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Nectar Flavonol Rhamnosides Are Floral Markers of Acacia (*Robinia pseudacacia*) Honey

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With the objective of finding floral markers for the determination of the botanical origin of acacia (robinia) honey, the phytochemicals present in nectar collected from *Robinia pseudacacia* flowers were analyzed by high-performance liquid chromatography−tandem mass spectrometry. Eight flavonoid glycosides were detected and characterized as kaempferol combinations with rhamnose and hexose. Acacia honey produced in the same location where the nectar was collected contained nectar-derived kaempferol rhamnosides. This is the first time that flavonoid glycosides have been found as honey constituents. Differences in the stability of nectar flavonoids during honey elaboration and ripening in the hive were shown to be due to hydrolytic enzymatic activity and to oxidation probably related to hydrogen peroxide (glucose-oxidase) activity. Acacia honeys contained propolis-derived flavonoid aglycones (468−4348 μg/100 g) and hydroxycinnamic acid derivatives (281−3249 μg/100 g). In addition, nectar-derived kaempferol glycosides were detected in all of the acacia honey samples analyzed (100−800 μg/100 g). These flavonoids were not detected in any of the different honey samples analyzed previously from different floral origins other than acacia. Finding flavonoid glycosides in honey related to floral origin is particularly relevant as it considerably enlarges the number of possible suitable markers to be used for the determination of the floral origin of honeys.

KEYWORDS: Flavonoids; floral markers; botanical origin; floral nectar; robinia; honey quality; HPLC-MS-MS

INTRODUCTION

The authenticity of honey can be evaluated under two different aspects: authenticity regarding production and authenticity regarding description. The first evaluation aims to recognize defects or adulterations during honey production and processing, including addition of sweeteners, removal of water, and use of excessive heat. It is performed through physicochemical analyses, some of which are requested by law (EU Council Directive 2001/110) like sugar content, moisture, electrical conductivity, free acidity, diastase activity, HMF (hydroxy-methyl-furfural), water-insoluble contents, and others used for the identification of specific anomalous components (sugar cane, maize syrups, beet sugar, products of fermentation, aging, and overheating).

The evaluation of authenticity regarding description aims to identify botanical and geographical origin of honey and to avoid possible misdescriptions. The classical approach to the evaluation of botanical origin is based on the integration of pollen analysis, sensory analysis, and determination of some physicochemical parameters: color, free acidity, sugar contents, diastase activity, electrical conductivity, and specific rotation. All of these methods are quite labor intensive and need specialized personnel for pollen and sensory analysis, but until now, they remain the methods of choice. New analytical methods have been developed (*1*−*3*), and others are going to be developed and

Table 1. Honey Samples Studied in the Present Work

sample code	botanical origin	geographical origin
SUC-001	sucrose syrup honey	Bologna (Italy)
R-001	*R. pseudacacia* L.	Bologna (Italy)
R-469	*R. pseudacacia* L	Bologna (Italy)
R-409	*R. pseudacacia* L	Bologna (Italy)
R-656	*R. pseudacacia* L.	Trento Valsugana (Italy)
R-466	*R. pseudacacia* L	Castello di Fiemme, Trento (Italy)
R-579	*R. pseudacacia* L	Frossasco, Torino (Italy)
R-655	*R. pseudacacia* L	Varese (Italy)
S-001	*R. pseudacacia* L.	Sebechleby (Slovakia)
S-004	*R. pseudacacia* L.	Bratislava (Slovakia)
S-011	*R. pseudacacia* L.	Bátorové Kosihy (Slovakia)
S-012	*R. pseudacacia* L.	Tupá (Slovakia)

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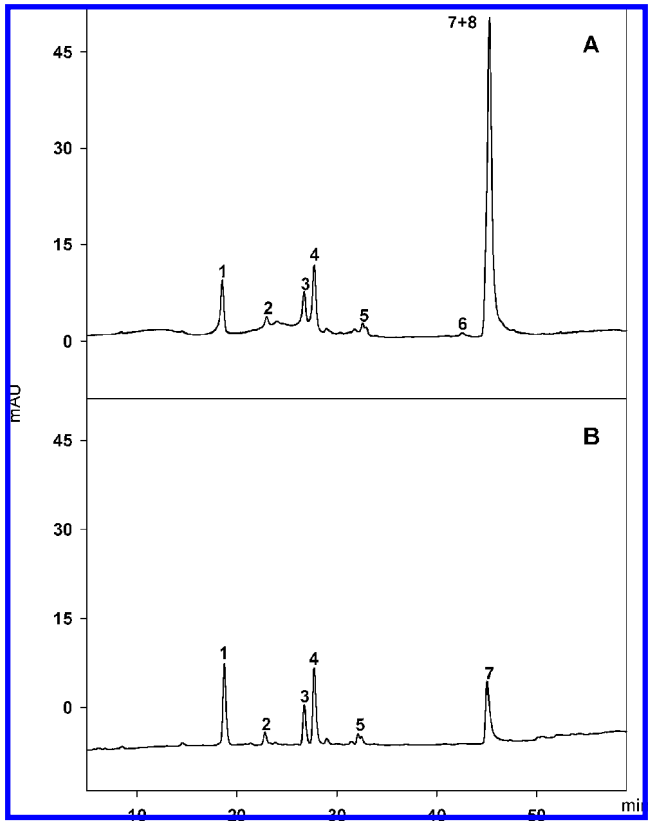

Figure 1. HPLC-DAD chromatogram (340 nm) of kaempferol-glycosides in *R. pseudacacia* nectar. (**A**) Freshly extracted. (**B**) Extracts after 6 days of treatment with H_2O_2. Compounds: **1**, kaempferol-3-*O*-(hexoxyl)robinoside-7-*O*-rhamnoside; **2**, kaempferol-3-*O*-(hexoxyl)robinoside; **3**, kaempferol-3-*O*-hexoside-7-*O*-rhamnoside; **4**, kaempferol-3-*O*-robinoside-7-*O*-rhamnoside; **5**, kaempferol-3-*O*-robinoside; **6**, kaempferol-7-*O*-robinoside; **7**, kaempferol-7-*O*-rhamnoside; and **8**, kaempferol-7-*O*-rhamnosyl (1→2) rhamnosyl (1→ 2) hexosyl (1→ 2) rhamnoside.

proposed for routine analysis. Among them, the determination of specific markers, such as phenolic compounds, is one of the most promising (*4*−*9*).

Phenolic compounds, and particularly flavonoids, have been considered especially appropriate among secondary metabolites, as markers to be used in plant chemotaxonomic studies (*10*). In fact, phenolics have been reported as suitable floral origin markers for citrus honey (hesperetin) (*11*), eucalyptus honey (myricetin, tricetin, quercetin, luteolin, and kaempferol mixtures) (*12*), rosemary honey (kaempferol) (*13*), and heather honey (ellagic acid) (*14*). Other compounds such as abscisic acid have been proposed as markers of heather and calluna honeys (*15*), although they have been reported in honeys from many other sources. In all of these studies, the analysis of either the floral nectar directly collected from the flowers or the content of bee sack has been found particularly useful to study the presence of specific markers for each floral origin.

Acacia honey, also known as robinia honey, is produced in different European Countries such as Italy, Germany, Croatia, Slovakia, etc. This is produced from *Robinia pseudacacia* blossoms, and it is highly appreciated by the consumers due to its clear aspect and mild flavor and aroma (*16*). Previous studies have analyzed the phenolic compounds present in robinia honey from Croatia, and several flavonoid aglycones were detected and quantified (*16*). The present work aims at the identification of the phenolic compounds present in *R. pseudacacia* floral nectar and their evaluation in acacia honey samples produced in Europe as possible floral origin markers.

MATERIALS AND METHODS

Reagents. Chlorogenic acid (5-*O*-caffeoylquinic acid), rutin (quercetin-3-*O*-rutinoside), quercetin, hesperetin, and *cis*−*trans*-abscisic acid were purchased from Sigma (St. Louis MO), and chrysin (5,7-dihydroxyflavone) was from Carl Roth OGH (Karlsruhe, Germany). Formic and acetic acid were of analytical grade, and methanol was high-performance liquid chromatography (HPLC) grade and supplied by Merck (Darmstadt, Germany). Milli-Q system (Millipore Corp., Bedford, MA) ultra pure water was used throughout this study.

Collection of Nectar. Robinia blossoms were picked from robinia trees in Bologna (Italy) and brought to the laboratory. During the same day, nectar was aspirated from flowers using a glass capillary and then collected in eppendorf test tubes and stored at −20 °C until analysis. About 10 mL of nectar was collected.

Collection of Honey Samples. Honey samples collected for the study are listed in **Table 1**.

Experimental Honeys. In the summer of 2006, two healthy colonies, originated from sister queens of *Apis mellifera ligustica*, were split to form two queenright and two queenless colonies. The latter, deprived of honey and pollen combs and provided with empty frames, were confined under a greenhouse in Bologna (Italy), not allowed to forage on flowers, and fed with 1 kg of sucrose syrup (1/1 ratio of sucrose/water) each every two days for 8 days. About 2 kg of "sucrose syrup honey" was centrifugally extracted from the two nuclei and collected into 500 mL glass pots with metal twist-off caps (SUC-001). The two queenright colonies, supplied with a supper and not supplementary feed, were brought to an area near Bologna (Italy) with flowering robinia trees (*R. pseudacacia*) to produce acacia (robinia) honey. Honey was centrifugally extracted from the supper and collected into 500 mL glass pots with metal twist-off caps (R-001).

Robinia Honeys. Ten additional samples of robinia honey were collected in different regions of Italy and Slovakia during the summers of 2006 and 2007, respectively, from apiaries of *A. m. ligustica* and *Apis mellifera carnica*. All of the honey samples were stored in the dark at 4 °C until analysis.

Certification of Honey Samples. All of the collected honey samples conformed to the requisites listed in the Council Directive 2001/110/EC: sugar content, moisture content, water-insoluble matter, electrical conductivity, free acid, diastase activity, and HMF. The botanical origin was certified by the traditional analysis method: sensorial and pollen analysis and physicochemical analyses (color and specific rotation, in addition to the previously listed ones).

Among the Italian robinia samples, R-001, R-409, R-466, R-655, and R-656 resulted in an excellent quality and respondent to the declared botanical origin for pollen content, physicochemical parameters, flavor, and taste typical for this kind of honey. Samples R-579 and R-469 were respondent to a robinia honey, but the quality was lowered by the presence of nectar of *Taraxacum*, which modified their sensory characteristics.

The four samples from Slovakia were respondent to the declared botanical origin for pollen content and physicochemical parameters; the sensory analysis revealed the presence of *Cruciferae*, confirmed also by the palynological analysis. The presence of *Cruciferae* nectar, which gives honey a typical taste, is a common characteristic of the robinia honeys produced in Eastern Europe.

The analysis of sucrose syrup honey (SUC-001) revealed a high content of water, because it was extracted before honeybees could dehumidificate it, an anomalous low content of fructose and glucose, and a high content of sucrose with respect to the normal content of honey, indicating that bees were not able to break down all of the sucrose into glucose and fructose.

Extraction of Phenolic Compounds from Nectar. *Robinia* nectar was diluted with ultra pure water and centrifuged at 7000 rpm for 10 min in a Centromix centrifuge (Selecta, Barcelona). The supernatant was filtered through a Sep-Pak solid phase extraction (SPE) cartridge (a reverse phase C_{18} cartridge; Waters Millipore, United States). This cartridge was previously activated with 10 mL of methanol and 10 mL of water. The supernatant was filtered through the cartridge and then was washed with 10 mL of water. The phenolics remaining in the cartridge were then eluted with 1 mL of methanol. The methanol

Table 2. R_t, UV, and −MS: $[M - H]^-$, −MS2$[M - H]^-$, and −MS3$[(M - H)\rightarrow(M - H - 146)]^-$ Data of *Robinia* Nectar Kaempferol-Glycoside Derivatives[a]

			kaempferol-3-*O*-glycosyl-7-*O*-rhamnosyl derivatives				
compounds[b]	R_t (min)	UV (nm)	$[M - H]^-$	−MS2$[M - H]^-$	−MS3$[(M - H)\rightarrow(M - H - 146)]^-$		
1 K-3-*O*-(Hx)Rob-7-*O*-Rh	17.1	266, 318sh, 348	901	755	593 (28)	575 (65)	285 (100)
3 K-3-*O*-Hx-7-*O*-Rh	25.4	266, 320sh, 348	593	447			285 (100)
4 K-3-*O*-Rob-7-*O*-Rh	26.4	266, 320sh, 348	739	593			285 (100)

			kaempferol-3-*O*-glycosyl/-7-*O*-glycosyl derivatives				
compounds[b]	R_t (min)	UV (nm)	$[M - H]^-$	−MS2$[M - H]^-$			
2 K-3-*O*-(Hx)Rob	22.0	266, 298sh, 348	755		593(25)	575(50)	285(100)
5 K-3-*O*-Rob	42.2	266, 322sh, 367	593				285(100)
6 K-7-*O*-Rob	45.0	266, 322sh, 367	593				285(100)
7 K-7-*O*- Rh	45.0	266, 322sh, 367[c]	431				285(100)
8 K-7-*O*-Rh-Rh-Hx-Rh	45.0		885	739(85)	593(35)	431(100)	285(44)

[a] Main observed fragments. Other ions were found, but they have not been included. [b] K, kaempferol; Hx, hexosyl; Rob, robinosyl; and Rh, rhamnosyl. [c] UV data obtained from the HPLC chromatogram in **Figure 1B**.

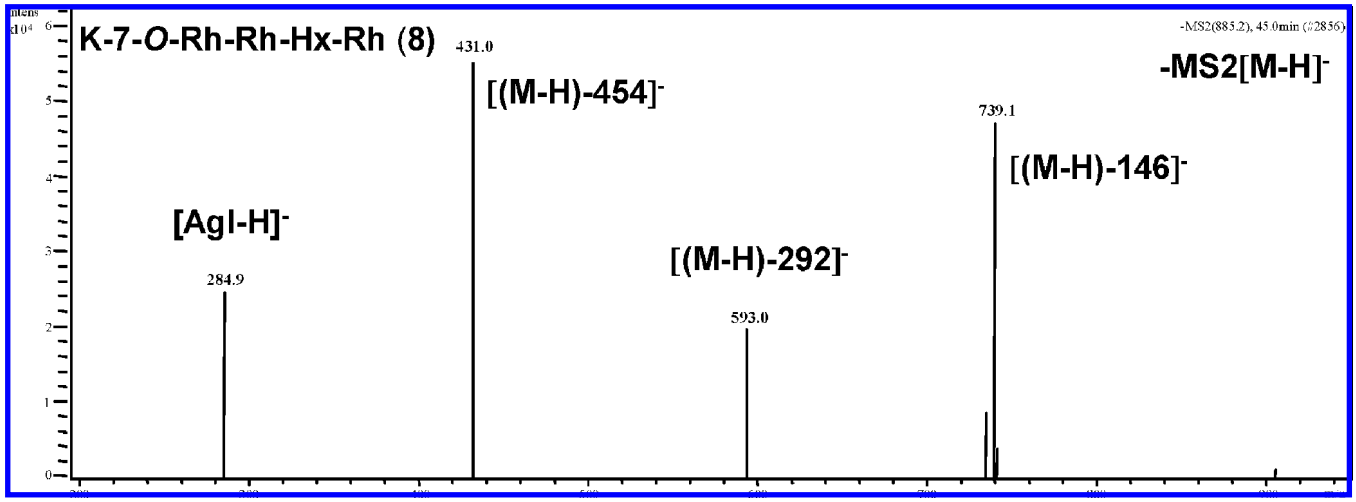

Figure 2. MS2 spectrum of kaempferol-7-*O*-rhamnosyl (1→2) rhamnosyl (1→2) hexosyl (1→2) rhamnoside (compound **8**).

fraction was filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp.) and stored at −20 °C until further analysis by HPLC-diode array detection-tandem mass spectrometry (DAD-MS-MS).

Extraction of Phenolic Compounds from Honey. *SPE Cartridge Method.* Honey samples (20 g) were dissolved with five parts of water (adjusted to pH 2 with HCl) until completely fluid. This solution (100 mL) was then filtered through a Sep-Pak cartridge, which was previously activated as described above. The cartridge was washed with 10 mL of water, and the phenolic compounds were eluted with 1 mL of methanol. The methanol fraction was filtered through a 0.45 μm filter and stored at −20 °C until further analyzed by HPLC-DAD-MS-MS.

Nonionic Polymeric Resin Amberlite XAD-2 Extraction Method. Extraction was carried out as described previously (*5*). Honey samples (ca. 50 g) were dissolved in five parts of water (adjusted to pH 2 with HCL) until completely fluid. The solution was mixed with 200 g of Amberlite XAD-2 resin (Supelco, Bellefonte; pore size, 9 nm; particle size, 0.3−1.2 mm) and stirred with a magnetic stirrer at room temperature for 10 min to adsorb phenolic compounds (*17*). The resin was packed into a glass column (55 cm × 4 cm), washed with acid water (pH 2 with HCl, 200 mL), and subsequently washed with ultra pure water (∼ 300 mL) to eliminate sugars and other honey polar compounds. The phenolic compounds were recovered with methanol (400 mL) and taken to dryness under reduced pressure (40 °C). The residue was resuspended in 5 mL of ultrapure water and extracted with diethyl ether (5 mL × 3) (*18*). Then, the extracts were combined, concentrated under reduced pressure, and redissolved in 1 mL of methanol. These methanol extracts were filtered through a 0.45 μm filter and stored at −20 °C until further analysis by HPLC-DAD-MS-MS.

Analysis of Phenolic Compounds by HPLC-DAD-MS-MS. The samples were analyzed using an Agilent HPLC 1100 Series instrument equipped with a diode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (G1312 A), an autosampler (G1313 A), a degasser (G1322 A), and DAD (G1315 B) controlled by software (v. A08.03). Separations of phenolic compounds were achieved on a C_{18} LiChroCART column (Merck, Darmstadt, Germany) (RP-18, 250 mm × 4 mm; 5 μm particle size) protected with a 4 mm × 4 mm C18 LiChroCART guard column. The mobile phase was water/acetic acid (99:1, v/v) (solvent A) and HPLC grade methanol (solvent B) at flow rate of 1 mL min^{-1}. Elution was performed with a gradient starting with 20% B in A, to reach 50% B in A at 40 min, 80% B in A at 55 min, and then became isocratic for 5 min. UV chromatograms were recorded at 290 and 360 nm.

The mass detector was an ion trap spectrometer (G2445A) equipped with an electrospray ionization (ESI) system and controlled by software (v. 4.1). The nebulizer gas was nitrogen. The pressure and the flow rate of the dryer gas were set at 65 psi and 11 L min^{-1}, respectively. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were measured from *m/z* 100 up to *m/z* 1000. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the negative mode.

Analysis of phenolic compounds of honey was achieved with same instrument, on the same column used in nectar analysis. In this case, the mobile phase used was water/formic acid (99:1, v/v) (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 mL min^{-1}.

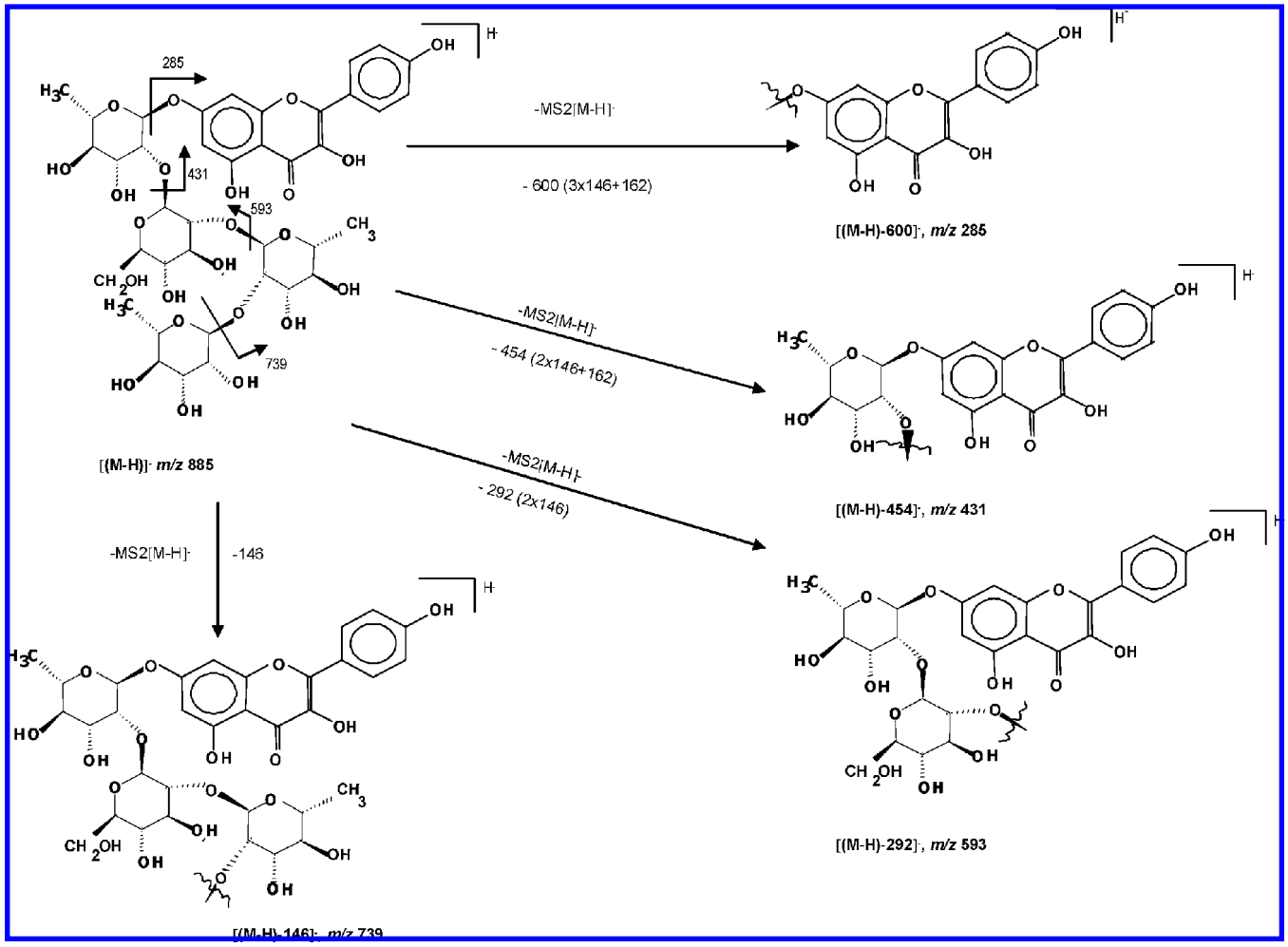

Figure 3. ESI-MS*n* fragmentation pathway of kaempferol-7-*O*-rhamnosyl (1→2) rhamnosyl (1→2) hexosyl (1→2) rhamnoside) (compound **8**).

Elution was performed with a gradient starting with 10% B in A to reach 30% B in A at 20 min, 45% B in A at 30 min, 60% B in A at 40 min, 80% B in A at 45 min, and 90% B in A at 60 min and then became isocratic for 5 min. Chromatograms were recorded at 290, 320, 340, and 360 nm.

The phenolic compounds were identified according to their UV spectra, molecular weights, retention times, and their MS-MS fragments, when possible, with commercially available standards. Hydroxycinnamic acid derivatives were quantified as chlorogenic acid at 320 nm, and flavonols, flavonol glycosides, and flavones were quantified as quercetin, rutin, and chrysin, respectively, at 340 nm. Flavanones were quantified as hesperetin and abscisic acid as *cis-trans*-abscisic acid at 290 nm.

Degradation of Nectar Flavonoids by Hydrogen Peroxide. The methanol flavonoid fraction (0.5 mL) obtained by extraction with SPE cartridge (Sep-Pak C_{18}) from *Robinia* nectar was mixed with two drops of diluted hydrogen peroxide. Commercial hydrogen peroxide (30%, Panreac, Barcelona) was diluted 10-fold with ultrapure water. This solution was incubated at room temperature in the dark, and 50 μL samples were taken for analysis at 0, 2, and 6 days. Samples (20 μL) were directly analyzed by HPLC-MS-MS under the conditions described above.

RESULTS AND DISCUSSION

Phenolic Compounds in *R. pseudacacia* Nectar. The HPLC-DAD analysis of nectar (**Figure 1A**) reveals the presence of at least seven different chromatographic peaks with UV spectra characteristic of flavonols. The UV spectra of the first five chromatographic peaks (**1**−**5**) indicated that they were 3-substituted flavonols, while peak **6** and the main chromatographic peak (**7** + **8**) showed the characteristic UV spectrum of flavonols with free hydroxyl at 3 (UV max at 367 nm in band I) (*19*). Their chromatographic behavior and retention times suggest that most of them are di-triglycosylated conjugates, consistent with previous studies on nectar flavonoid constituents (citrus, rosemary) in which the presence of flavonoid glycosides had been reported (hesperetin 7-rutinoside and quercetin and kaempferol 3-sophorosides) (*11, 13*). The flavonoid profile of *Robinia* nectar was, however, much more complex than those previously studied, as these generally presented just one main flavonoid. The MS data showed that compounds **1**, **3**, and **4** (**Table 2**) were glycosylated on two different phenolic hydroxyls of the flavonoid nucleus (*20*). Their UV spectra indicate that the hydroxyl in the 3-position is blocked (*19*) and the shoulder at ca. 320 nm suggests that these flavonoids have a double glycosylation at 3- and 7-positions of the kaempferol molecule (*21*). Their MS/MS studies provide information on the type of sugars and position linkages in these glycosides. Thus, the MS2[M − H]$^-$ of compounds **1**, **3**, and **4** show in all cases a first loss of a rhamnosyl residue (loss of 146 m.u.), leaving the aglycone with an additional glycosidic residue. This behavior is characteristic of compounds with 3,7-di-*O*-glycosylation in which the sugar residue at the 7-position is released first (*20*) (**Table 2**), indicating that these flavonoids are 3-*O*-glycosyl-7-*O*-rhamnosyl derivatives. The MS3[(M − H) − (M − H − 146)]$^-$ of compound **4** showed that no intermediate fragment was observed between [M − H − 146] (*m*/*z* 593) and the aglycone (*m*/*z* 285), and this indicates that the glycoside at position 3 is a rhamnosyl(1→6)hexoside (*20*)

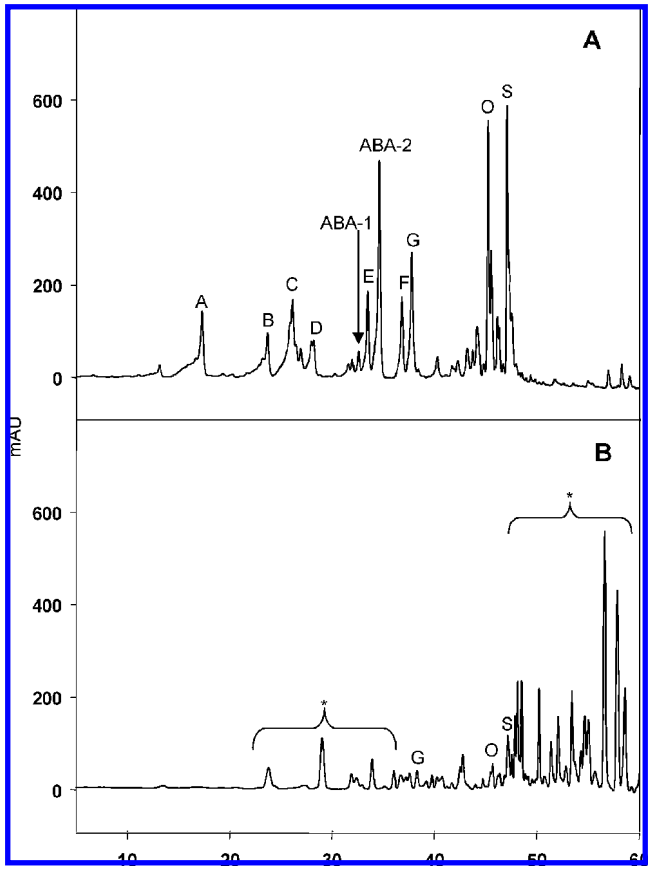

Figure 4. HPLC-DAD chromatograms (290 nm) of phenolic compounds in acacia honey extracted with Amberlite XAD-2. (**A**) Acacia honey and (**B**) sucrose syrup honey. Peaks: A, caffeic acid; B, *p*-coumaric acid; C, ferulic acid; D and E, hydroxycinnamic acid derivative (caffeic or ferulic acid); F, isosakuranetin; G, pinobanksin; O, pinocembrin; S, chrysin; ABA-1 *trans*−*trans* abscisic acid; ABA-2, *cis*−*trans* abscisic; and *, bee-origin unidentified peaks.

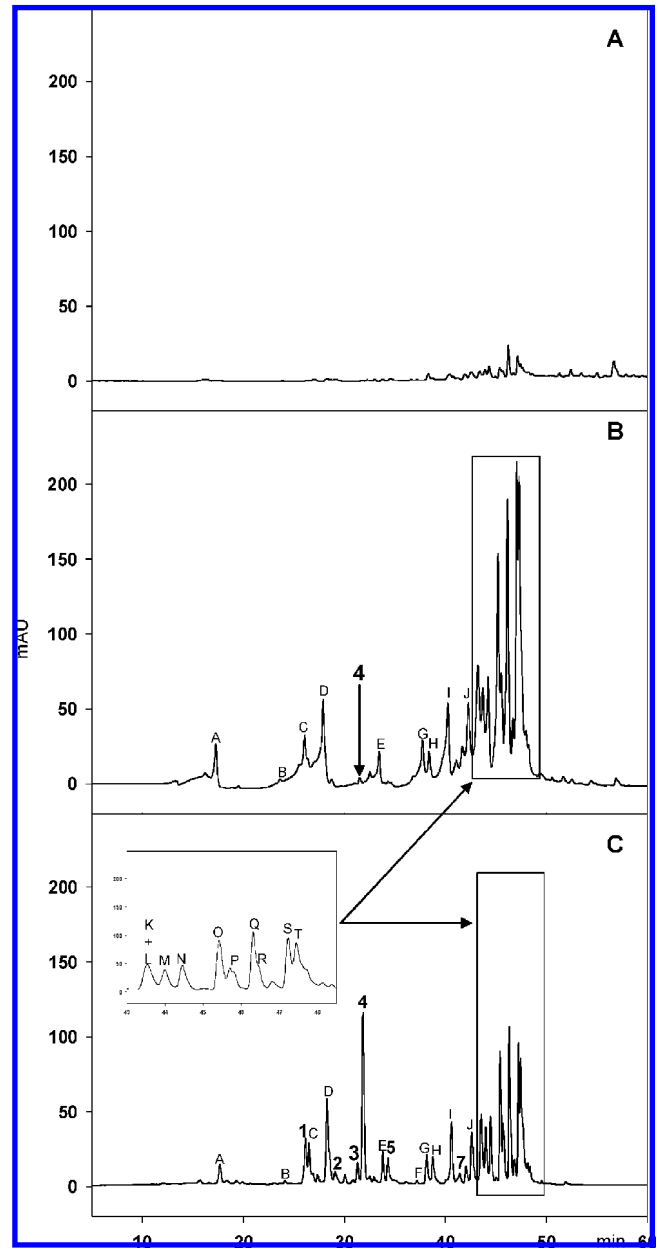

Figure 5. HPLC chromatograms (360 nm) of phenolic compounds and kaempferol-glycosides in acacia honey: (**A**) sucrose syrup honey extracted with Amberlite XAD-2, (**B**) acacia honey extracted with Amberlite XAD-2, and (**C**) acacia honey extracted with C_{18} SPE cartridge. Peaks: F, isosakuranetin (4′-methoxy-5,7-dihydroxyflavanone); G, pinobanksin (3,5,7-trihydroxyflavanone); O, pinocembrin (5,7-dihidroxyfavanone); P, unidentified flavanone; A, caffeic acid; B, *p*-coumaric acid; C, ferulic acid; D and E, hydroxycinnamic acid derivative (caffeic or ferulic acid); N, dimethyl-allyl-caffeate; R, phenylethyl caffeate; H, quercetin (3,5,7,3′,4′-pentahydroxyflavone); I, unidentified flavonol; J, kaempferol (3,5,7,4′-tetrahydroxyflavone); K + L, apigenin (5,7,4′-trihydroxyflavone) + isorhamnetim (3,5,7,4′-tetrahydroxy-3′-methoxyflavone); M, acacetin (5,7-dihydroxy-4′-methoxyflavone); Q, methylquercetin (3,5,7,3′-tetrahydroxy-4′-methoxyflavone, tentatively); S, chrysin (5,7-dihydroxyflavone); T, galangin (3,5,7-trihydroxyflavone). Compounds: **1**, kaempferol-3-*O*-(hexoxyl)robinoside-7-*O*-rhamnoside; **2**, kaempferol-3-*O*-(hexoxyl)robinoside; **3**, kaempferol-3-*O*-hexoside-7-*O*-rhamnoside; **4**, kaempferol-3-*O*-robinoside-7-*O*-rhamnoside; **5**, kaempferol-7-*O*-robinoside; and **7**, kaempferol 7-*O*-rhamnoside.

and most likely a robinoside (rhamnosyl-galactoside, the characteristic disaccharide previously reported in *Robinia* leaves flavonoid glycosides) (*22*); therefore, **4** was tentatively identified as robinin (kaempferol-3-*O*-rhamnosyl-galactoside-7-*O*-rhamnoside; kaempferol 3-*O*-robinoside-7-*O*-rhamnoside) (*23*). Compound **1** showed a molecular weight 162 m.u. higher than **4**, suggesting that this was a derivative of **4** with an additional hexosyl residue on the robinoside linked at position 3. The MS3 analysis of compound **1** showed losses of 162 and 180 m.u., leading to relevant fragments (**Table 2**), and this indicates that the additional hexose is linked through a (1→2) interglycosidic linkage to the hexose or the rhamnose of the robinoside linked at position 3. Compound **3** is a simpler flavonol glycoside characterized as kaempferol 3-hexoside-7-rhamnoside. The MS of the other five compounds (**2** and **5**−**8**) indicate that they are glycosylated on a single phenolic hydroxyl (**Table 2**) (*20*). Compounds **7** and **8** coelute under a single chromatographic peak, and they were only detected as a mixture of two compounds after the MS analysis using the ion trap. Their UV spectra show that the hydroxyl in **3** is free in compounds **6** and **7** and probably in **8** (UV, BI max at 367 nm), while this is blocked in compounds **2** and **5** (UV, BI max at 348 nm) (*19*). The MS of compound **2** indicates that this is a triglycoside of kaempferol (two hexosyl and one rhamnosyl residues), and its MS2 fragmentation is similar to that observed for the MS3 $[(M - H) - (M - H - 146)]^-$ fragmentation of compound **1** (**Table 2**), in agreement with a derivative of compound **1** in which the rhamnosyl at position 7 has been removed

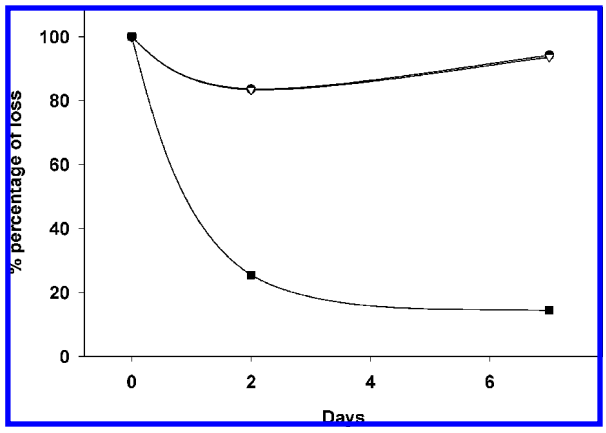

Figure 6. Degradation of *Robinia* nectar flavonoids by hydrogen peroxide oxidation [values were measured as a percentage of decrease in absorbance (360 nm) of the chromatographic peaks]: Compound **3** (kaempferol-3-*O*-hexoside-7-*O*-rhamnoside) (●); compound **4** (kaempferol-3-*O*-robinoside-7-*O*-rhamnoside) (△); compound **7** + **8** (kaempferol-7-*O*-rhamnoside + kaempferol-7-*O*-rhamnosyl (1→ 2) rhamnosyl (1→ 2) hexosyl (1→ 2) rhamnoside (■).

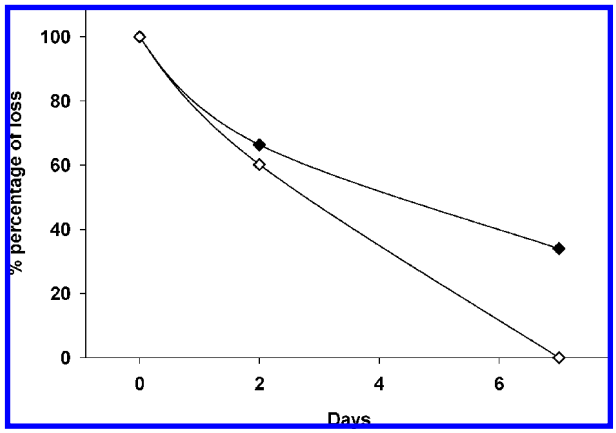

Figure 7. Degradation of *Robinia* nectar flavonoids **7** and **8** by hydrogen peroxide oxidation [values were measured as a percentage of decrease in total ions registered for the extracted ions at *m*/*z* 431 and *m*/*z* 885]: compound **7** (*m*/*z* 431) (kaempferol-7-*O*-rhamnoside) (◆); compound **8** (*m*/*z* 885) (kaempferol-7-*O*-rhamnosyl (1→2) rhamnosyl (1→2) hexosyl (1→2) rhamnoside) (◇).

(kaempferol-3-*O*-hexosyl-robinoside). Compound **5** shows a deprotonated molecular ion at *m*/*z* 593 consistent with a kaempferol rhamnosyl-hexoside. Both sugars are linked to one phenolic hydroxyl as the disaccharide moiety is released in one single fragment. The same mass and fragmentation behavior are observed for compound **6**, showing that both compounds were isomers. The lack of intermediate fragments also shows that the interglycosidic linkage is more likely (1→6). The UV spectrum of **5** indicates that the hydroxyl in C-3 of the flavonoid nucleus is blocked, while this is free in compound **6**. All of these data indicate that **5** is kaempferol 3-*O*-rhamnosyl (1→6) hexoside and most likely 3-robinoside, while **6** is kaempferol 7-*O*-robinoside. Compounds **7** and **8** coelute in one single chromatographic peak and have deprotonated molecular ions at *m*/*z* 431 and 885, respectively. Compound **7** is identified as kaempferol 7-rhamnoside (free hydroxyl at C-3 and loss of 146 m.u. in the MS2 experiment to yield kaempferol aglycone). Compound **8** is more complex, and its MS analysis indicates that this is a kaempferol tetraglycoside, in which three rhamnoses and one hexose are linked to the aglycone in one single hydroxyl at C-7 (tentatively). After its MS-MS analysis, the MS2 fragmentation (**Figures 2** and **3** and **Table 2**), it seems clear that one of the rhamnosyl residues is directly linked to the aglycone moiety (fragment at *m*/*z* 431). The high relative abundance of the ion at *m*/*z* 739 (85%) ($[M - H - 146]^-$) indicates that another rhamnose is a terminal sugar, and this is not linked to the hydroxyl at C-6 of the hexose, and that its interglycosidic linkage is more likely (1→2) (*20*). The presence of an ion at *m*/*z* 593 (kaempferol + rhamnosyl + hexosyl) (loss of two rhamnoses) with high relative abundance indicates that none of them is linked by (1→6) linkage and that the hexosyl residue is linked to a rhamnose that is directly linked to the aglycone. In addition, the lack of MS2 resulting in fragment losses of 162 or 180 (162 + 18) indicates that the hexosyl is substituted; therefore, the structure of this complex compound **8** can be tentatively characterized as kaempferol 7-*O*-rhamnosyl (1→2) hexosyl (1→2) rhamnosyl (1→2) rhamnoside. Thus, *R. pseudacacia* nectar is characterized by a mixture of kaempferol glycosides, and no other UV absorbing metabolite is detected in the chromatograms.

Phenolic Compounds in Experimental Acacia and Sucrose Honeys. Experimental acacia honey (sample R-001) was produced in the same location and at the same time where nectar had been collected and when only robinia flowers were available for nectar collection. In addition, honey samples produced by the same bee colony but only from sucrose syrup (SUC-001) were also produced as a control to evaluate those compounds incorporated into honey either from the bee or from the hive environment (beeswax, propolis, etc.) but not from robinia flowers. When the phenolics present in the experimental acacia honey were extracted using the Amberlite XAD-2 resin adsorption methodology (*18*), the HPLC chromatogram showed a phenolic profile characterized by propolis-derived compounds (**Figure 4A**). These included the flavonoid aglycones chrysin, pinocembrin, and pinobanksin, as well as several hydroxycinnamic acid derivatives (caffeic, *p*-coumaric, and ferulic acids). In addition, abscisic acid isomers were detected. When the phenolics of experimental honey samples produced from sucrose syrup were extracted and analyzed, only the propolis-derived flavonoids pinocembrin, pinobanksin, and chrysin were detected but in very small amounts (**Figure 4B**). The presence of propolis-derived flavonoid aglycones in the sucrose syrup honey indicates that during the elaboration/ripening process, a migration of propolis phenolics from the beeswax toward honey occurs. Alternatively, the bee could directly incorporate these propolis polyphenols into honey through its secretions, as it was shown that the bee ingests propolis, since the characteristic propolis polyphenols were already found in beeswax scales just after being secreted by bees (*24*).

Because kaempferol glycosides are present as relevant constituents in robinia nectar, the aglycone kaempferol should be searched as a potential marker to look for in honeys of this floral origin. This is based in previous works reporting that nectar glycosides were hydrolyzed by the bee enzymes to render the aglycones that were the metabolites detected in honey (*11, 13*). In acacia honey, however, kaempferol aglycone (J) was just detected as a minor constituent in the HPLC analysis of honey polyphenols (**Figure 5**), indicating that the *Robinia* nectar flavonoids were not hydrolyzed during honey elaboration/ripening. This prompted us to evaluate the presence of the native nectar flavonoid glycosides in honey. In the analysis of the acacia honey phenolics after extraction

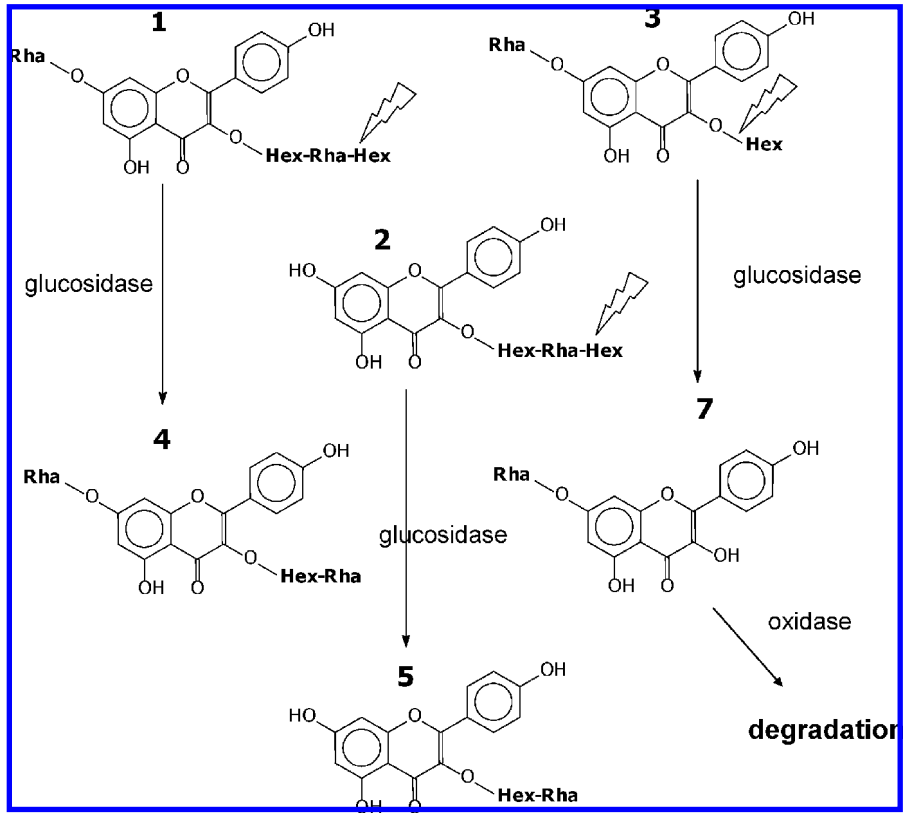

Figure 8. Transformation of *Robinia* nectar flavonoids during honey elaboration/ripening. Effect of hydrolytic enzymes (glucosidases) and oxidative enzymes (glucose oxidase) that releases hydrogen peroxide (oxidative degradation).

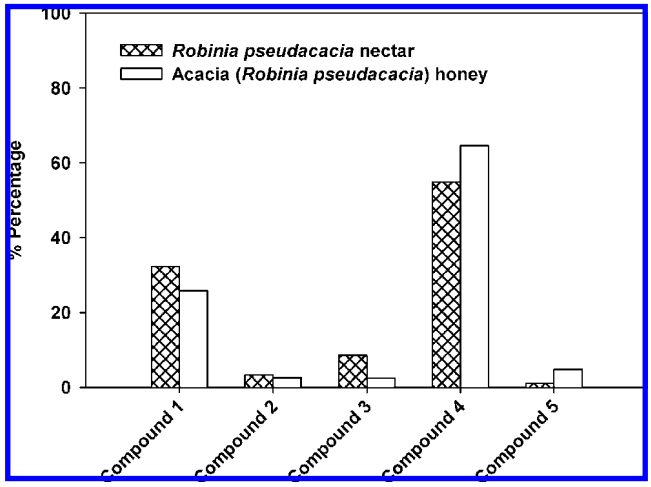

Figure 9. Relative percentage of flavonoids **1**−**5** in *R. pseudacacia* nectar and in acacia honey.

using the Amberlite XAD-2 resin methodology (*18*), the glycoside **4** from *Robinia* nectar was detected as a minor constituent by HPLC-MS-MS (**Figure 5B**). This indicates that the XAD-2 extraction methodology that uses a final extraction with ethylic ether was not appropriate for the extraction of these polar glycosidic flavonoids, and then, the extraction procedure was modified to optimize the potential detection of flavonoid glycosides. Thus, honey was directly filtered through reversed-phase SPE cartridges (C-18) after dilution in water. The HPLC analysis of the honey phenolics extracted using this SPE system showed that the flavonoid glycosides present in *Robinia* nectar were the main peaks in the chromatogram of acacia honey extracts recorded at 360 nm (**Figure 5C**). These were characterized by HPLC-MS-MS. However, compounds **6** and **8** were not detected in honey, and **7** was only present as a minor constituent in this chromatogram, in spite of compounds **7** and **8** being the major ones in the nectar chromatogram (**Figure 1A**). Therefore, a study of the fate of nectar flavonoids during honey elaboration and ripening was set up.

Stability of Nectar Flavonoids in Honey. In previous studies, the conversion of the flavonoid glycosides present in nectar to the flavonoid aglycones detected in honey was explained by the activity of hydrolytic enzymes in the bee saliva and/or honey (glucosidases). This explained the occurrence of kaempferol in rosemary honey while nectar contained kaempferol 3-sophoroside (kaempferol 3-diglucoside) (*13*). In *Robinia*, however, all of the nectar flavonoids are rhamnosides, and this is most likely the reason why they were not hydrolyzed by the enzymes present in honey and bee saliva, as rhamnosidases have not been reported in honey bee secretions (*25*−*28*). This would explain the occurrence of glycosides in acacia honey but would not explain the absence of compounds **6**−**8** in the honey chromatograms in spite of **7** and **8** being the main constituents in nectar. The common feature of these three "sensitive" flavonoids is the presence of a free hydroxyl at position 3, which is readily observed by their UV spectra, which has a band I wavelength maximum around 367 nm. These compounds with free hydroxyl in **3** are known to be rather unstable in mild alkaline conditions (*19*) and are also rather sensitive to oxidation in the presence of mild oxidants. Hydrogen peroxide is known to be one of the main antimicrobial compounds in honey and is produced by the action of glucose oxidase from bee secretions (*29, 30*). Thus, hydrogen peroxide could be responsible for the observed degradation of these flavonoids. To evaluate this possibility, *Robinia* nectar was incubated with diluted hydrogen peroxide, and samples were taken and analyzed after 2 and 6 days at room temperature. The results obtained are shown in the chromatogram **B** of **Figure 1**, showing that compounds **6**−**8**

Table 3. Phenolic Compounds and Abscisic Acid Contents in Acacia Honeys[a]

	R-001	R-469	R-409	R-656	R-466	R-579	R-655	S-001	S-004	S-011	S-012
						flavonoids					
F	105.38	711.60	330.53	282.65	84.31	345.03	323.37	58.18	40.86	71.32	119.44
G	173.36	959.62	600.91	412.55	79.76	342.92	299.88	242.56	343.05	310.19	492.47
O	206.40	881.36	678.84	372.34	100.94	317.91	392.66	165.00	192.81	196.24	323.45
P	119.01	366.90	179.82	121.09	63.51	101.06	182.97	45.25	23.30		122.16
H	12.13	67.28	32.19	12.75	3.95	12.53	17.39	11.76	0.00	11.87	21.76
I	31.84	51.95	84.21	42.19	7.58	44.88	51.54	41.53	34.12	14.92	33.46
J	21.77	160.95	71.16	28.31	8.24	29.74	39.64	37.66	8.82	34.94	48.02
K + L	39.99	188.80	104.46	50.58	13.82	37.50	64.32	31.03	13.55	31.75	57.44
M	27.19	122.80	70.00	34.22	4.78	23.99	41.75	11.78	7.55	17.93	31.99
Q	53.68	183.32	93.49	48.18	7.06	21.12	51.31	3.32	0	3.87	0.00
S	85.34	334.19	257.96	138.96	60.24	103.28	155.99	71.54	115.24	70.62	99.15
T	53.68	297.87	191.89	98.98	34.51	94.72	116.82	17.32	24.27	18.80	27.75
total	929.78	4348.24	2695.46	1642.80	468.7	1474.68	1737.64	736.93	803.57	782.45	1377.09
						hydroxycinnamic derivatives					
A	173.61	852.90	424.65	168.14	20.47	202.50	124.34	32.57	47.93	95.38	74.08
B	66.54	525.92	323.34	340.37	587.68	384.05	402.27	117.99	129.37	77.62	93.56
C	109.48	420.99	265.78	251.58	274.37	404.51	290.29	116.55	176.12	0.00	72.52
D	35.80	266.80	168.26	94.89	82.35	148.35	70.08	0.00	38.08	0.00	48.49
E	120.80	642.56	331.28	182.24	30.23	101.03	170.15	20.61	36.68	43.94	43.81
N	70.79	336.08	131.54	122.86	25.25	101.99	80.57	27.56	33.00	64.34	70.88
R	33.67	204.00	82.87	95.46	30.70	31.83	85.39	15.82	24.76	0.00	43.01
total	610.69	3249.25	1727.61	1255.54	1051.05	1374.26	1223.09	331.10	485.94	281.28	446.35
						abscisic acid					
ABA-1	5.34	81.90	22.70	14.87	8.74	49.71	14.68				
ABA-2	70.30	299.55	188.56	148.14	96.66	222.40	125.81	83.52	85.56	64.69	59.30

[a] Values are μg 100 g^{-1} honey. Honey samples were extracted using the Amberlite XAD-2 methodology (*14*). F, isosakuranetin; G, pinobanksin; O, pinocembrin; P, unidentified flavanone; H, quercetin; I, unidentified flavonol; J, kaempferol; K + L, apigenin + isohamnetin; M, acacetin; Q, methylquercetin (tentatively); S, chrysin; T, galangin; A, caffeic acid; B, *p*-coumaric acid; C, ferulic acid; D and E, caffeic acid derivatives; N, dimethyl-allyl-caffeate; R, phenyl-ethyl caffeate; ABA-1, *trans*−*trans* abscisic acid; and ABA-2, *cis*−*trans* abscisic acid.

Table 4. Kaempferol Glycosides in Acacia Honey[a]

	kaempferol glycosides						
	1 (*m*/*z* 901)	**2** (*m*/*z* 755)	**3** (*m*/*z* 593)	**4** (*m*/*z* 736)	**5** (*m*/*z* 593)	**7** (*m*/*z* 431)	total
R-001	48.23	14.50	17.86	147.71	37.09	8.88	274.27
R-469	25.70	17.54	35.87	81.50	36.26	22.96	219.84
R-409	101.93	22.20	49.41	252.83	58.77	16.25	501.39
R-656	32.02	4.79	12.68	101.64	15.45	8.40	174.98
R-466	97.98	10.02	22.30	163.56	32.46	7.84	334.16
R-579	140.39	29.85	64.76	417.51	106.40	32.46	791.37
R-655	67.31	12.67	24.15	203.01	42.96	12.37	362.47
S-001	33.80	8.41	15.12	106.71	24.01	14.26	202.31
S-004	25.89	3.25	19.78	44.49	6.23	1.48	101.12
S-011	26.98	3.71	13.31	47.07	12.17	4.28	107.52
S-012	25.86	17.73	32.21	77.70	15.49	9.01	178.00

[a] Values are μg 100 g^{-1} honey. Honey samples were extracted using C_{18} SPE cartridge. Compounds: **1**, kaempferol-3-*O*-(hexoxyl) robinoside-7-*O*-rhamnoside; **2**, kaempferol-3-*O*-(hexoxyl)robinoside; **3**, kaempferol-3-*O*-hexoside-7-*O*-rhamnoside; **4**, kaempferol-3-*O*-robinoside-7-*O*-rhamnoside; **5**, kaempferol-3-*O*-robinoside; and **7**, kaempferol-7-*O*-rhamnoside.

are readily degraded, while the rest of flavonols remain stable under these mild oxidative conditions. The degradation kinetics of *Robinia* nectar flavonoids (**Figure 6**) show that peak **7** + **8** is readily degraded, while the other flavonoids remain stable. The individual stability of compounds **7** and **8** was evaluated using the MS-MS extracted ion chromatogram analysis, as this was not possible using UV absorbance detection as both compounds coeluted. This shows that compound **8** degrades much faster than compound **7** (**Figure 7**), and this could explain why small amounts of **7** are detected in honey, while compound **8** was not detected at all. In addition, compound **7** could be produced from compound **3** by the action of glucosidases (**Figure 8**), and this could also contribute to its detection in honey in spite of its sensitivity to oxidation.

When the percentage of each flavonoid glycoside was evaluated both in the nectar and in the experimental acacia honey (without taking into consideration the flavonoids showing free hydroxyl at 3 that are degraded by oxidative methods as demonstrated), a decrease in compounds **1** and **3** was observed, while compounds **4** and **5** increased their content in honey (**Figure 9**). This could be explained by the effect of bee glucosidases that can release the terminal hexose in compounds **1**−**3** leading to compounds **4**−**7** (**Figure 8**). However, an increase in compound **7** is not observed due to its oxidative degradation. This shows that the nectar flavonoid profile can be modified during honey elaboration/ripening by both hydrolytic enzymes (glucosidases) and hydrogen peroxide (released by glucose-oxidase) present in bee secretions.

Analysis of Robinia Nectar Flavonoids in Acacia Honeys from Italy and Slovakia. Eleven acacia (*R. pseudacia*) honey samples were analyzed to detect the presence of the nectar flavonoid markers (**Table 1**). They were extracted both using the Amberlite XAD-2 method for the detection of flavonoid

aglycones and propolis-derived phenolics (**Table 3**) and by SPE extraction to evaluate the presence of flavonoid glycosides from nectar (**Table 4**). These analyses show that all 11 samples contain flavonoid aglycones (468−4348 μg/100 g honey) and hydroxycinnamic acid derivatives (281−3249 μg/100 g honey) characteristic from propolis and that most probably originate by diffusion from beeswax (*7, 31*). In addition, smaller amounts of abscisic acid isomers were also detected (**Table 3**). The propolis-derived compounds have been recognized as useful markers for the geographical origin of honey samples produced in temperate areas (*31*). In addition, the analyses confirm that the flavonoid glycosides from *Robinia* nectar were detected in all of the acacia honey samples analyzed (100−800 μg/100 g) (**Table 4**). This suggests that the analysis of these flavonoid glycosides could be useful to help in the determination of botanical origin of acacia honeys. These flavonoid glycosides were not detected in honey samples from different floral origins previously studied in our group (*5−8, 11−15*).

These results are particularly relevant as they indicate that flavonoid glycosides are detected for the first time in honey, and this enlarges considerably the number of possible suitable markers to be used for the determination of the floral origin of honeys. A re-examination of those honey samples studied previously should be carried out using SPE extraction combined with HPLC-DAD-MS-MS detection to locate possible flavonoid glycoside markers in other floral origin honeys.

The described method is suitable for the evaluation of the botanical origin of acacia honey. The certification of the botanical origin of honey is a very important criterion in adding value, since it assures the consumer the quality and authenticity of the product. Furthermore, the possibility of discriminating between flower origin honey and experimental sucrose honey enables the identification of adulteration by sugar syrups in the process of quality control and verification of origin.

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