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ARTICLE

# Identification of Botanical Biomarkers in Argentinean *Diplotaxis* Honeys: Flavonoids and Glucosinolates

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To select and establish floral biomarkers of the botanical origin of *Diplotaxis tenuifolia* honeys, the flavonoids and glucosinolates present in bee-deposited nectar collected from hive combs (unripe honey) and mature honey from the same hives fron which the unripe honey samples were collected were analyzed by LC-UV-PAD-ESI-MS$^n$. Glycosidic conjugates of the flavonols quercetin, kaempferol, and isorhamnetin were detected and characterized in unripe honey. *D. tenuifolia* mature honeys contained the aglycones kaempferol, quercetin, and isorhamnetin. The differences between the phenolic profiles of mature honey and freshly deposited honey could be due to hydrolytic enzymatic activities. Aliphatic and indole glucososinolates were analyzed in unripe and mature honeys, this being the first report of the detection and characterization of glucosinolates as honey constituents. Moreover, these honey samples contained different amounts of propolis-derived flavonoid aglycones (1765−3171 $\mu$g/100 g) and hydroxycinnamic acid derivatives (29−1514 $\mu$g/100 g). Propolis flavonoids were already present in the freshly deposited nectar, showing that the incorporation of these compounds to honey occurs at the early steps of honey production. The flavonoids quercetin, kaempferol, and isorhamnetin and the glucosinolates detected in the samples could be used as complementary biomarkers for the determination of the floral origin of Argentinean *Diplotaxis* honeys.

**KEYWORDS: *Diplotaxis tenuifolia*; floral markers; botanical origin; nectar; LC-UV-PAD-ESI-MS$^n$**

## INTRODUCTION

Honey characterization is traditionally carried out by palynological, physicochemical, and sensory analyses (*1*−*3*), although the botanical origin remains difficult to determine in some cases. Because honey botanical and geographical origins influence consumer preference, it is of great interest to find new methods for honey origin determination. The identification of floral markers to certify the botanical origin of a given honey is important for correct and legal labeling. In the past decade different compounds have been suggested as floral markers including the phenolic compounds kaempferol and ellagic acid or the group myricetin, tricetin, and luteolin as floral markers for rosemary, heather, and eucalyptus honeys, respectively (*4*−*8*). All of these markers were detected either in the floral nectar directly collected from the flowers or in the content of the bee honey sac, which has been found particularly useful in the study of the presence of specific markers for each floral origin (*4*, *8*, *9*). In these nectars, glucosidic conjugates of flavonoid compounds were generally detected, and these include sophorosides [glucosyl(1→2)glucosides] and gentiobiosides [glucosyl(1→6)glucosides]. These flavonoid glucosides are readily hydrolyzed by the bee saliva enzymes to render the corresponding aglycones that are the metabolites detected in most mature honeys (*4*, *8*). However, in a recent study, different flavonoid rhamnosides and rutinosides were found in some flower nectars and in the corresponding honeys, as was the case of kaempferol rhamnosides in acacia honey (*9*). It was suggested that these glycosidic compounds cannot be hydrolyzed by the glucosidase activity of the bee saliva (*8*, *9*), and therefore they remain as glycosides in honey as no rhamnosidase activity has been reported in bees either.

In the Pampas region of Argentina, *Diplotaxis tenuifolia* (L.) DC. ("wild rocket") was introduced as a melliferous species in 1923 (*10*). This plant became a widely spread weed and nowadays is used by honey bees (*Apis mellifera* L.) as an important source of nectar (*11*). *D. tenuifolia* is a herbaceus cruciferous plant that contains a wide range of bioactive phytochemicals such as natural antioxidants (vitamin C, carotenoids, and phenolics) and glucosinolates (*12*, *13*). Glucosinolates are $\beta$-thioglucoside *N*-hydroxysulfates containing a side chain and a $\beta$-D-glucopyranosyl moiety. The side chain determines whether the glucosinolate is defined as aliphatic, aromatic, or indole. Glucosinolates are hydrolyzed by the enzyme myrosinase (a glucosidase), producing bioactive breakdown products such as isothiocyanates and indoles. These compounds are known to confer the disease-preventive and health-promoting effects of cruciferous foods on human

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health (*14*−*16*). More than 120 glucosinolates have been identified in Brassicaceae, and the concentration and type of glucosinolates may vary depending on the development stage and also in different organs within the plant (leaves, flowers, seeds, and roots). Several unifloral honeys from different species belonging to the Brassicaceae family are known in Europe (*17*).

The main objective of this study is the phytochemical characterization of flavonoid glycosides and glucosinolates in *D. tenuifolia* honey, the determination of the changes occurring in these phytochemicals during the transformation of nectar into honey in the hive during honey ripening, and the potential use of these phytochemicals as honey floral markers.

## MATERIALS AND METHODS

**Honey Collection.** Three sets of honey samples were obtained from bee yards located nearby Saavedra (37° 46′ S, 62° 20′ W), Tornquist (38° 5′ S, 62° 12′ W), and Estación Napostá (38° 26′ S, 62° 15′ W) localities in southern Buenos Aires Province (Argentina). In the region prevail agricultural fields and cattle feed areas, in which *D. tenuifolia* is a widely spread perennial weed. Natural vegetation consists of herbaceous stratum, the most representative family being Poaceae accompanied by native shrubs in some areas. The most important crop is wheat (*Triticum aestivum* L.), and the main woody plant, generally located close to houses in the field, is *Eucalyptus* sp. A major annual weed, flowering after the harvest within the wheat fields, is *Centaurea solstitialis* L.

The botanical origin of honeys was confirmed by pollen analysis (*18*). Twenty-seven pollen types were identified. *D. tenuifolia* pollen content was dominant (>45%) and averaged 70.71% in the studied honeys. *Eucalyptus* sp. and *Centaurea* sp. pollens were present in all samples as minor pollens and *Oxalis* sp. and Heliantheae type as traces. Samples were stored at 4 °C in the dark for further analysis.

**Unripe Honey Collection.** Five samples were taken from the same beehives during the last week of March 2009. During an important nectar influx, unripe honey samples (freshly deposited nectar) were taken with a syringe from new combs and rapidly frozen.

**Extraction of Markers from Unripe Honey.** Phytochemical compounds from five different unripe *D. tenuifolia* honey samples were isolated using a solid-phase extraction cartridge (Sep-Pack SPE, Waters Millipore, USA). Briefly, the samples were diluted with ultrapure water (Milli-Q system, Millipore Corp., Bedford, MA) and centrifuged at 5000*g* for 10 min, in a Centromix centrifuge (Selecta, Barcelona, Spain). The supernatants were filtered through a Sep-pack cartridge previously activated with methanol Milli-Q water (10:10, v/v). The compounds that remained adsorbed in the cartridge were eluted with 1 mL of pure methanol. The aqueous phases were lyophilized and redissolved in 2 mL of Milli-Q water for detection of glucosinolates. All fractions from all samples were filtered through a 0.45 $\mu$m membrane filter Millex-$HV_{13}$ (Millipore Corp., USA) and stored at −80 °C until further analyzed by LC-UV-PDA-ESI-$MS^n$.

**Extraction of Flavonoids from *D. tenuifolia* Pollen.** A pollen sample (300 mg) was extracted with 1 mL of methanol/water (1:1; v/v) at room temperature for 10 min and directly filtered through a 0.45 mm filter; 20 $\mu$L was analyzed by HPLC under the same conditions as honey samples.

**Extraction of Markers from Honey.** Analysis of phytochemical compounds was determined according to the methodology previously reported (*9*), with some modifications. Honey samples (10 g) were homogenized in Milli-Q water (1:5, w/v) at pH 2 adjusted with HCl until complete dissolution. The solutions were then extracted using a Sep-Pack $C_{18}$ cartridge, which was previously activated as described above. After the cartridge had been rinsed with 10 mL of Milli-Q water, the flavonoid fraction was eluted with 2 mL of pure methanol. The aqueous phases for detection of glucosinolates were analyzed as described above.

**Analysis of Flavonoid Compounds by HPLC-PDA-Tandem Mass Spectrometry (MS-MS).** Chromatographic separations of unripe honey extracts were carried out in a 250 mm × 4.6 mm i.d., 5 $\mu$m, $C_{18}$ Phenomenex Luna column with a Phenomenex "Securityguard" precolumn with a C18 cartridge (Macclesfield, Cheshire, U.K.), using Milli-Q water (A) and pure methanol (B) as solvents (99.9%, HPLC gradient grade; Merck, Darmstadt, Germany). The flavonoids were separated using a linear gradient starting with 0% B at 0−5 min, reaching 17% B at 15 min, 25% B at 22 min, 35% B at 30 min, 50% B at 35 min, 99% B at 50 min, and 0% B at 55−65 min. The flow rate was 1 mL $min^{-1}$ and the injection volume 20 $\mu$L. All chromatograms were recorded at 270 and 330 nm.

The LC-UV-PDA-ESI-$MS^n$ analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode 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 and was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted at 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 $min^{-1}$, respectively. The full scan mass covered the range from $m/z$ 100 to 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in the negative ionization mode. $MS^n$ is carried out in the automatic mode on the more abundant fragment ion in MS ($n - 1$).

The phenolic compounds were identified and quantified according to their UV spectra, molecular weights, retention time, and MS/MS fragments, when possible, with commercially available standards: flavonols as quercetin-rutinoside at 330 nm, flavones as chrysin at 330 nm, and flavanones as hesperetin at 290 nm. All of these external standards were purchased from Sigma (St. Louis MO) except chrysin (Carl Roth, OGH, Karlsruhe, Germany).

**Analysis of Glucosinolates by HPLC-PAD-Tandem Mass Spectrometry (MS/MS).** Analysis of glucosinolates in unripe and mature honey samples was achieved using the same instruments, column, and gradient. In this case, the mobile phase was a mixture of (A) trifluoroacetic acid (TFA) 0.01% and (B) acetonitrile/TFA (99.9:0.01). These compounds were separated and identified by their UV spectra, molecular weights, and characteristic MS fragment ions following a previously reported LC-MS method (*19*, *20*).

## RESULTS AND DISCUSSION

**Flavonoids in Unripe *D. tenuifolia* Honey.** LC-UV-PDA-ESI-$MS^n$ screening of the five unripe honeys (nectars freshly deposited in the comb) showed similar chromatographic profiles for all samples (**Figure 1B**; **Table 1**). The major compound detected was a kaempferol derivative with a substituted hydroxyl in position 3 (**6**) (UV spectra) (**Table 2**) (*21*). Its deprotonated molecular ion at $m/z$ 609 $[M - H]^-$ indicated the presence of two hexoses and the $MS^2$ fragmentation showed losses of hexosyl (−162) and hexosyl plus water (−180) from $[M - H]^-$, which corresponded to $m/z$ 447 and 429, respectively (**Table 2**), and the deprotonated ion of kaempferol ($m/z$ 285) as base peak. This fragmentation is characteristic of a dihexoside with interglycosidic linkage 1→2 (*22*). The presence of glucose as the only hexose in a previous study of *D. tenuifolia* (*23*) leaves suggests that compound **6** is kaempferol-3-*O*-sophoroside (kaempferol-3-*O*-(1→2) diglucoside).

Compound **4** is a dihexoside of quercetin, the $MS^n$ fragmentation of which is similar to that of compound **6** (**Figure 1B**; **Table 2**). The UV spectrum of compound **4** could not be recorded due to coelution with compound **5** in a single chromatographic peak. All of these data, and its relative $t_R$ with respect to that of **6**, suggested that **4** could be tentatively characterized as quercetin-3-*O*-sophoroside. This compound, when observed, was detected in only trace amounts. The UV spectrum of peaks 4 + 5 was consistent with that of kaempferol-3-*O*-substituted (*21*). In the $MS^2$ fragmentation of compound **5** a base peak at $m/z$ 285 corresponding to the deprotonated aglycone ion $[kaempferol - H]^-$ was observed, indicating that only one phenolic hydroxyl was glycosylated (*22*). Another minor ion with a relative abundance of 20% corresponding to the loss of a hexosyl radical was also observed (*22*). This fragmentation pattern differs from that of gentiobiosides

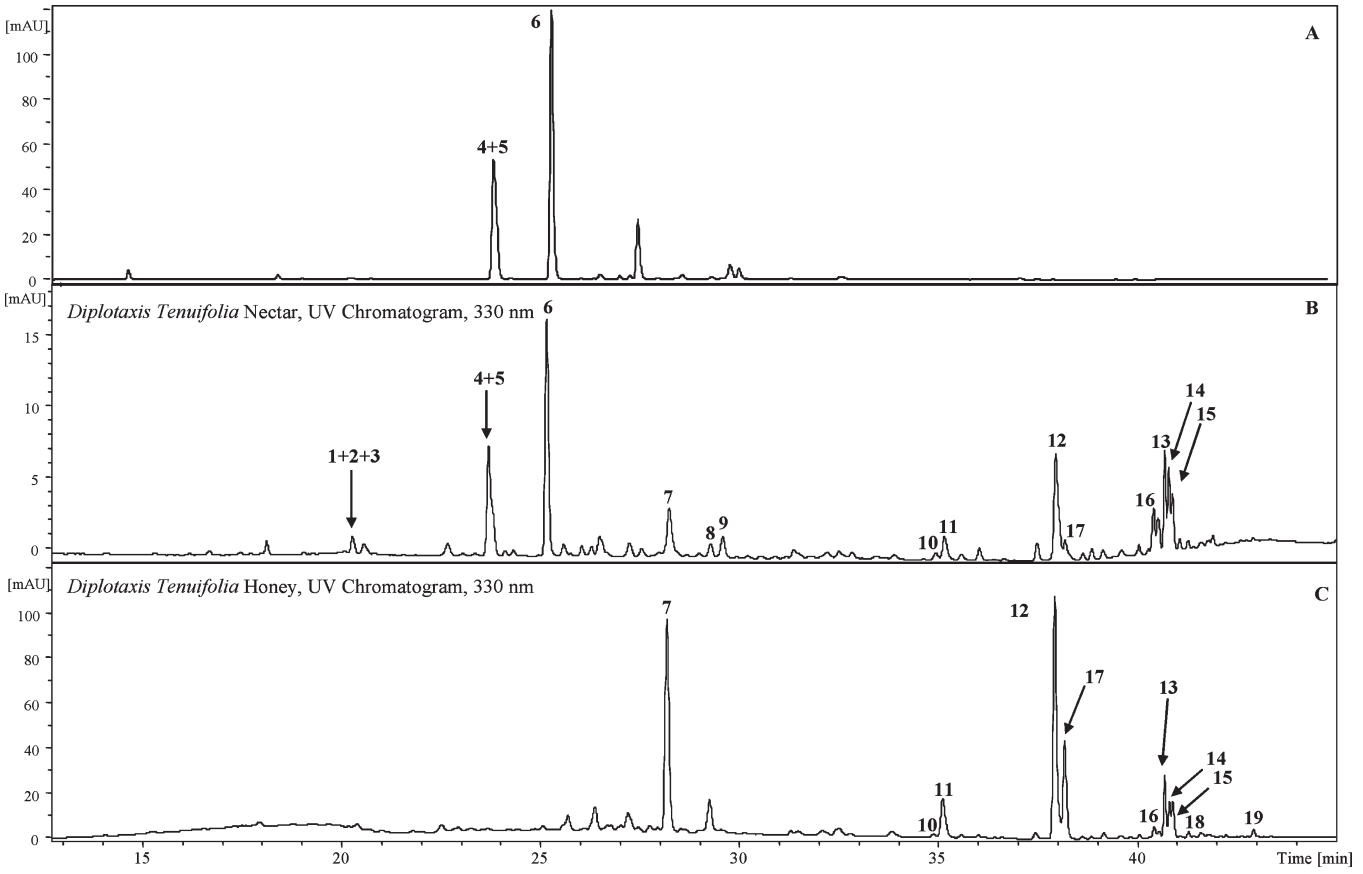


**Figure 1.** HPLC-UV chromatogram of *Diplotaxis tenuifolia* pollen (**A**), unripe honey (**B**), and *D. tenuifolia* mature honey (**C**) at 330 nm. Peaks: 1, quercetin-3,3′,4′-tri-*O*-glucoside; 2, isorhamnetin-3-*O*-glucoside-4′-*O*-gentiobioside; 3, quercetin-3,4′-di-*O*-glucoside; 4, quercetin-3-*O*-sophoroside; 5, kaempferol-3-*O*-diglucoside isomer; 6, kaempferol-3-*O*-sophoroside; 7, isorhamnetin-4′-*O*-gentiobioside; 8, isorhamnetin-4′-*O*-glucoside; 9, kaempferol-4′-*O*-glucoside; 10, pinobanksin; 11, quercetin; 12, kaempferol; 13, isorhamnetin; 14, dimethylallyl caffeate; 15, chrysin; 16, pinocembrin; 17, pinostrobin; 18, pinocembrim 3-acetate; 19, tectochysin.

**Table 1.** Phenolic Compound Contents in *Diplotaxis tenuifolia* Unripe Honeys from Different Localities in Argentina[a]

| peak | compound | DU[b]-1 | DU-2 | DU-3 | DU-4 | DU-5 |
|---|---|---|---|---|---|---|
| 1 + 2 + 3 | quercetin-3,3′,4′-triGlc[c] + isorhamnetin-3-Glc-4′-gentiobioside + quercetin-3,4′-diGlc | 0.65 | 0.31 | 1.28 | 0.85 | 0.82 |
| 4 + 5 | quercetin-3-sophoroside + kaempferol-3-diGlc isomer | 5.13 | 3.51 | 6.13 | 5.99 | 6.77 |
| 6 | kaempferol-3-sophoroside | 7.36 | 5.27 | 8.52 | 7.97 | 9.22 |
| 7 | isorhamnetin-4′-gentiobioside | 2.51 | 1.82 | 3.11 | 3.39 | 2.74 |
| 8 | isorhamnetin-4′-Glc | 0.52 | 0.39 | 0.68 | 0.84 | 0.88 |
| 9 | kaempferol-4′-Glc | 0.70 | 0.36 | – | 0.72 | 0.60 |
| 10 | pinobanksin | 4.93 | 3.09 | 5.41 | 6.52 | 6.19 |
| 11 | quercetin | 0.97 | 0.44 | 1.07 | 1.07 | 1.03 |
| 12 + 13 | kaempferol + isorhamnetin | 3.18 | 2.30 | 3.96 | 4.08 | 3.52 |
| 14 | dimethylallyl caffeate | 0.85 | 1.10 | 1.90 | 2.15 | 2.24 |
| 15 | chrysin | 1.86 | 5.74 | 3.18 | 0.67 | 6.28 |
| 16 | pinocembrin | 5.61 | 0.52 | 1.19 | 1.14 | 1.11 |
| 17 | pinostrobin | 1.04 | 0.46 | 1.07 | 1.17 | 0.70 |
| | **total** | **35.31** | **25.31** | **37.50** | **36.56** | **42.10** |

[a] Values are $\mu$g/100 g of unripe honey; –, not detected; tr, trace amounts. [b] DU, *Diplotaxis tenuifolia* unripe honey. [c] Glc, glucoside.

[glucosyl(1→6)glucosides] (*24*), and the $t_R$ of **5** is shorter than that of the sophoroside (**6**), indicating that **5** has an interglycosidic linkage, either (1→3) or (1→4), but the available data do not allow a more specific assignment.

Compounds **1** and **2** (**Figure 1B**; **Table 2**) coeluted under a single chromatographic peak, and MS analysis indicated that they were quercetin and isorhamnetin trihexosides, $[M - H]^-$ at $m/z$ 787 and 801, respectively. The $MS^2$ $[787]^-$ and $MS^3$ $[787 \rightarrow 625]^-$ fragmentations of **1** showed the ions at $m/z$ 625 and 463 as base peaks, respectively (**Table 2**; **Figure 2**). This fragmentation pattern is consistent with that of quercetin-3,3′,4′-tri-*O*-glucoside that was previously described in *D. tenuifolia* leaves (*23*). This compound was not detected in all of the unripe honey samples analyzed. When it was absent, a quercetin di-*O*-hexoside (**3**) was observed instead (**Table 1**), the $MS^2$ fragmentation (**Table 2**) of which was characteristic of a quercetin-di-*O*-hexoside (glycosylation in different phenolic hydroxyl positions). As compounds **1** and **3** show the same retention time ($t_R$), this suggests that

**Table 2.** $t_R$, UV, MS $[M - H]^-$, and $-MS^2$ $[M - H]^-$ Data of Flavonoids from *Diplotaxis tenuifolia* Unripe Honeys[a]

| | | | | | $-MS^2$ $[M - H]^-$ (*m*/*z*) (%) | | | |
|---|---|---|---|---|---|---|---|---|
| peak | compound[b] | $t_R$ (min) | UV (nm) | $[M - H]^-$ | −162 | −180 | −324 | $[Aglc-H/2H]^-$ |
| 1 | Querc-3,3′,4′-triGlc[c] | 22.3 | 255, 267sh,[d] 353[e] | 787 | 625 (100) | | 463 (10) | |
| 2 | Isrhmn-3-Glc-4′diGlc[c] | 22.3 | 255, 267sh, 353[e] | 801 | 639 (96) | | 477 (100) | 315 (33) |
| 3 | Querc-3,4′-diGlc | 22.3 | 255, 267sh, 353[e] | 625 | 463 (100) | | | 301 (30) |
| 4 | Querc-3-diGlc | 23.1 | 265, 295sh, 347[f] | 625 | 463 (10) | 445 (27) | | 300 (100) |
| 5 | Kaempf-3-diGlc isomer | 23.1 | 265, 295sh, 347[f] | 609 | 447 (20) | | | 285 (100) |
| 6 | Kaempf-3-diGlc | 25.6 | 265, 299sh, 347 | 609 | 447 (6) | 429 (60) | | 285 (100) |
| 7 | Isrhmn-4′-diGlc | 27.1 | 255, 267sh, 370 | 639 | | | | 315 (100) |
| 8 | Isrhmn-4′-Glc | 28.2 | 255, 266sh, 325sh, 365 | 477 | | | | 315 (100) |
| 9 | Kaempf-4′-Glc | 28.3 | 267, 315sh, 363 | 447 | | | | 285 (100) |
| 10 | pinobanksin | 34.9 | | 271 | | | | |
| 11 | quercetin | 35.1 | | 301 | | | | |
| 12 | kaempferol | 38.2 | 253sh, 267, 300sh, 320sh, 367 | 285 | | | | |
| 13 | isorhamnetin | 38.9 | | 315 | | | | |
| 14 | dimethylalylcaffeate | 40.3 | | 247 | | | | |
| 15 | chrysin | 41.9 | 267, 313 | 253 | | | | |
| 16 | pinocembrin | 42.1 | 289, 331sh | 255 | | | | |
| 17 | pinostrobin | 42.3 | 293, 333sh | 269 | | | | |

[a] Main observed fragments. Other ions were found but they have not been included. [b] Isrhmn, isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone); Querc, quercetin (3,5,7,3′,4′-pentahydroxyflavone); Kaempf, kaempferol (3,5,7,4′-tetrahydroxyflavone); chrysin, 5,7-dihydroxyflavone; pinocembrin, 5,7-dihydroxyflavanone; pinostrobin, 5-hydroxy-7-methoxyflavanone. [c] $MS^3$ [787→625]$^-$, 463 (100); $MS^3$ [787→463]$^-$, 301 (100); $MS^3$ [801→639]$^-$, 315 (100); $MS^3$ [801→477]$^-$, 315 (100). [d] sh, shoulder. [e] UV spectra of 1 + 2 + 3. [f] Compounds **4** and **5** coeluted, and the UV spectrum recorded was that of the major compound (**5**).

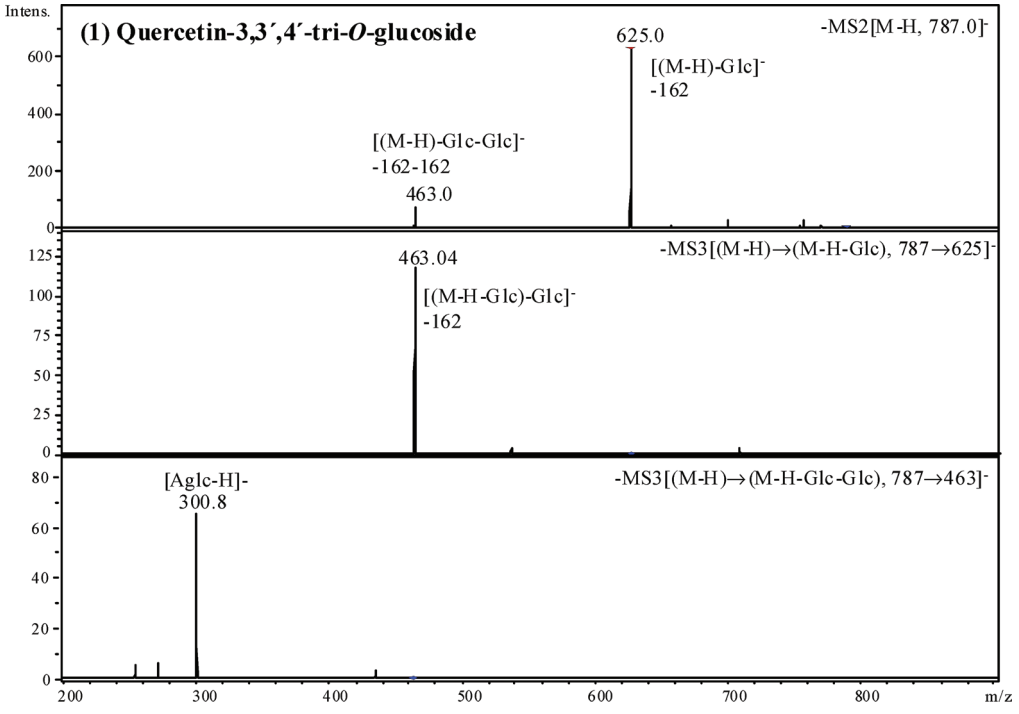


**Figure 2.** $MS^2$ and $MS^3$ spectra of quercetin-3,3′,4′-tri-*O*-glucoside (compound **1**).

compound **3** might be an artifact generated from **1** in the ion-transfer process.

The $MS^2$ analysis of compound **2** showed ions at the *m*/*z* 639 and 477 as main peaks and the deprotonated aglycone ion $[Aglc-H]^-$ in minor abundance (**Table 2**; **Figure 3**). These results pointed out the glycosylation on two different phenolic hydroxyls of the flavonoid nucleus (*22*). In a previous study on the leaves of *Diplotaxis* spp., flavonols with a free hydroxyl at position 7 and glycosylations at 3- and 4′-positions of the flavonoid nucleus were identified (*23*). In addition, the $MS^3$ [801→639]$^-$ fragmentation (**Figure 3**) and the absence of any other fragment ions indicate that compound **2** has an interglycosidic linkage (1→6), suggesting that **2** could be characterized as isorhamnetin-3-*O*-glucoside-4′-*O*-gentiobioside.

Compounds **7**, **8**, and **9** showed characteristic UV spectra of flavonols with a free hydroxyl at position 3. Compound **7** showed a deprotonated molecular ion at *m*/*z* 639, and its $MS^2$ fragmentation showed only one peak corresponding to the deprotonated aglycone ion *m*/*z* 315 without intermediate ions. This indicates that **7** is a gentiobioside. The position of the glycosylation should be at 4′ (*23*); therefore, compound **7** can be characterized as isorhamnetin-4′-*O*-gentiobioside. For the same reasons, compounds **8** and **9** were identified as isorhamnetin and kaempferol monohexosides and could be characterized as isorhamnetin-4′-*O*-glucoside and kaempferol-4′-*O*-glucoside, respectively. The UV spectra, MS, and $t_R$ for compound **12** suggest that this was kaempferol aglycone, and this was confirmed by comparison with

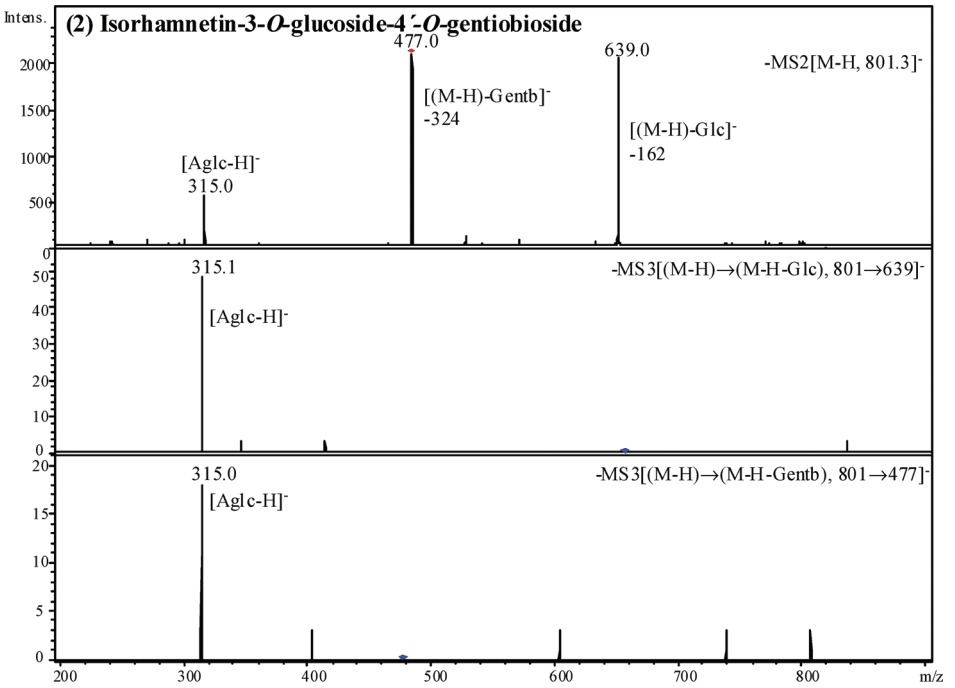


**Figure 3.** $MS^2$ and $MS^3$ spectra of isorhamnetin-3-*O*-glucoside-4′-*O*-gentiobioside (compound **2**).

an authentic sample. Some nectar samples obtained from flowers of *D. tenuifolia* should be analyzed in the future to confirm the flavonoid glucoside profiles detected in the present study.

Although *D. tenuifolia* nectar was not available, *D. tenuifolia* pollen was extracted and HPLC-MS analyzed (**Figure 1A**). Pollen contained mainly compounds **4**, **5**, and **6**, which are the main flavonoid glycosides detected in the unripe honey samples. Compounds **4** and **6** are quercetin and kaempferol sophorosides, which are characteristic flavonoids of pollens from different botanical origins. The unripe honey flavonoid profile is a combination of *D. tenuifolia* pollen flavonoids and flavonoid glycosides that have been previously found in *D. tenuifolia* leaves (flavonoids **1**, **2**, **3**, **7**, **8**, and **9**) (*23*). This suggests that the flavonoid glycosides present in the unripe honeys can originate in *D. tenuifolia* nectar, although this has not been analyzed and should be studied in the future.

In addition, the HPLC chromatograms recorded at 330 nm of unripe honeys showed a series of lipophilic flavonoid aglycones and other phenolics characteristic of propolis known to be present in honeys produced in temperate regions, being often the main flavonoids in honey (*25*, *26*). The lipohilic flavonoid fingerprints are similar for unripe and mature honeys (**Figure 1B**,**C**). The profiles were similar to those obtained from propolis samples from northern Argentina (results not shown), which are characterized by the presence of phenolic acids and flavonoid aglycones (*27*, *28*). The flavanones pinocembrin (**16**), pinobanksin-acetate (**18**), and pinostrobin (**17**), the flavones chrysin (**15**) and tectochrysin (**19**), and the phenolic acid dimethylallyl caffeate (**14**) were detected in all honey samples (unripe or mature) in different amounts, as shown in **Table 3**. The presence of these compounds in the unripe and mature honey samples is quite variable as it is thought to be related to the degree of propolis contamination in the hive and beeswax (*25*, *26*). The occurrence of propolis flavonoids in the unripe honey samples and in levels similar to those found in mature honeys is of interest because it suggests that the propolis-derived flavonoids are transferred to honey at the early steps of nectar deposit in the comb (**Figure 1B**). A question that remains to be answered is if propolis flavonoids diffuse passively from the beeswax to the honey matrix, or if they are actively incorporated into honey by the bee during the harvest, transport, and deposit of nectar in the hive. The results of this study show no difference between the content of propolis-derived flavonoids in unripe and mature honey, suggesting that passive diffusion is not the main mechanism for propolis incorporation into honey and that an active function of the bee cannot be disregarded. The occurrence of propolis flavonoids in the wax scales freshly secreted by the bee, which suggested the intake of propolis flavonoids by the bee before the transfer to the wax scales (*29*), is another argument in favor of the active incorporation of these phenolics rather than a passive contamination in the hive.

**Flavonoids in *D. tenuifolia* Honeys.** The HPLC-PDA chromatogram of honey methanolic extracts revealed the presence of quercetin (**11**), kaempferol (**12**), and isorhamnetin (**13**) in all samples (**Figure 1C**). In previous studies of the flavonoids in different *Brassica* spp., these flavonol aglycones were always detected in leaves, roots, and flowers (*12*, *23*, *30*−*32*). However, this is the first time that this type of compound is studied in honeys from wild cruciferous plants and particularly from *D. tenuifolia*. The flavonoid glucosides present in unripe honey (**Figure 1B**) were not detected in the mature honey samples (**Figure 1C**). These results are in agreement with previous studies, where the flavonoid glucosides present in nectar were hydrolyzed to give the corresponding aglycones. This was attributed to the glucosidase activity present in bee saliva (*4*, *8*). This phenomenon could explain the occurrence of kaempferol, isorhamnetin, and quercetin in mature honey, whereas unripe honey contained mainly their flavonoid glucosides. However, isorhamnetin 4′-diglucoside (**7**) was detected in different proportions in two of the analyzed mature honey samples (data not shown). This variability could suggest a different degree of ripening for the analyzed honey samples or contamination of the honey samples with other floral origin. However, quercetin (**11**), kaempferol (**12**), and isorhamnetin (**13**) were the main phenolic metabolites present in all of the analyzed honeys. These compounds, however, have been previously found in honeys from different geographical and botanical origins (*4*, *25*, *26*, *35*) and have recently been identified in Cuban honey samples (*36*), suggesting that their potential use as botanical origin markers of honey can be rather limited.

**Table 3.** Phenolic Compound Content in *Diplotaxis tenuifolia* Mature Honeys[a]

| peak | compound | $t_R$ | DM[b]-1 | DM-2 | DM-3 |
|---|---|---|---|---|---|
| | | | **Flavonoids** | | |
| 7 | isorhamnetin-4′-diGlc | 28.1 | 1396.97 ± 267.75 | 307.70 ± 68.03 | nd |
| 10 | pinobanksin | 35.0 | 43.57 ± 24.26 | 220.75 ± 3.00 | 739.12 ± 45.01 |
| 11 | quercetin | 35.1 | 165.76 ± 45.19 | 332.87 ± 67.25 | 189.74 ± 0.19 |
| 12 | kaempferol | 37.8 | 787.93 ± 229.39 | 647.77 ± 99.77 | 210.45 ± 33.17 |
| 13 | isorhamnetin | 38.1 | 449.72 ± 8.94 | 99.88 ± 25.85 | 113.79 ± 1.65 |
| 15 | chrysin | 40.3 | 56.46 ± 4.72 | 54.72 ± 13.45 | 80.48 ± 13.14 |
| 16 | pinocembrin | 40.7 | 146.73 ± 39.56 | 12.77 ± 26.44 | 102.04 ± 42.46 |
| 17 | pinostrobin | 41.1 | 94.64 ± 23.27 | 66.87 ± 18.78 | 63.53 ± 0.00 |
| 18 | pinobanksin-3-acetate | 41.8 | 15.87 ± 4.94 | 9.08 ± 1.69 | 5.56 ± 0.00 |
| 19 | tectochrysin | 42.3 | 10.23 ± 0.89 | 12.43 ± 0.60 | 16.81 ± 0.09 |
| | **total** | | **3170.88** | **1764.84** | **1521.52** |
| | | | **Hydroxycinnamic Acids** | | |
| OH1 | chlorogenic acid | 19.9 | | 36.94 ± 10.44 | 250.21 ± 15.21 |
| OH2 | unidentified caffeic acid derivative | 22.5 | | | 165.55 ± 0.93 |
| OH3 | *p*-coumaric acid | 26.2 | | | 256.45 ± 12.30 |
| OH4 | unidentified caffeic acid derivative | 27.3 | | | 244.59 ± 10.54 |
| OH5 | ferulic acid | 28.6 | 15.54 ± 0.00 | 25.85 ± 2.08 | 575.51 ± 156.43 |
| 14 | dimethylallyl caffeate | 40.4 | 14.42 ± 0.00 | 208.18 ± 61.67 | 21.75 ± 0.40 |
| | **total** | | **29.96** | **270.99** | **1514 06** |

[a] Values are μg/100 g of honey; nd, not detected; tr, trace amounts. [b] DM, *Diplotaxis tenuifolia* mature honey.

**Glucosinolates in Unripe and Mature *D. tenuifolia* Honeys.** The study of $MS^2$ $[M - H]^-$ fragmentation of the aqueous extracts showed different glucosinolate-derived peaks corresponding to a specific product ion at *m*/*z* 259, which corresponds to the fragment ions from the aglycone side chain and to the sulfate group, respectively, that are considered glucosinolate maker ions as reported elsewhere (**Figures 4** and **5**) (*20*, *30*, *33*). In the unripe honey samples the aliphatic glucosinolates glucoiberin (*m*/*z* 422), glucobrassicanapin (*m*/*z* 386), and glucoraphanin (*m*/*z* 436) and the indolic glucosinolates 4-hydroxyglucobrassicin (*m*/*z* 463), glucobrassicin (*m*/*z* 447), and neoglucobrassicin (*m*/*z* 477) were detected. The nature and structure of the glucosinolates were confirmed by their $t_R$, UV spectra, molecular masses, and MS/MS fragmentation as well as the characteristic product ions cited above (*20*, *30*, *33*, *34*) (**Table 4**).

Glucoraphanin and glucoerucin have been previously reported as chemotaxonomic markers of *D. tenuifolia* (*12*, *30*). Although glucoerucin was not detected in the samples analyzed, all of the other characteristic glucosinolates of the Brassicaceae, including glucoraphanin and glucoiberin, as well as the indolic compounds glucobrassicin, glucobrassicanapin, and neoglucobrassicin, were detected in *D. tenuifolia* unripe honey. These results could indicate that Brassicaceae honeys could share some characteristic chemical markers of the glucosinolate family.

In the study of LC-UV-PAD-ESI-$MS^n$ of the aqueous extract of mature honey, the aliphatic glucosinolates glucoiberin and glucobrassicanapin were detected. Indolic glucosinolates are known to be chemically less stable than aliphatic glucosinolates (*12*, *14*) and, during the ripening of honey in the hive, the indolic glucosinolates could be degraded to isothiocyanates, indoles, and other unstable compounds as a result of the action of glucohydrolases as well as nonenzymatic conversion to other products (*12*, *14*). The indolic glucosinolates could be more affected by abiotic stresses and changes upon different environmental conditions during maturation or storage of the honey, which could explain how the glucosinolate composition varied with losses of the more sensitive compounds (**Table 4**) (*14*, *15*). Therefore, the presence or absence of these nitrogen−sulfur compounds might be used as bioindications of the freshness of a given honey, but this preliminary finding should be confirmed in more detailed studies.

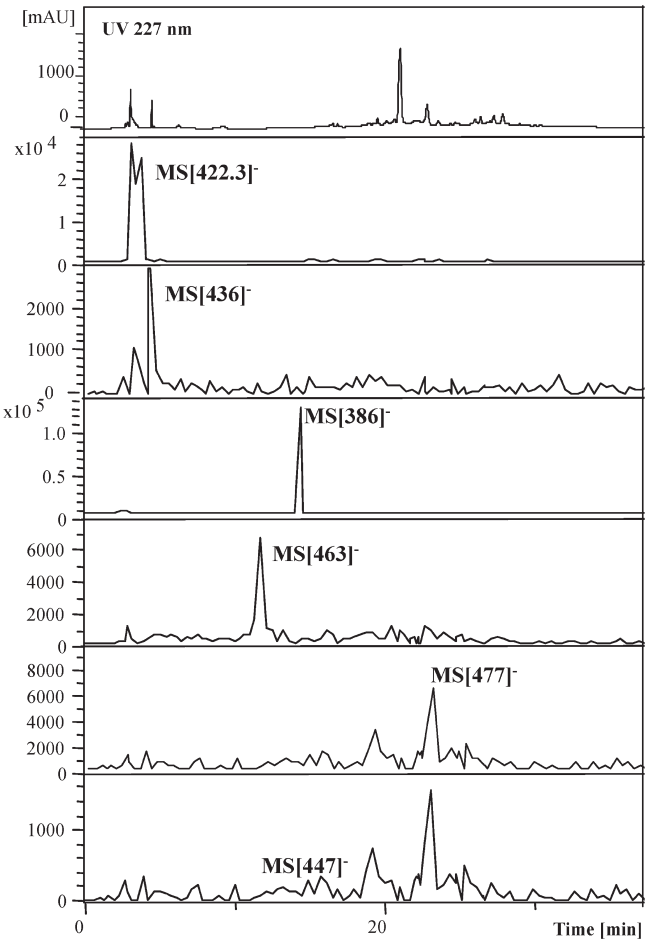


**Figure 4.** HPLC-MS EIC (extracted ion chromatogram) of glucosinolates in aqueous extracts from unripe honey (nectar) of *D. tenuifolia*.

This is the first report, to the best of our knowledge, of glucosinolates being detected and identified in "nectar" and honey produced from Brassicaceae species and particularly from

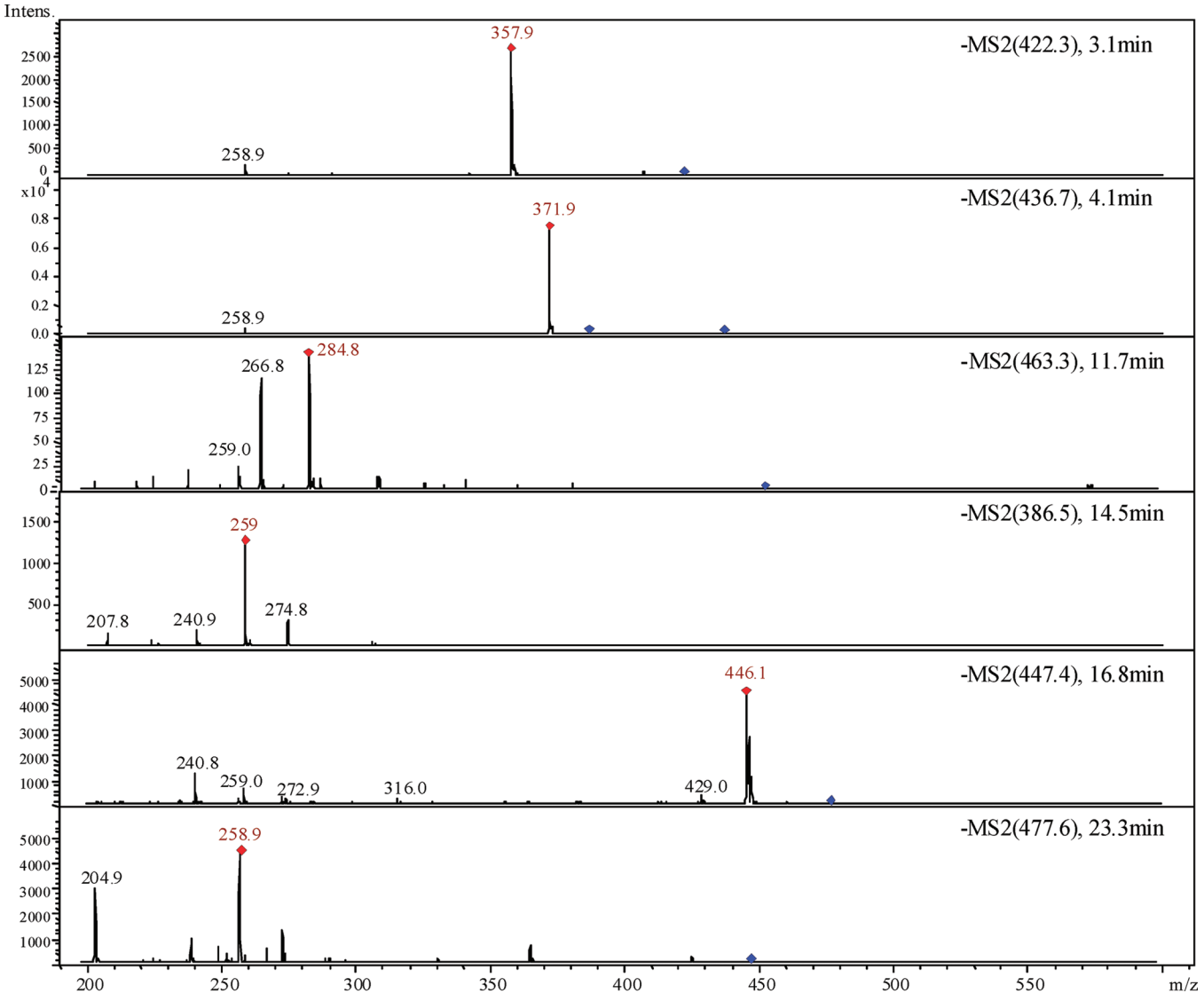


**Figure 5.** $MS^2$ spectra of glucosinolates in unripe honey (nectar) of *D. tenuifolia*.

**Table 4.** Identified Glucosinolates in *Diplotaxis tenuifolia* Unripe Honey and Mature Honey, with Their Retention Times and MS Data

| compound | $t_R$ (min) | $[M-H]^-$ | $MS^2$ $[M-H]^-$, *m/z* |
|---|---|---|---|
| unripe honey (nectar) | | | |
| glucoiberin | 3.0 | 422 | 358, 259 |
| glucoraphanin | 4.0 | 436 | 375, 294, 259 |
| 4-hydroxyglucobrassicin | 11.7 | 463 | 383, 285, 267, 259 |
| glucobrassicanapin | 15.0 | 386 | 285, 259 |
| glucobrassicin | 16.8 | 447 | 275, 259, 251 |
| neoglucobrassicin | 24.6 | 477 | 477, 466, 284, 259 |
| mature honey | | | |
| glucoiberin | 3.0 | 422 | 358, 259 |
| glucobrassicanapin | 15.0 | 386 | 285, 259 |

*D. tenuifolia*. However, quantification of the glucosinolate concentration was not possible due to the limited amount available of each sample.

As a conclusion, it could be highlighted that the phytochemical profiling of flavonoid-*O*-glucosides of kaempferol, quercetin, and isorhamnetin detected in unripe honey and mature honeys of *D. tenuifolia* as well as the corresponding aglycones of these flavonoids, together with the differences in abundance and composition of aliphatic and indole glucosinolates in nectars and honeys, could represent a potential tool of chemical markers for the determination of the botanical origin of Argentinian *Diplotaxis* honeys, as well as to evaluate the degree of honey maturation.

## LITERATURE CITED

(1) Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.* **1998**, *63*, 549−562.

(2) Bogdanov, S.; Ruoff, K.; Persano Oddo, L. Physicochemical methods for characterization of unifloral honeys. A review. *Apidologie* **2004**, *35*, S4−S17.

(3) Piana, M. L.; Persano Oddo, L.; Bentabol, A.; Bruneau, E.; Bogdanov, S.; Guyot Declerck, C. Sensory analysis applied to honey: state of the art. *Apidologie* **2004**, *35*, S26−S37.

(4) Gil, M. I.; Ferreres, F.; Ortiz, A.; Subra, E.; Tomás-Barberán, F. A. Plant phenolic metabolites and floral origin of rosemary honey. *J. Agric. Food Chem.* **1995**, *43*, 2833−2838.

(5) Ferreres, F.; Andrade, P.; Gil, M. I.; Tomás-Barberán, F. A. Floral nectar phenolics as biochemical markers for the botanical origin of heather honey. *Eur. Food Res. Technol.* **1996**, *202*, 40−44.

(6) Andrade, P.; Ferreres, F.; Amaral, M. T. Analysis of honey phenolic acids by HPLC, its application to honey botanical characterization. *J. Liq. Chromatogr. Relat. Technol.* **1997**, *20*, 2281−2288.

(7) Martos, I.; Ferreres, F.; Tomás-Barberán, F. A. Identification of flavonoid markers for the botanical origin of eucalyptus honey. *J. Agric. Food Chem.* **2000**, *48*, 1498−1502.

(8) Truchado, P.; Ferreres, F.; Tomás-Barberán, F. A. Liquid chromatography−tandem mass spectrometry reveals the widespread occurrence of flavonoid glycosides in honey, and their potential as floral origin markers. *J. Chromatogr., A* **2009**, *1216*, 7241−7248.
(9) Truchado, P.; Ferreres, F.; Bortolotti, L.; Sabatini, A. G.; Tomás-Barberán, F. A. Nectar flavonol rhamnosides are floral markers of acacia (*Robinia pseudacacia*) honey. *J. Agric. Food Chem.* **2008**, *56*, 8815−8824.
(10) Valle, A.; Andrada, A.; Aramayo, E.; Gallez, L.; Lamberto, S. Mieles de la Región Periserrana del Sistema de Ventania. *Invest. Agrar.* **2001**, *16*, 341−354.
(11) Valle, A.; Andrada, A.; Aramayo, E.; Gil, M.; Lamberto, S. A melissopalynological map of the south and southwest of the Buenos Aires Province, Argentina. *Span. J. Agric. Res.* **2007**, *5*, 172−180.
(12) Fahey, J. W.; Zalcmann, A. T.; Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocianates among plants. *Phytochemistry* **2001**, *56*, 5−51.
(13) McNaughton, S. A.; Marks, G. C. Development of a food composition database for the estimation of dietary intakes of glucosinolates, the biologically active constituents of cruciferous vegetables. *Br. J. Nutr.* **2003**, *90*, 687−697.
(14) Halkier, B. A.; Gershenzon, J. Biology and biochemistry of glucosinolates. *Annu. Rev. Plant. Biol.* **2006**, *57*, 303−333.
(15) Mithen, R. F.; Dekker, M.; Verkerk, R.; Rabot, S.; Johnon, I. T. The nutritional significance, biosynthesis and bioavailability of glucosinolates in human foods. *J. Sci. Food Agric.* **2000**, *80*, 967−984.
(16) Surh, Y. J.; Kundu, J. K.; Na, H. K. Nrf2 as a master redox switch in turning on the cellular signalling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med.* **2008**, *74*, 1526−1539.
(17) Persano Oddo, L.; Piana, L.; Bogdanov, S.; Bentabol, A.; Gotsiou, P.; Kerkvliet, J.; Martin, P.; Morlot, M.; Ortiz Valbuena, A.; Ruoff, K.; Von Der Ohe, K. Botanical species giving unifloral honey in Europe. *Apidologie* **2004**, *35*, S82−S93.
(18) Louveaux, J.; Maurizio, A.; Vorwohl, G. Methods of Melissopalynology by International Commission for Bee Botany of IUBS. *Bee World* **1978**, *59*, 139−157.
(19) Martínez-Sánchez, A.; Allende, A.; Bennett, R. N.; Ferreres, F.; Gil, M. I. Microbial, nutritional and sensory quality of rocket leaves as affected by different sanitizers. *Postharvest Biol. Technol.* **2006**, *42*, 86−97.
(20) Francisco, M.; Moreno, D. A.; Cartea, M. E.; Ferreres, F.; García-Viguera, C.; Velasco, P. Simultaneous identification of glucosinolates and phenolic compounds in a representative collection of vegetable *Brassica rapa*. *J. Chromatogr., A* **2009**, *1216*, 6611−6619.
(21) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer: New York, 1970.
(22) Ferreres, F.; Llorach, R.; Gil-Izquierdo, A. Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* **2004**, *39*, 312−321.
(23) Martínez-Sánchez, A.; Llorach, R.; Gil, M. I.; Ferreres, F. Identification of new flavonoid glycosides and flavonoid profiles to characterize rocket leafy salads (*Eruca vesicaria* and *Diplotaxis tenuifolia*). *J. Agric. Food Chem.* **2007**, *55*, 1356−1363.
(24) Cuyckens, F.; Rozenberg, R.; Hoffmann, E.; Claeys, M. Structure characterization of flavonoid *O*-diglycosides by positive and negative nano-electrospray ionization ion trap mass spectrometry. *J. Mass Spectrom.* **2001**, *36*, 1203−1210.
(25) Tomás-Barberán, F. A.; Ferreres, F.; Garcia-Viguera, C.; Tomas-Lorente, F. Flavonoids in honey of different geographical origin. *Z. Lebensm.-Unters. Forsch.* **1993**, *196*, 38−44.
(26) Tomás-Barberán, F. A.; Martos, I.; Ferreres, F.; Radovic, B. S.; Anklam, E. HPLC Flavonoid profiles as markers for the botanical origin of European unifloral honeys. *J. Sci. Food Agric.* **2001**, *81*, 485−496.
(27) Gardana, C.; Scaglianti, M.; Pietta, P.; Simonetti, P. Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *45*, 390−399.
(28) Lima, B.; Tapia, A.; Luna, L.; Fabani, M. P.; Schmeda-Hirschmann, G.; Podio, N. S.; Wunderlin, D. A.; Feresin, G. E. Main flavonoids, DPPH activity, and metal content allow determination of the geographical origin of propolis from the Province of San Juan (Argentina). *J. Agric. Food Chem.* **2009**, *57*, 2691−2698.
(29) Tomas-Barberan, F. A.; Ferreres, F.; Tomas-Lorente, F.; Ortiz, A. Flavonoids from *Apis mellifera* beeswax. *Z. Naturforsch.* **1993**, *48C*, 68−72.
(30) Bennett, R. N.; Rosa, A. S. E.; Mellon, F. A.; Kroon, P. A. Ontogenic profiling of glucosinolates, flavonoids and other secondary metabolites in *Eruca sativa* (salad rocket), *Diplotaxis erucoides* (wall rocket), *Diplotaxis tenuifolia* (wild rocket), and *Bunias orientalis* (Turkish rocket). *J. Agric. Food Chem.* **2006**, *54*, 4005−4015.
(31) Bennett, R. N.; Mellon, F. A.; Kroon, P. A. Screening crucifer seeds as sources of specific intact glucosinolates using ion-pair high-performance liquid chromatography negative ion electrospray mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 428−438.
(32) Martínez-Sánchez, A.; Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F. A comparative study of flavonoid compounds, vitamin C, and antioxidant properties of baby leaf Brassicaceae species. *J. Agric. Food Chem.* **2008**, *56*, 2330−2340.
(33) Fabre, N.; Poinsot, V.; Debrauwer, L.; Vigor, C.; Tulliez, J.; Fourasté, I.; Moulis, C. Characterisation of glucosinolates using electrospray ion trap and electrospray quadrupole time-of-flight mass spectrometry. *Phytochem. Anal.* **2007**, *18*, 306−319.
(34) Millán, S.; Sampedro, M. C.; Gallejones, P.; Castellón, A.; Ibargoitia, M. L.; Goicolea, M. A.; Barrio, R. J. Identification and quantification of glucosinolates in rapeseed using liquid chromatography−ion trap mass spectrometry. *Anal. Bioanal. Chem.* **2009**, *394*, 1661−1669.
(35) Martos, I.; Cossentini, M.; Ferreres, F.; Tomas-Barberan, F. A. Flavonoid composition of Tunisian honey and propolis. *J. Agric. Food Chem.* **1997**, *45*, 2824−2829.
(36) Alvarez-Suarez, J. M.; Gonzalez-Paramas, A. M.; Santos-Buelga, C.; Battino, M. Antioxidant characterization of native monofloral Cuban honeys. *J. Agric. Food Chem.* **2010**, *58*, 9817−9824.

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