartridge previously activated with methanol (10 mL) followed by water (10 mL). Following this, the phytochemicals that remained adsorbed in the cartridge were eluted with 1 mL methanol. The methanol fractions were filtered through a 0.45 μm membrane filter and stored at −20°C until further analyzed by HPLC-DAD-MS^n/ESI (Truchado et al. 2011).

33.3 Analysis of Honey Flavonoids Using Advanced HPLC-MS Methods

Analysis of honey flavonoid glycosides and aglycones was carried out in an Agilent HPLC 1100 series equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface, controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted to 350°C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full scan mass covered the range from *m*/*z* 100 up to *m*/*z* 2,000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage camping cycles from 0.3 to 2.0 V. Mass spectrometry data were acquired in the negative ionization mode. MSn was carried out in the automatic mode on the more abundant fragment ion in $MS^{(n-1)}$. Chromatographic analyses were carried out on a LiChroCART column (250mm × 4 mm, RP-18, 5 μm particle size, LiChrospher®100 stationary phase, Merck, Darmstadt, Germany) protected with a LiChroCART guard column (4 mm × 4 mm, RP-18, 5 μm particle size, Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: water–formic acid (1%) (A) and methanol (B) (99.9%, HPLC grade; Merck, Darmstadt, Germany), starting with 10% B and using a linear gradient to obtain 30% at 20 min, 60% at 40 min, 70% at 45 min, and 90% at 60 min. The flow rate was 1 mL/min, and the injection volume 20 μL. Spectral data from all peaks were accumulated in the range of 240–600 nm, and chromatograms were recorded at 280, 320, 330, 360, or 520 nm. The phenolic compounds were identified according to their UV spectra, molecular weights, retention times, and their MS–MS fragments, and whenever possible, with commercially available standards.

33.4 Flavonoids Observed in Honey from Combs and Pots

33.4.1 Apis mellifera Comb Honey

This type of honey contains flavonoid aglycones and other lipophylic compounds as the main plant secondary metabolites. Some honey samples of specific floral origin contain metabolites that may be considered biomarkers of the particular plant, as is the case of the flavanone hesperetin for citrus honey (Ferreres et al. 1993) and the alkaloid kinurenic acid for chestnut honey (Truchado et al. 2009a). Other honey samples contain specific compounds that are common to a number of different plant species, as in the case of the flavone tricetin and the flavonol myricetin in eucalyptus honey (Martos et al. 2000) and ellagic acid and abscisic acid in heather honey (Ferreres et al. 1996a).

Some *A. mellifera* honey contains relatively high amounts of flavonoid aglycones from propolis (poplar bud exudates collected by bees) (Fig. 33.1) including the flavones chrysin, galangin and techtochrysin, the flavanones pinocembrin and pinobaknsin and the caffeic acid derivatives dimethyl-allyl-caffeate and phenyl-ethyl-caffeate. Some of these compounds have also been reported in beeswax and in freshly secreted wax scales. It is suggested that bees may ingest propolis to incorporate these flavonoid metabolites in the secreted wax (Tomás-Barberán et al. 1993c).

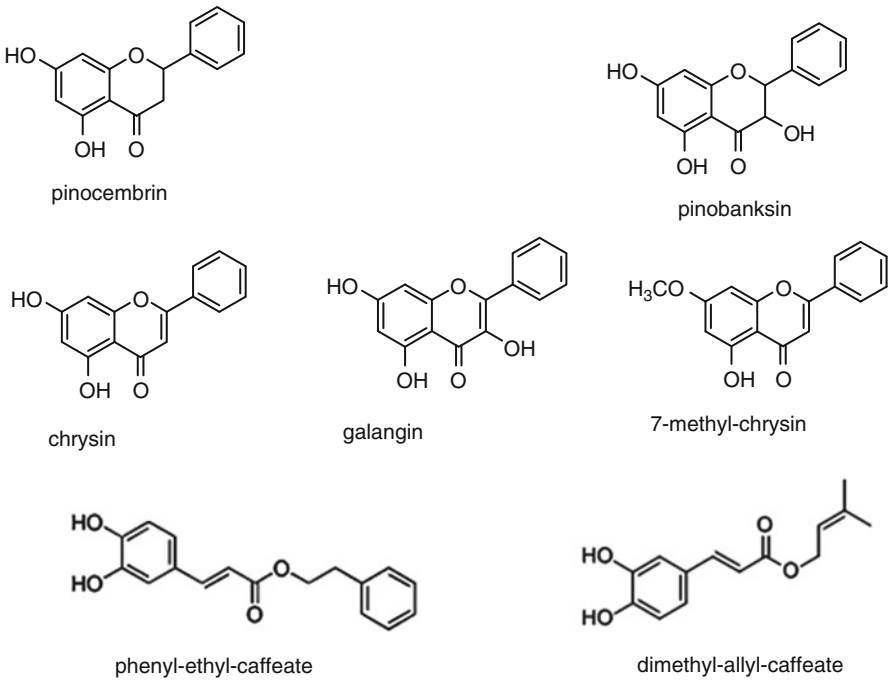

Fig. 33.1 Propolis-derived flavonoids and other phenolic compounds from *Apis mellifera* honey

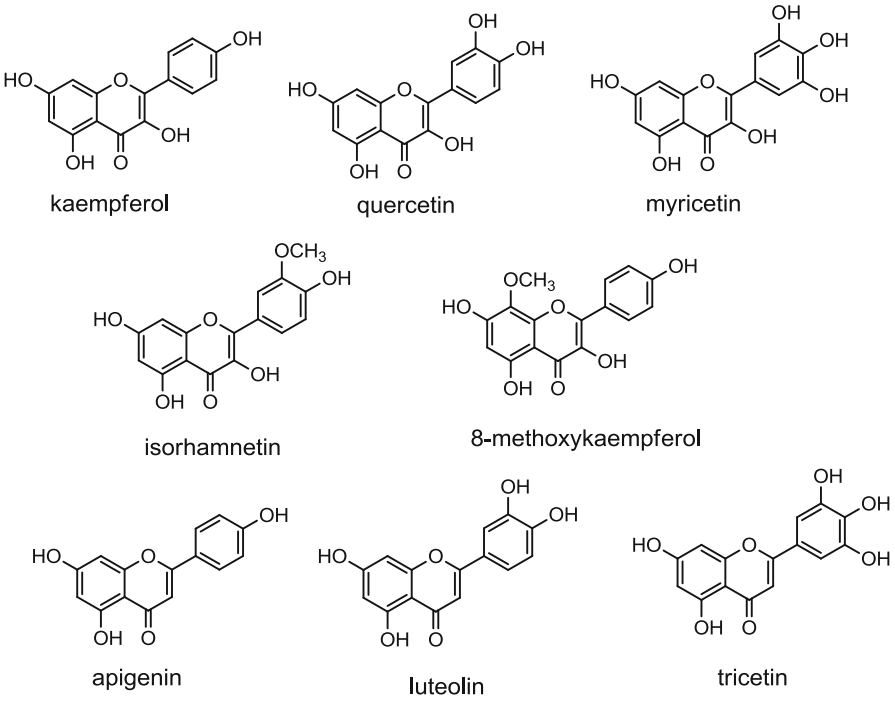

Fig. 33.2 Nectar and pollen derived flavonoid aglycones in honey and pot-honey

In addition, *A. mellifera* honey contains a large number of flavonoid aglycones derived from the naturally occurring flavonol-glycosides present in nectar, and probably pollen, from hydrolysis caused by bee saliva enzymes. These flavonoid aglycones include mainly polyhydroxylated flavones, but also their mono methyl ethers (i.e., isorhamnetin and 8-methoxykaempferol) and flavanones like hesperetin (Fig. 33.2).

A good example to illustrate hydrolytic activity of bee saliva is found in eucalyptus nectar and honey which clearly shows the presence of flavonol glucosides and diglucosides in nectar, and the transformation of these polar metabolites into the corresponding aglycones in mature honey (Fig. 33.3) (Truchado et al. 2009b).

When flavonoid rhamnosides or rhamnosyl-glucosides are present in nectar, those glycosides are not hydrolyzed by bee enzymes, as the bee does not have rhamnosidases in its saliva, and therefore the natural plant nectar glycosides are found in mature honey (Fig. 33.4). This occurs with *Robinia pseudacacia* honey, reported to contain mainly nectar flavonoid glycosides that bees cannot hydrolyze (Truchado et al. 2008).

When the transformation of nectar flavonoid glycosides is followed during the maturation of nectar in the comb to produce mature honey, the original flavonoid glycosides that are present in freshly deposited nectar are hydrolyzed sequentially, This process releases the aglycones found in mature honey, as demonstrated in *Diplotaxis tenuifolia* (Brassicaceae) honey (Truchado et al. 2010) (Fig. 33.5).

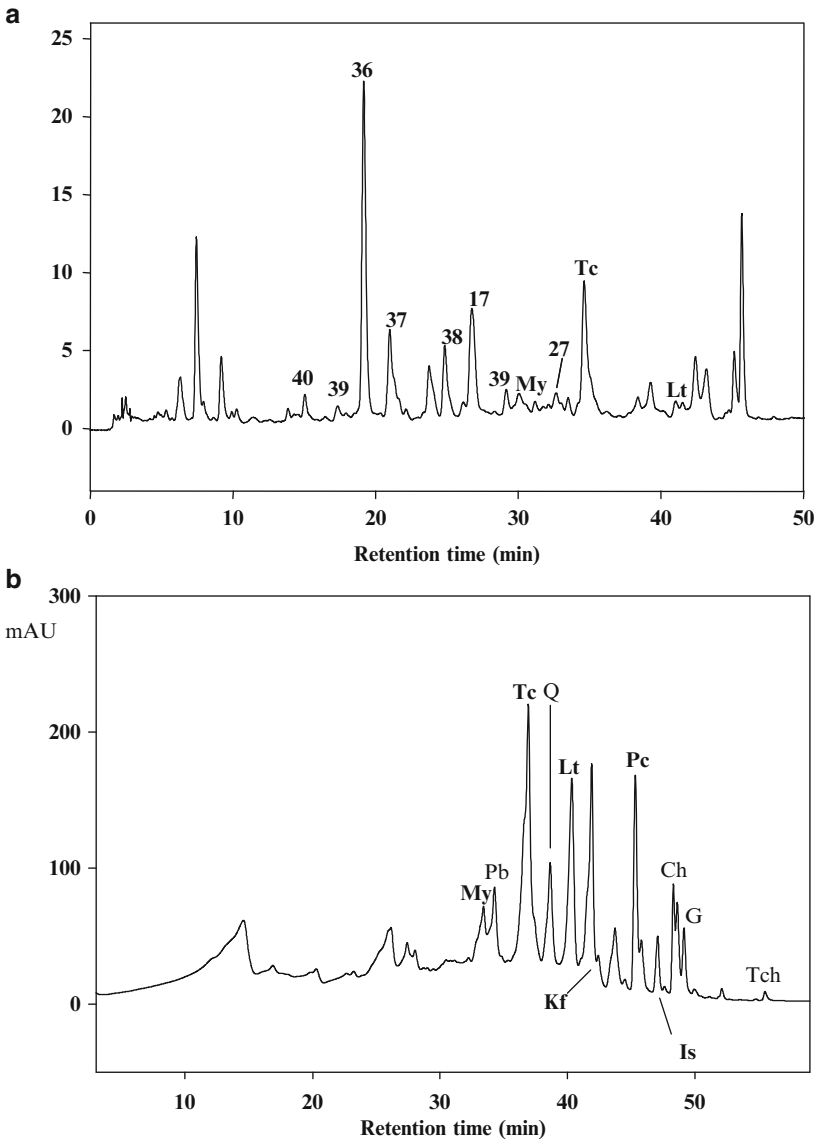

Fig. 33.3 Nectar (**a**) and honey (**b**) flavonoid profiles of *Apis mellifera* Eucalyptus honey. For flavonoid identification see Table 33.1

It can be concluded that, as a general rule, mature *A. mellifera* honey contains a larger amount of flavonoid aglycones than glycosides, although some specific honeys maintain large fractions of the original flavonoid glycosides, particularly when rhamnosides are present.

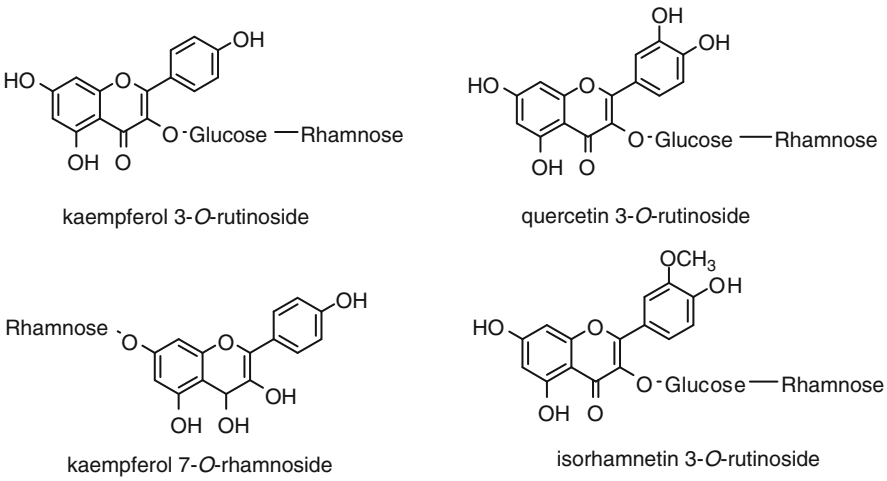

Fig. 33.4 *Apis mellifera* honey representative flavonoid glycosides

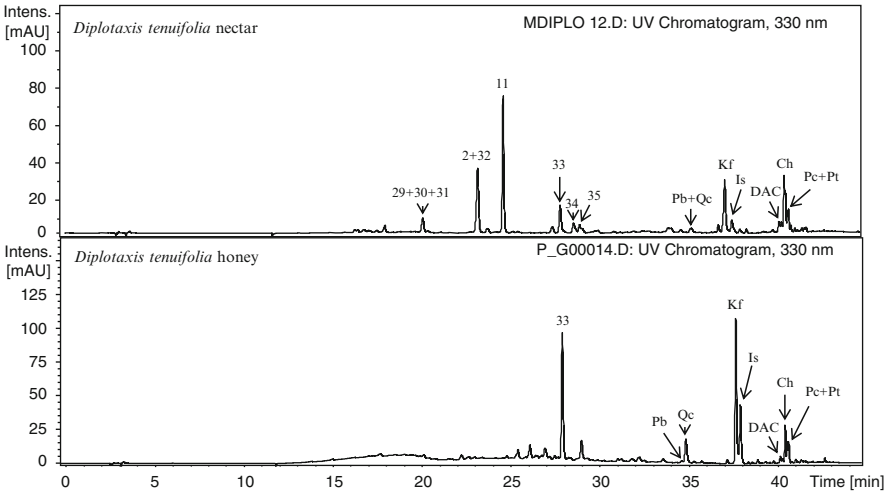

Fig. 33.5 HPLC/DAD (330 nm) phenolic profile of *Diplotaxis tenuifolia* honey from Argentina. The chromatogram from nectar is immature honey. For compound identification see Table 33.1

33.4.2 Stingless-Bee Pot-Honey

Pot-honey is generally characterized by a higher content of flavonoid glycosides than *A. mellifera* honey. This characteristic difference might be explained by the very low diastase activity of stingless bees compared to *Apis* (Persano Oddo et al. 2008). Recent studies report the occurrence of flavone di-*C*-glycosides and flavonoid *O*-glycosides in stingless-bee honey (Truchado et al. 2011) (Fig. 33.6).

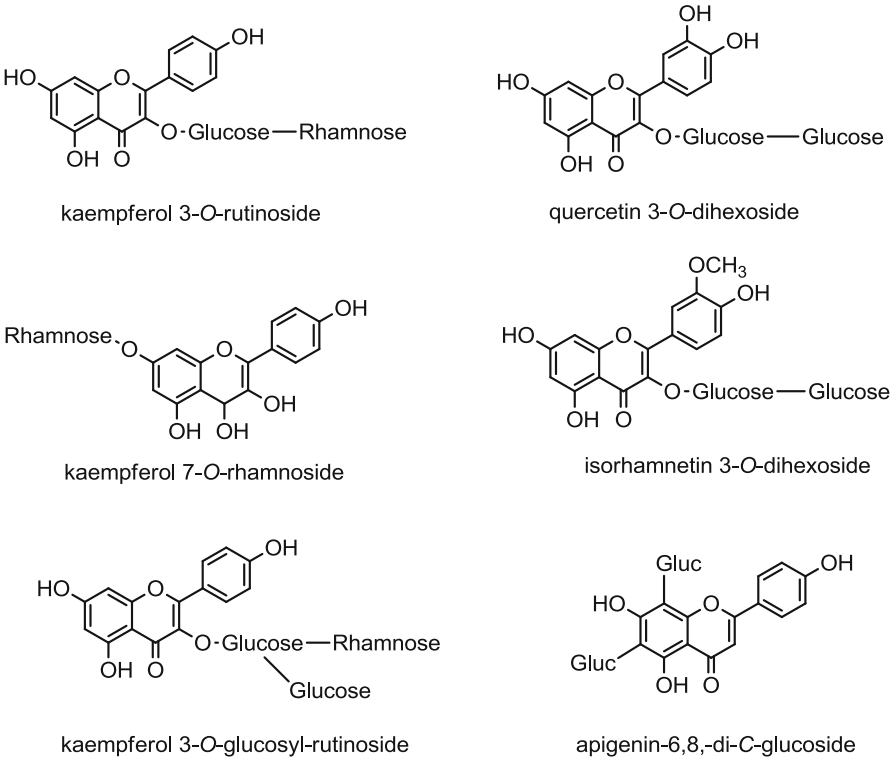

Fig. 33.6 Stingless-bee honey representative flavonoid glycosides

A collection of eight *Tetragonula carbonaria* honey samples collected from nests in various locations around Brisbane (Queensland, Australia), in suburban areas where the flora was composed mainly of ornamental shrubs and flowering trees (Persano Oddo et al. 2008) was studied to evaluate the content of flavonoid compounds. This screening showed a similar chromatographic profile for all samples (Fig. 33.7a), in which flavonoid aglycones [tricetin (*Tc*), pinobanksin (*Pb*), luteolin (*Lt*), kaempferol (*Kf*), apigenin (*Ap*), isorhamnetin (*Is*), and pinocembrin (*Pc*)], were identified together with large number of flavonoid glycosides derived from quercetin, kaempferol, and isorhamnetin and a possible tetrahydroxydihydroflavone (*H*). Six flavonoid triglycosides, namely, one flavonoid trihexoside (**1**), two compounds with a −3-*O*-(2-hexosyl, 6-rhamnosyl)hexoside substitution (**3**, **9**), another two with a −3-*O*-(2,6-di-rhamnosyl)hexoside substitution (**5**, **14**), and another compound isomeric of **3** and **9** with a tentative −3-*O*-(2-hexosyl, 3-rhamnosyl)hexoside substitution (**7**), were detected. In the same way several flavonoid diglycosides derived from the triglycosides mentioned above and with −3-*O*-(2-hexosyl)hexoside (**2**, **4**, **11**), −3-*O*-(2-rhamnosyl)hexoside (**6**, **15**, **16**) (Fig. 33.7a), and −3-*O*-(6-rhamnosyl)hexoside (**17**) substitutions were, as well as two −3-*O*-(2-pentosyl)hexosides (**10**, **13**) and one tentative −3-*O*-(3-pentosyl)hexoside (**18**), detected (Table 33.1).

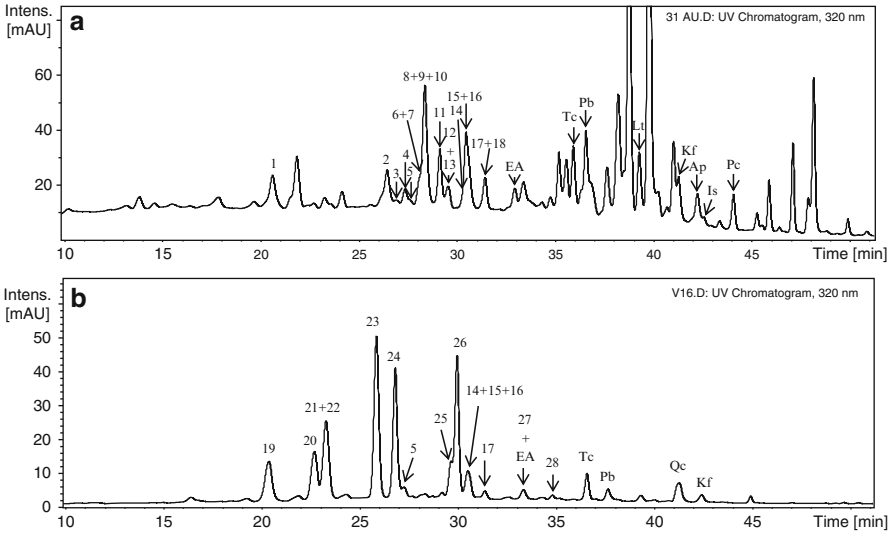

Fig. 33.7 HPLC/DAD (320 nm) phenolic profile of stingless-bee honeys (**a**) *Tetragonula carbonaria* honey from Australia, (**b**) *Melipona favosa* honey from Venezuela. For compound identification see Table 33.1

In the same way, 12 stingless-bee (*Melipona favosa*) honey samples from Venezuela collected in the arid climate area of Moruy were analyzed. The vegetation of this area was rich in Cactaceae and Mimosaceae species (Truchado et al. 2011) and all of them showed a similar chromatographic profile (Fig. 33.7b). The samples were characterized by the occurrence of five flavonoid di-*C*-glycosides: three apigenin 6,8-di-*C*-hexoside isomers (**19**, **20**, **21**), apigenin 6-*C*-pentoside-8-*C*-hexoside (**23**), and apigenin 6-*C*-hexoside-8-*C*-pentoside. Compounds with this *C*-glycosylation type had not been reported in honey (Truchado et al. 2011). In addition, these honey samples contained flavonol 3-*O*-glycosides, similar or identical to those reported from Australian stingless-bee honey described above. Compounds **5** and **14** and kaempferol 3-*O*-(2,6-di-rhamnosyl)hexoside (**26**) with a similar glycosylation to that of compound **5**, the diglycosides **15** and **16,** and the 3-*O*-(6-rhamnosyl)hexoside derivatives **17**, **27,** and **28**, in which only the aglycone was different, were detected and quantified. In addition, some propolis-derived aglycones, ellagic acid (*EA*), a flavonoid tetraglycoside [kaempferol 3-*O*-(2-hexosyl) rhamnosyl, 6-rhamnosyl)hexoside] (**25**), and a pentahydroxy-dihydroflavone, most likely dihydroquercetin (**22**), were detected (Fig. 33.7b).

Several stingless-bee honeys from Bolivia were also studied [“erereú choca” *Melipona brachychaeta* Moure, 1950; “erereú barcina” *Melipona grandis* Guérin, 1834; “obobosí” *Scaptotrigona depilis* (Moure, 1942); “suro negro” *Scaptotrigona polysticta* Moure, 1950; “suro choco” *Scaptotrigona* sp., *aff. xanthotricha* Moure, 1950; “señorita” *Tetragonisca fiebrigi* (Schwarz, 1938)] from Parque Nacional Amboró at different geographical areas with different vegetation. Only one honey

Table 33.1 Flavonoids from nectar and honey samples from *Tetragonula carbonaria* (T), *Melipona favosa* (M), *Apis mellifera* (*Diplotaxis tenuifolium*) (D) and *Apis mellifera* (*Eucalyptus globulus*) (E)

No.	Compound	T	M	D	E
1	Quercetin-*O*-trihexoside[a]	×			
2	Quercetin-3-*O*-sophoroside[a]	×		×	
3	Isorhamnetin-3-*O*-(2-hexosyl, 6-rhamnosyl)hexoside[a]	×			
4	Isorhamnetin-3-*O*-sophoroside[a]	×			
5	Quercetin-3-*O*-(2,6-di-rhamnosyl)hexoside[a]	×	×		
6	Quercetin-3-*O*-(2-rhamnosyl)hexoside[a]	×			
7	Isorhamnetin-3-*O*-(2-hexosyl, 3-rhamnosyl)hexoside[a]	×			
8	Tetrahydroxydihydroflavone[b]	×			
9	Kaempferol-3-*O*-(2-hexosyl, 6-rhamnosyl)hexoside[a]	×			
10	Quercetin-3-*O*-(2-pentosyl)hexoside[a]	×			
11	Kaempferol-3-*O*-sophoroside[a]	×		×	
12	Isorhamnetin-3-*O*-(hexosyl)hexosideisomer[a]	×			
13	Kaempferol-3-*O*-(2-pentosyl)hexoside[a]	×			
14	Isorhamnetin-3-*O*-(2,6-di-rhamnosyl)hexoside[a]	×	×		
15	Kaempferol-3-*O*-(2-rhamnosyl)hexoside[a]	×	×		
16	Isorhamnetin-3-*O*-(2-rhamnosyl)hexoside[a]	×	×		
17	Quercetin-3-*O*-(6-rhamnosyl)hexoside[a]	×	×		×
18	Quercetin-3-*O*-hexoside[a]	×			
19	Apigenin-6,8-di-*C*-hexoside[c]		×		
20	Apigenin-6,8-di-*C*-hexoside isomer[c]		×		
21	Apigenin-6,8-di-*C*-hexoside isomer[c]		×		
22	Dihydroquercetin[b]		×		
23	Apigenin-6-*C*-pentoside-8-*C*-hexoside[c]		×		
24	Apigenin-6-*C*-hexoside-8-*C*-pentoside[c]		×		
25	Kaempferol-3-*O*-(2-hexosyl)rhamnosyl, 6-rhamnosyl)hexoside[a]		×		
26	Kaempferol 3-*O*-(2,6-di-rhamnosyl)hexoside[a]		×		
27	Kaempferol-3-*O*-(6-rhamnosyl)hexoside[a]		×		×
28	Isorhamnetin-3-*O*-(6-rhamnosyl)hexoside[a]		×		
29	Quercetin-3,3′,4′-*O*-triglucoside[a]			×	
30	Isorhamnetin-3-*O*-glucoside-4′-*O*-gentiobioside[a]			×	
31	Quercetin-3,4′-*O*-diglucoside[a]			×	
32	Kaempferol-3-*O*-diglucoside isomer[a]			×	
33	Isorhamnetin 4′-*O*-gentiobioside[a]			×	
34	Isorhamnetin 4′-*O*-glucoside[a]			×	
35	Kaempferol-4′-*O*-glucoside[a]			×	
36	Tricetin 7-*O*-sophoroside (diglucoside)[a]				×
37	Tricetin 7,4′-di-*O*-glucoside[a]				×
38	Quercetin 3-*O*-glucuronide[a]				×
39	Myricetin 3,7-di-*O*-glucoside[a]				×
40	Myricetin 3-*O*-sophoroside (diglucoside)[a]				×
EA	Ellagic acid[d]		×		
DAC	Dimethylallylcaffeate[d]			×	
My	Myricetin[b]				×
Qc	Quercetin[b]		×	×	×

(continued)

Table 33.1 (continued)

No.	Compound	T	M	D	E
Lt	Luteolin[b]	×			×
Kf	Kaempferol[b]	×	×	×	×
Ap	Apigenin[b]	×			
Is	Isorhamnetin[b]	×		×	×
G	Galangin[b]				×
Ch	Chrysin[b]			×	
Tch	Techtochrysin[b]				×
Tc	Tricetin[b]	×	×		×
Pb	Pinobanksin[b]	×	×	×	
Pc	Pinocembrin[b]	×		×	×
Pt	Pinostrobin[b]			×	×

[a]*O*-glycoside
[b]Aglycone
[c]*C*-glycoside
[d]Phenolic acid derivative

sample from each stingless-bee species was available for analysis and suggests strong limitations of this study. The flavonoid profile observed was not as consistent as observed in the pot-honey from Venezuela and Australia. Nevertheless, the flavonoid glycosides detected which were also derivatives of quercetin, kaempferol, and isorhamnetin showed a glycosidic combination similar to those reported above for other stingless-bee honeys: normally hexosyl-hexosides although the second sugar could also be rhamnose or a pentose. Flavonoid triglycosides were also detected and in this case the additional sugar was often rhamnose. Several of these glycosides are common to all the analyzed samples, and in some cases flavonoid aglycones were also observed.

Several stingless-bee samples from Brazil were also analyzed: seven from "tiúba" *Melipona fasciculata*, four from "uruçú" *M. scutellaris*, and three from "jandaíra" *M. subnitida*, two from "mandaçaia" *M. quadrifasciata* and one from "uruçú amarela" *M. rufiventris*. All of them are characterized by having a very limited number of flavonoids, and in a very low quantity. These samples do not show a similar or common flavonoid profile, even for the same bee species, although this could be explained by different localities and therefore different floral origin. Some of them, and particularly the three samples from *M. subnitida*, have an abundant content of *tt* and *ct*-abscisic acid. In other samples they contained very small amounts of di-*C*-glycosyl flavonoids. Among the flavonoid *O*-glycosides, isorhamnetin and kaempferol derivatives, with a similar structure to those reported above, were detected, as well as other derivatives with glycosylations in the 3 and 7 positions. The aglycones pinobanksin and kaempferol were also detected.

A recent study reports the flavonoid glycoside content of stingless-bee honey (2.7 mg/100 g honey) is considerably higher than the content of aglycones (0.3 mg/100 g) (Truchado et al. 2011), and this differs from previous studies on *A. mellifera*, with much higher aglycone content and smaller flavonoid-glycoside content.

33.5 Conclusions and Further Research

Although the flavonoid content of *A. mellifera* honey has been extensively studied for potential use in determining botanical and geographical origin and also considering potential health benefit, the composition of stingless-bee honey is still largely unknown. An appealing topic of research is thus available due to the large number of bee species and the many and diverse plant sources used for honey production. The transformation of nectar flavonoids by bee enzymes is less relevant for the Meliponini, and therefore honey may better preserve the natural plant compounds. This observation deserves exploration in more detail. The fact that pot-honey is processed in storage pots containing resins may cause a transfer from the food container to the stored food which has never been measured, but certainly would add to its phytochemical spectra and bioactivity.

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