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Liquid chromatography–tandem mass spectrometry reveals the widespread occurrence of flavonoid glycosides in honey, and their potential as floral origin markers

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ABSTRACT

HPLC-MS–MS analysis of unifloral honey extracts has shown the occurrence of flavonoid glycosides in most of the analyzed samples. These compounds are not present in large amounts, but can reach up to 600 μg/100 g honey in canola and rapeseed honeys. Rhamnosyl-hexosides (tentatively rutinosides and neohesperidosides) and dihexosides (hexosyl(1→2)hexosides and hexosyl(1→6)hexosides) of flavonols such as quercetin, kaempferol, isorhamnetin and 8-methoxykaempferol, are the main flavonoid glycosides found in honey. However, flavonoid triglycosides and monoglycosides are also detected in some floral origins. Eucalyptus and orange blossom nectars were collected and analyzed showing that nectar flavonoid glucosides, as is the case of eucalyptus flavonoids, can be readily hydrolyzed by the bee saliva enzymes, while flavonoid rhamnosyl-glucosides, as is the case of citrus nectar flavonoids, are not hydrolyzed, and because of these reasons the flavonoid glycoside content of citrus honey is higher than that of eucalyptus honey that contains mainly aglycones. The flavonoid glycoside profiles detected in honeys suggest that this could be related to their floral origin and the results show that the HPLC-MSn ion trap analysis of flavonoid glycosides in honey is a promising analytical method to help in the objective determination of the floral origin of unifloral honeys.

1. Introduction

Honey phytochemical composition depends on its floral origin, the contamination with propolis, a plant resin collected by bees for different purposes within the hive, and the type of honeybee subspecies. In addition, external factors such as climate, geographical origin and processing conditions can also affect honey phytochemical composition. Unifloral honeys are appreciated by the consumers as they are considered higher quality products with characteristic sensory properties. Unifloral honey production is, however, limited. For this reason, the objective determination of the floral origin of honeys has become a very important issue regarding honey quality.

The traditional technique used to identify honey botanical origin is the melissopalynological method (EU Council Directive 2001/110). However, several new analytical methodologies have been recently explored to help with the determination of both the botanical and geographical origins. These include gas chromatography (GC) [1], capillary electrophoresis (CE) [2–4] and HPLC-PAD [5–8]. In particular, HPLC-PAD allows the identification of different phytochemical compounds that can be use as botanical markers for the identification of the honey floral origin [9,10]. Currently, specific phytochemicals, particularly phenolic compounds, can be related to the floral origin of several honeys as is the case of kaempferol for rosemary honey [11], ellagic acid, benzoic acid, phenylacetic acid, mandelic acid and β-phenylactic acid for heather honey [12,13], abscisic acid for calluna and heather honeys [14], methyl springhare for manuka honey [15], kynurenic acid and 3-aminoacetophenone and 1-phenyl-ethanol for chestnut honey [16,17], terpenoid acids for linden honey [18], myricetin, tricetin and luteolin for eucalyptus honey [19,20] and hesperetin for citrus honey [21]. These metabolites are generally lipophilic and bee saliva enzymes have been suggested as the responsible for the transformation of the polar phytochemicals present in floral nectar into the metabolites detected in honey. A recent study has demonstrated the occurrence of kaempferol rhamnosides and rhamnosyl-glucosides in acacia honey [22] and indicated the inability of bee enzymes to hydrolyze specific glycosidic combinations present in plant nectars, as is the case of rhamnosides and rutinosides. This finding enlarged considerably the potential number of floral markers for the botanical origin of honey. Previous studies of honey phytochemicals were addressed to the study of lipophilic metabolites (flavonoid aglycones, terpenoids, alkaloids) while the occurrence of glycosidic phytochemicals had been neglected. With the development of HPLC-MSn equipments, the detection and identification of small

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Table 1
Honey samples.

	Samples	Floral origin	Location
1	Canola	*Brassica napus*	Bologna – Italy
2	Cherry blossom-1	*Prunus avium*	Frossaco – Italy
3	Cherry blossom-2	*Prunus avium*	Tornareccio-CH – Italy
4	Eucalyptus-1	*Eucalyptus camaldulensis*	Roma – Italy
5	Eucalyptus-2	*Eucalyptus* spp.	CI – Spain
6	Eucalyptus-3	*Eucalyptus* spp.	MK – Spain
7	Eucalyptus-4	*Eucalyptus* spp.	Callosa Segura – Spain
8	Linden-1	*Tilia* ssp.	Sebecheleby – Slovakia
9	Linden-2	*Tilia* ssp.	Bratislava – Slovakia
10	Linden-3	*Tilia* ssp.	Banská – Slovakia
11	Lucerne-1	*Mendicago sativa*	Bologna – Italy
12	Lucerne-2	*Mendicago sativa*	Bologna – Italy
13	Lavender	*Lavandula* ssp.	Puimoisson – Italy
14	Orange blossom-2	*Citrus* spp.	Roccascalegna-CH – Italy
15	Orange blossom-2	*Citrus* spp.	Tornareccio-CH – Italy
16	Rapeseed	Rapeseed (*Brassica campestris*)	Sebechleby – Slovakia
17	Rhododendron-1	*Rhododendron* spp.	Brescia – Italy
18	Rosemary-1	*Rosmarinus officinalis*	Tornareccio-CH – Italy
19	Rosemary-2	*Rosmarinus officinalis*	Manfredonia-FG – Italy
20	Sunflower	*Heliamtus annus*	Bologna – Italy
21	Taraxacum-1	*Taraxacum officinalis*	Bologna – Italy
22	Taraxacum-2	*Taraxacum officinalis*	Rivolta d′ Adda – Italy
23	Tilia-1	*Tilia* ssp.	Bologna – Italy
24	Tilia-2	*Tilia* ssp.	Minerbio-BO – Italy
25	Tilia-3	*Tilia* ssp.	Torino – Italy
26	Tilia-4	*Tilia* ssp.	Bologna – Italy
27	Tilia-5	*Tilia* ssp.	Bologna – Italy

amounts of glycosidic phytochemicals in a complex food matrix as honey has become possible. The present study aims at the determination of the occurrence of flavonoid glycosides in unifloral honeys, using HPLC-MSn. In some cases, the floral nectars have also been studied in order to evaluate the metabolic changes occurring during bee manufacturing and honey maturation, and to evaluate the suitability of these phytochemicals as potential markers of honey floral origin.

2. Experimental

2.1. Honey collection

Twenty-seven experimental and commercial honey samples from 12 different floral origins and produced in different localities of Italy, Slovakia and Spain were selected for this study. Experimental honeys were provided and certified by the Agricultural Research Council (CRA-API, Bologna, Italy) and the Institute of Molecular Biology (Slovak Academy of Sciences, Bratislava, Slovakia). Commercial honeys used for this study were purchased in a local supermarket (Table 1). All samples were stored at 4 °C in dark until analysis. The botanical origin was certified by the traditional analyses: sensory and pollen analyses and physicochemical analyses.

2.2. Nectar collection

2.2.1. Eucalyptus blossom nectar

Eucalyptus blossoms were collected in Espinardo (Murcia, Spain) during June 2007. Eucalyptus nectar has a high density and it remains in the lower parts of the pistil, inside the shaft. To obtain the nectar, the stamens were removed using forceps, leaving only the shaft of the pistil. Then, one drop of Milli-Q water was placed in and recovered together with the nectar using a Pasteur pipette. The nectar was kept in an eppendorf test tube and stored at −20 °C until analysis.

2.2.2. Orange blossom nectar

Orange floral nectar was collected in Santomera (Murcia, Spain) in spring 2007. The droplets of nectar from orange blossoms, situated in the concave sepals, were aspirated using a glass capillary, then collected in eppendorf test tubes and stored at −20 °C until analysis

2.3. Extraction of phytochemicals from nectar

Phytochemical compounds of eucalyptus, and orange nectars were extracted using a solid phase extraction (SPE) cartridge (a Sep-Pak reversed phase C_{18} cartridge; Waters Milipore, USA). Nectar samples were diluted with ultra pure water (Milli-Q system, Millipore Corp., Bedford, MA), and centrifuged at 7000 rpm for 10 min, in a Centromix centrifuge (Selecta, Barcelona). The supernatants were filtered through a SPE cartridge previously activated with methanol (10 mL) followed by water (10 mL). Then, the phytochemicals that remained adsorbed in the cartridge were eluted with 1 mL methanol. The methanol extracts were filtered through a 0.45 μm membrane filter Millex-HV_{13} (Millipore Corp., USA) and stored at −20 °C until further analysis by HPLC-PAD-MSn.

2.4. Extraction of phytochemicals from honey

Honey samples (10 g) were dissolved with five parts of water (adjusted to pH 2 with HCl) until completely fluid. This solution (50 mL) was then filtered through a Sep-Pak C_{18} cartridge, which was previously activated as described above. The cartridge was washed with 10 mL water and the phytochemical compounds eluted with 2 mL 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.

2.5. HPLC-PAD-tandem mass spectrometry (MSn)

Chromatographic separations of eucalyptus and orange nectars were carried out 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 C_{18} LiChroCART guard column, with 1% acetic acid (A) and methanol (B) as solvents (99.9%, HPLC grade; Merck, Darmstadt, Germany). Starting with 20% B, to reach 50% B at 40 min, 80% B at 55 min, and then became isocratic for 5 min.

Analysis of phytochemical compounds of honey samples was achieved with the same instrument, and on the same column used in nectar analyses. In this case, the mobile phase used was water/formic acid (99:1, v/v) (solvent A) and HPLC grade methanol (solvent B) (99.9%, HPLC grade; Merck, Darmstadt, Germany) and the elution was performed with a gradient starting with 10% B to reach 30% B at 20 min, 45% B at 30 min, 60% B at 40 min, 70% B at 45 min, 90% B at 60 min and then became isocratic for 5 min. The flow rate of 1 mL min^{-1} and all chromatograms were recorded at 290, 320, 340 and 360 nm.

The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC system consisted of a binary pump (G1312 A), an auto sampler (G1313 A) a degasser (G1322 A), and photodiode-array detector (G1315 B) controlled by software (v. A08.03). 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 full scan mass covered the range from m/z 100–1000 collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 to 2 V. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively.

Mass spectrometry data were acquired in the negative mode. The phenolic compounds were identified according to their UV spectra, molecular weights, retention times and their MSn fragments, and whenever possible, by chromatographic comparisons with authentic standards. Flavonols, flavones and flavanones were quantified as quercetin, chrysin and hesperetin at 340 and 290 nm, respectively. Flavonoids glycosides were quantified as rutin at 340 nm. Calibration curves were prepared for the UV detector. The calibration curves were linear in the range of 5–500 μM for rutin and were characterized by correlation coefficients >0.99. The limit of detection was 0.61 μg/L, and the limit of quantification was 1.52 μg/L. Quercetin, rutin and hesperidin were purchased from Sigma (St. Louis MO) and chrysin from Carl Roth OGH (Karlsruhe, Germany). The results were expressed as micrograms per 100 g of honey.

3. Results

3.1. HPLC-MS–MS analyses of flavonoid glycosides in unifloral honeys

The HPLC-PAD-MSn study of the extracts obtained from unifloral honeys of different botanical origins (Table 1) revealed the presence of different flavonoid glycosides of quercetin (3,5,7,3′,4′-pentahydroxyflavone; [aglycone-H]$^-$ fragment at *m*/*z* 301); kaempferol (3,5,7,4′-tetrahydroxyflavone, *m*/*z* 285), 8-methoxykaempferol (3,5,7,4′-tetrahydroxy-8-methoxyflavone, *m*/*z* 315) and isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone, *m*/*z* 315). The occurrence of flavonol triglycosides (**1**, **14**–**17**) and flavonol diglycosides (**2–13**) was detected in different honey samples (Fig. 1). In addition the flavanone hesperetin 7-rutinoside (hesperidin) (**11**) was found in citrus honey. These compounds are generally minor constituents in the HPLC chromatograms, while hydroxycinnamates derived from propolis were the main constituents (Fig. 1). Flavonoid aglycones characteristic of propolis (chrysin, galangin, pinocembrin, pinobanksin and quercetin and kaempferol methyl ethers) were also detected generally in higher proportions than the flavonoid glycosides (Table 2).

HPLC-MSn analysis can be used to establish the nature of the sugars, the interglycosidic linkage and the position of glycosylation in flavonoid glycosides [21]. The analysis of the available samples revealed the occurrence in honeys of flavonoid hexosyl (1 → 2) hexosides, rhamnosyl (1 → 2) hexosides (tentatively neohesperidosides), rhamnosyl(1 → 6)hexosides (tentatively rutinosides), hexosyl (1 → 6) hexosides and pentosides, hexosides and rhamnosides depending on the floral origin of the honey sample analyzed. The flavonoid triglycosides had always a similar glycosylation patters with glycosidic substitutions at the hydroxyls in the 3- and 7-positions of the flavonoid nucleus. The MS2[M−H]$^-$ spectra of these compounds was characterized by the presence of only one ion as a result of the loss of the glycosyl residue in position 7 [M−H-Gly]$^-$ that it is known to be released first than the glycosyl residue at 3-position, during the MS fragmentation [23]. In compounds **1** and **14**, the glycosidic residue at 7 position was a hexosyl residue as a relevant ([M−H-162]$^-$) ion was observed, and in compounds **15–17** the glycosidic fraction at position 7 was a deoxyhexosyl residue (most likely rhamnose) as a relevant ([M−H-146]$^-$) ion was observed instead (Table 3).

In all the triglycosides studied, with the exception of compound **16**, the MS3 event [(M−H) → (M−H-Gly7)]$^-$ showed that the fragmentation of the glycosidic fraction in the position 3 of the aglycone produced only one ion corresponding to the deprotonated aglycone [Aglc-H]$^-$ (Table 3). The absence of other fragment ions as [M−H-162]$^-$ and/or [M−H-180]$^-$, indicates for all these compounds an interglycosidic linkage (1 → 6). However, the fragmentation of **16** showed an intermediate ion [(M−H)-162]$^-$ suggesting that in this

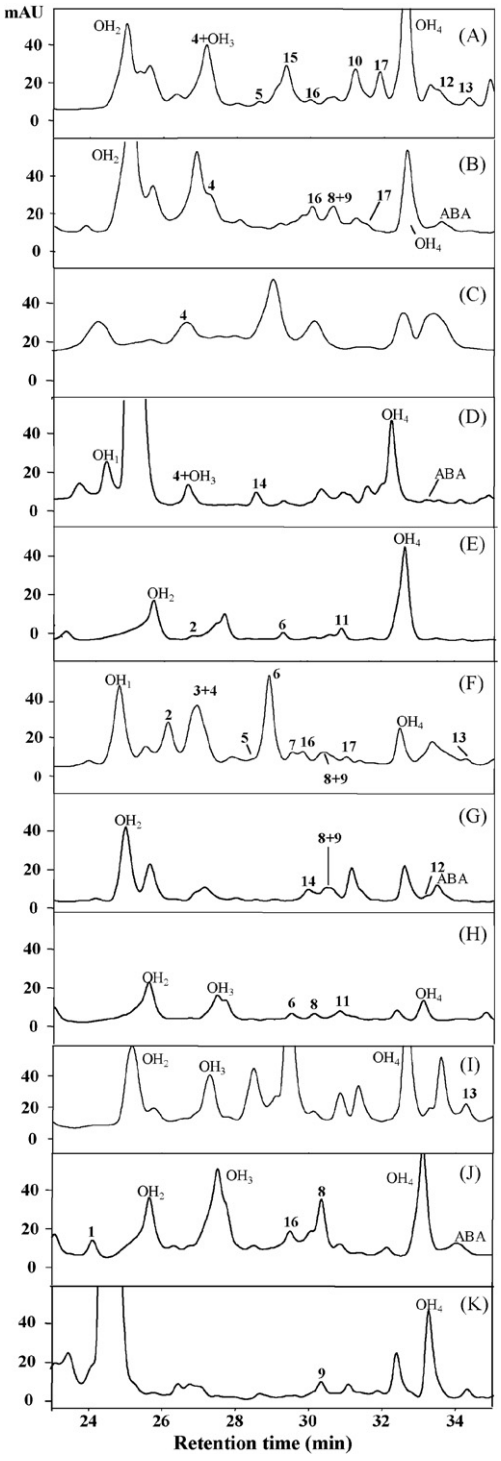

Fig. 1. HPLC-DAD chromatogram (340 nm) of selected unifloral honey. (A) Canola, (B) cherry blossom 2, (C) eucalyptus 2, (D) lucerne, (E) orange 2, (F) rapeseed, (G) rhododendron, (H) rosemary 1, (I) sunflower 1, (J) taraxacum 2, (K) tilia 1. Tentative identification of flavonoid glycosides (**1**) isorhamnetin-3-*O*-(pentosyl-hexoside)-7-*O*-hexoside; (**2**) Quercetin-3-*O*-hexosyl (1 → 2)hexoside; (**3**) kaempferol-3,4′-di-*O*-hexoside; (**4**) 8-Methoxykaempferol-3-*O*-hexosyl (1 → 2)hexoside; (**5**) 8-Methoxykaempferol-3-*O*-neoheperidoside; (**6**) kaempferol-3-*O*-hexosyl (1 → 2)hexoside; (**7**) isorhamnetin-3-*O*-hexosyl (1 → 2)hexoside; (**8**) kaempferol-3-*O*-neoheperidoside; (**9**) isorhamnetin-3-*O*-neoheperidoside; (**10**) Quercetin-3-*O*-rutinoside; (**11**) hesperidin; (**12**) kaempferol-3-*O*-rutinoside; (**13**) isorhamnetin-3-*O*-hexosyl (1 → 6)hexoside; (**14**) isorhamnetin-3-*O*-rutinoside-7-*O*-hexoside; (**15**) quercetin-3-*O*-rutinoside-7-*O*-rhamnoside; (**16**) kaempferol-3-*O*-hexosyl (1 → 2)hexoside-7-*O*-rhamnoside; (**17**) kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside; (ABA) abscisic acid; (OH_1) *p*-coumaric acid; (OH_2) ferulic acid; (OH_3) and (OH_4) hydroxycinnamic acid derivatives.

Table 2
Flavonoid aglycones content in unifloral honeys[a].

Samples	Aglycones														Total
	Iso	Pb	Pc	25[b]	27	28	Q	K	Is + Ap	M	M-Q	Ch	G	Tch	
Canola-1	501.2	608.2	788.9	–	–	184.2	101.5	259.8	188.0	132.1	171.8	376.9	194.2	38.3	3545.1
Cherry B-1	–	591.6	–	–	–	–	–	102.1	–	–	53.54	194.2	108.5	17.04	1066.9
Cherry B-2	262.8	545.6	330.2	–	–	–	–	54.03	77.5	32.5	30.9	128.6	67.1	10	1539.2
Eucalyptus-1	–	794.6	634.5	294.4	628.9	511.6	297.0	78.4	–	–	51.6	122.2	79.0	–	3492.2
Eucalyptus-2	–	282.3	204.2	158.3	269.3	255.1	227.1	100.5	73.8	–	35.4	72.9	36.1	–	1715
Eucalyptus-3	–	399.0	756.4	142.7	354.7	360.8	156.2	21.7	59.3	–	14.0	35.1	11.0	–	2310.9
Eucalyptus-4	–	581.5	150.2	327.5	504.8	127.9	–	67.4	107.0	–	51.6	122.2	79.0	–	2119.1
Lavender	–	403.1	220.9	–	–	29.5	–	26.1	93.3	26.8	19.2	126.3	70.1	–	1015.3
Linden-1	–	437.4	321.4	–	–	88.1	–	67.8	174.4	30.4	20.6	125.0	62.1	–	1327.2
Linden-2	405.6	291.7	402.2			7.5	–	37.6	57.5	27.4	14.4	60.47	–	–	1304.3
Linden-3	–	621.6	691.7	–		–	–	33.4	33.4	14.4	–	192.4	114.9	–	1701.8
Lucerne-1	623.2	956.9	610.5	–	–	–	179.6	287.9	228.6	119.1	213.1	401.1	310.9	–	3930.9
Lucerne-2	215.9	364.8	624.5	–	–	–	–	47.9	62.87	94.2	79.2	158.7	90.8	15.3	1753.9
Orange-1	–	–	208.2				17.8	22.2	22.1	15.7	18.9	90.5	57.78	–	453.2
Orange-2	130.0	320.0	370.1	–		17.4	38.2	51.3	62.9	42.1	55.6	182.2	99.3	11.8	1380.9
Rapeseed-1	256.3	768.1	362.2				93.4	265.3	108.4	33.4	14.3	107.35	65.3	11.38	2085.4
Rhododendron-1		331.2	194.3	–	–	7.59	–	31.2	11.0	10.8	–	52.3	–	–	638.3
Rosemary-1	–	151.3	143.2	–	–	–	8.7	35.2	20.8	9.6	6.6	55.4	33.1	–	463.9
Rosemary-2	131.8	313.1	338.9	–	–	–	25.9	62.4	41.5	42.2	49.4	173.0	97.1	10.4	1285.7
Taraxacum-1	465.0	77.7	411.2	–	–	–	140.7	122.7	166.7	90.1	188.3	367.8	177.0	25.9	2233.1
Taraxacum-2	601.3	465.8	642.2	–	–	60.15	76.9	107.5	128.21	83.7	101.6	323.2	261.9	26.3	2878.7
Tilia-1	182.4	415.1	568.7	–	–	–	–	–	–	–	228.3	216.4	23.7	9.35	1643.9
Tilia-2	533.0	1054.3	523.7	–	–	–	27.3	276.1	194.4	111.9	191.7	395.2	244.1	44.5	3596.2
Tilia-3	–	682.1	1229.7	–	–	–	–	–			121.9	115.6	53.8	–	2203.1
Tilia-4	457.2	768.6	1560.2	–	–	–	111.8	69.31	76.78	72.79	365.7	346.9	147.9	40.3	4017.4
Tilia-5	402.7	825.3	–	–	–	–	99.07	128.4	85.2	80.7	359.6	340.9	155.8	30.6	2508.2
Sunflower-1		732.2	463.1				141.7	111.9	163.8	65.1	22.3	227.2	195.9	–	2123.2

Iso, isosakuranetin; Pb, pinobanksin; Pc, pinocembrin.

[a] Values are μg/100 g of honey.

[b] (**25**) Myricetin; (**27**) Tricetin; (**28**) Luteolin; Q, quercetin; K, kaempferol; Is + Ap, apigenin + isorhamnetin; M, acacetin (tentatively); M-Q, methylquercetin (tentatively); Ch, chrysin; G, galangin; Tch, tectochrysin

Table 3
R_t, UV and -MS: $[M-H]^-$, -MS2$[M-H]^-$, -MS3$[(M-H)\rightarrow(M-H\text{-}Gly7)]^-$, data of flavonoid glycosides detected in unifloral honeys.

Peak	Compounds[a]	R_t (min)	UV (nm)	$[M-H]^-$	-MS2$[M-H]^-$ m/z (%)				
					−15	−162	−146-18	−162-18	[Aglc-H]$^-$
2	Q-3-*O*-hex(1 → 2)hex	26.9	259, 265sh, 299sh, 355	625				445 (20)	301 (100)
3	K-3-4ī-di-*O*-hexoside[b]	26.7	–	609		447 (40)			285 (100)
4	8-*O*MeK-3-*O*-hex(1 → 2)hex[b]	27.0	–	639	624 (19)	477 (10)		459 (59)	315 (100)
5	8-*O*MeK-3-*O*-neoh[b]	28.2	–	623	608 (18)		459 (21)		315 (100)
6	K-3-*O*-hex(1 → 2)hex	29.6	265, 296sh, 349	609		447 (7)		429 (85)	285 (100)
7	I-3-*O*-hex(1 → 2)hex[b]	29.8	–	639				459 (59)	315 (100)
8	K-3-*O*-neoh[b]	30.8	–	593			429 (20)		285 (100)
9	I-3-*O*-neoh[b]	30.9	–	623			459 (14)		315 (100)
10	Q-3-*O*-rut	31.0	258, 260sh, 291sh, 349	609					301(100)
11	Hesperidin (hesperetin 7-*O*-rutinoside)	31.9	283, 326sh	609					301 (100)
12	K-3-*O*-rut[b]	33.1	–	593					285 (100)
13	I-3-*O*-hex(1 →6)hex	34.5	255, 265sh, 305sh, 355	639		447 (2)			315 (100)

Peak	Compounds[a]	R_t (min)	UV (nm)	$[M-H]^-$	-MS2$[M-H]^-$ m/z (%)		-MS3$[(M-H)\rightarrow(M-H\text{-}Gly7)]^-$ m/z (%)	
					-146	-162	-162	[Aglc-H]$^-$
14	I-3-*O*-rut-7-*O*-glc[b]	28.4	–	785		623 (100)		315 (300)
15	Q-3-*O*-rut-7-*O*-rham[b]	29.2	–	755	609 (100)			301 (100)
16	K-3-*O*-soph-7-*O*-rham	30.0	265, 305sh, 351	755	609 (100)		447(38)	285(100)
17	K-3-*O*-rut-7-*O*-rham[b]	31.6	265, 317sh, 351	739	593 (100)			285 (100)
1	I-3-*O*-(pent-glc)-7-*O*-glc[b]	24.00	–	771		609 (100)		315 (100)

(Q) Quercetin; (K) Kaempferol; (8-*O*MeK) 8-Methoxykaempferol; (I) Isorhamnetin.

[a] Hex(1 → 2)hex (hexosyl (1 → 2)hexoside); neoh, neohesperidoside: rham, rhamnoside; glc, glucoside; rut, rutinoside; hex(1 → 6)hex (hexosyl (1 → 6)hexoside).

[b] Compounds hidden by others or in traces. Their UV spectra have not been properly recorded.

case the interglycosidic linkage is (1 → 2) (hexosyl (1 → 2)hexose) [23].

The MS2$[M-H]^-$ and MS3$[(M-H)\rightarrow(M-H\text{-}Gly7)]^-$ fragmentations of the honey diglycosides and triglycosides, showed in several cases a base peak corresponding to the deprotonated aglycone ion [Aglc-H]$^-$ (Table 3) at m/z 315 corresponding to a tetrahydroxy-monomethoxyflavone. These flavonol aglycones were identified either as isorhamnetin (3,5,7,4′-tetrahydroxy-3′-methoxyflavone) (**1**, **7**, **9**, **13** and **14**) or 8-methoxykaempferol (3,5,7,4′-tetrahydroxy-8-methoxyflavone) (**4** and **5**) as the spectra of the 8-methoxykaempferol derivatives showed a characteristic ion $[M-H-15]^-$ and this ion was not detected in the case of isorhamnetin derivatives. This could be explained by an easier loss of the methyl ether on the hydroxyl at 8 position than that at the hydroxyl at the 3′ position [24]. In addition, compound **4** has been tentatively identified as 8-methoxykaempferol-3-*O*-sophoroside

by direct chromatographic comparison with an authentic marker previously isolated and identified from almond pollen [25]. For other flavonoids a characteristic fragment ion at *m/z* 301 (pentahydroxyflavone or trihydroxy-methoxyflavanone) revealed that the flavonoid was a quercetin glycoside (**2**, **10** and **15**) or a hesperetin glycoside (**11**) and they were readily identified by the characteristic UV spectra of the flavanone (hesperetin) that is very different from that of flavonols (quercetin) [26]. In other flavonoids a fragment ion at *m/z* 285 (tetrahydroxyflavone), together with a characteristic UV spectrum of flavonols, indicated that the glycoside was a derivative of kaempferol (**3**, **6**, **8**, **12**, **16**, and **17**) (Table 3).

In the MS2[M−H]$^-$ of the honey flavonoid diglycosides **2** and **4–9**, abundant ions corresponding to the loss of the terminal sugar and/or terminal sugar + H_2O were observed, which indicate that the interglycosidic linkage should be 1 → 2, either with a hexosyl residue [M−H-162]$^-$/[M−H-180]$^-$ or a rhamnosyl residue [(M−H)-146]-/[(M−H)-164]$^-$) as terminal sugars [27]. Thus, compounds **2**, **4**, **6** and **7** should be tentatively identified as flavonoid hexosyl (1 → 2)hexosides, and **5**, **8** and **9** as flavonoid rhamnosyl(1 → 2)hexosides (tentatively neohesperidosides; rhamnosyl(1 → 2)glucosides). These ions were not observed in compounds **10**, **11** and **12** which indicated the presence of a interglycosidic linkage 1 → 6 either a rhamnosyl (1 → 6) hexoside (tentatively rutinoside; rhamnosyl (1 → 6) glucoside) or a hexosyl (1 → 6) hexoside in the case of compound **13** [23].

In the MS/MS analysis of **3** (an isomer of compound **6**, kaempferol 3-hexosyl (1 → 2)hexoside) a high abundance of the ion [M−H-162]$^-$ was observed, but in this case the fragment ion [M−H-180]$^-$ was not detected this being a clear difference with the fragmentation of compound **6**. This suggested that compound **3** should be identified either as an isomeric kaempferol-3-*O*-dihexoside (with different hexoses) or a kaempferol-di-*O*-hexoside. This should be most likely identified as kaempferol-3,4′-di-*O*-hexoside as the fragmentation of kaempferol-3,7-di-*O*-glucoside shows as base peak the ion [M−H-162]$^-$ [21] and this fragment was not present here.

3.2. Distribution of flavonoid glycosides in unifloral honeys

The flavonoid glycoside profiles of the analyzed honeys are shown in Fig. 1. Flavonoid glycosides were detected in all the samples analyzed with the exception of eucalyptus sample (eucalyptus 2) and lavender. The amount of these compounds, quantified as rutin (quercetin 3-rutinoside), ranged between 8 and 408 μg/100 g (Table 4), and some samples contained traces of glycosides that were detected but could not be quantified in the UV chromatogram. The total amount of flavonoid glycosides ranged 0–600 μg/100 g honey, with canola and rapeseed being those honeys with higher flavonoid glycoside content. The largest amounts found were similar to those previously reported in robinia (acacia) honey [22]. The available *Brassicaceae* honeys (canola and rapeseed) were those containing more flavonoid glycosides both in number and total quantity. Thus, seven different flavonoid glycosides were detected in canola honey and nine in rapeseed honey (Table 4). Both canola and rapeseed belong to *Brassica napus*, but they are different cultivars. Both have in common the occurrence of a high number of glycosides but differ in the type of glycosides found. A consistent flavonoid glycoside composition was detected in tilia, taraxacum, rosemary, lucerne, linden and cherry blossom honeys. Of other unifloral origins, only one honey sample was available for analysis, and therefore the glycosides found could not be associated to the floral origin. Some more honeys samples of canola, rapeseed, rhodo-

Table 4
Flavonoid glycoside content in unifloral honeys[a].

Samples	Glycosides																	Total
	1[b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
Canola-1	–[c]	–	–	263.5*	20.5	–	–	–	–	140.8	–	64.1	41.9	–	192.7	27.2	101.7	588.9
Cherry B-1	–	–	–	318.1	–	–	–	95.3*	–	–	–	–	–	–	–	116.1	t	434.2
Cherry B-2	–	–	–	–	–	–	–	8.3	11.9	–	–	–	–	–	–	9.4	–	29.6
Eucalyptus-1	–	–	–	–	–	–	–	151.0	–	–	–	–	–	–	–	–	–	151
Eucalyptus-2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Eucalyptus-3	–	–	–	44.1	–	–	–	–	–	–	–	–	–	–	–	–	–	44.1
Eucalyptus-4	–	–	–	–	–	–	–	191.8	–	–	–	–	–	–	–	–	–	191.8
Linden-1	–	–	36.0	–	–	–	–	–	–	–	–	–	–	–	–	–	–	36
Linden-2	–	–	255.8	–	–	126.7	–	–	–	–	–	–	–	–	–	–	–	382.5
Linden-3	–	–	–	–	–	36.0	–	–	–	–	–	–	–	–	–	–	–	36
Lucerne-1	–	–	–	t	–	–	–	–	–	–	–	–	–	25.3	–	–	–	25.3
Lucerne-2	–	–	–	–	–	–	–	–	–	–	–	–	–	27.3	–	–	–	27.3
Orange-1	–	t	–	–	–	7.3	–	–	–	–	24.6	–	–	–	–	–	–	31.9
Orange-2	–	t	–	–	–	15.1	–	–	–	–	26.4	–	–	–	–	–	–	41.5
Rapeseed-1	–	130.9	252.3*	–	t	209.5	23.6	–	44.9*	–	–	–	t	–	–	27.8	14.7	406.5
Rhododendron-1	–	–	–	–	–	–	35.3	46.3*	–	–	–	t	–	–	–	–	–	35.3
Rosemary-1	–	–	–	–	–	10.0	–	12.7	–	–	12.4	–	–	–	–	–	–	35.1
Rosemary-2	–	–	–	–	–	11.3	–	11.7	–	–	23.3	–	–	–	–	–	–	46.3
Taraxacum-1	83.0	–	–	–	–	–	–	61.8	11.1	–	–	–	–	–	–	64.9	–	220.8
Taraxacum-2	38.5	–	–	–	–	–	–	198.0	–	–	–	–	–	–	–	3.3	–	239.8
Tilia-1	–	–	–	–	–	–	8.8	–	82.4	–	–	–	–	–	–	–	–	91.2
Tilia-2	–	–	–	–	–	–	24.3	–	357.5	–	–	–	–	–	–	–	–	381.8
Tilia-3	–	–	–	–	–	–	–	–	50.9	–	–	–	–	–	–	–	–	50.9
Tilia-4	–	–	–	–	–	–	–	–	407.0	–	–	–	–	–	–	–	–	407.0
Tilia-5	–	–	–	–	–	–	–	–	122.8	–	–	–	–	–	–	–	–	122.8
Sunflower-1	–	–	–	–	–	–	–	–	–	–	–	–	215.5	–	–	–	–	215.5

t, traces.

* Peak overlapping unidentified compound.

[a] Values are μg/100 g of honey.

[b] (**1**) Isorhamnetin-3-*O*-(pentosyl-hexoside)-7-*O*-hexoside; (**2**) quercetin-3-*O*-hexosyl(1 → 2)hexoside; (**3**) kaempferol-3,4′-di-*O*-hexoside; (**4**) 8-Methoxykaempferol-3-*O*-hexosyl(1 → 2)hexoside; (**5**) 8-Methoxykaempferol-3-*O*-neoheperidoside; (**6**) kaempferol-3-*O*-hexosyl(1 → 2)hexoside; (**7**) isorhamnetin-3-*O*-hexosyl(1 → 2)hexoside; (**8**) kaempferol-3-*O*-neoheperidoside; (**9**) isorhamnetin-3-*O*-neoheperidoside; (10) quercetin-3-*O*-rutinoside; (**11**) hesperidin; (**12**) kaempferol-3-*O*-rutinoside; (**13**) isorhamnetin-3-*O*-hexosyl(1 → 6)hexoside; (**14**) isorhamnetin-3-*O*-rutinoside-7-*O*-hexoside; (**15**) quercetin-3-*O*-rutinoside-7-*O*-rhamnoside; (**16**) kaempferol-3-*O*-hexosyl(1 → 2)hexoside-7-*O*-rhamnoside; (**17**) kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside.

[c] Not detected.

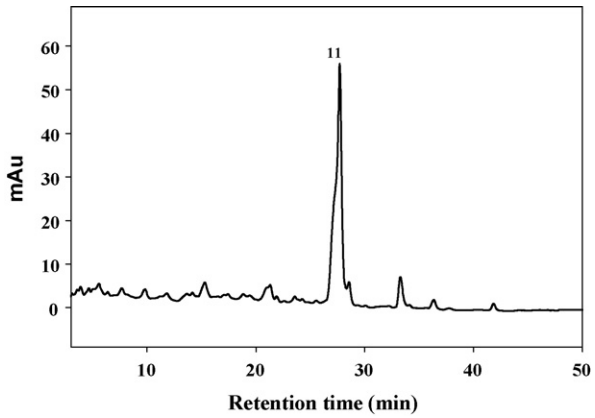

Fig. 2. HPLC-DAD chromatogram (290 nm) of orange nectar. (11) Hesperidin.

dendrom and sunflower honey should be analyzed in the future to confirm the flavonoid profiles detected as potential floral origin markers.

Two isorhamnetin triglycosides were detected in the honeys samples analyzed. One was tentatively identified as isorhamnetin-3-*O*-pentosyl-glucoside-7-*O*-glucoside (**1**), present only in one of the taraxacum honey samples (taraxacun 1). The other (compound **14**, isorhamnetin 3-*O*-rutinoside-7-*O*-hexoside**)** was only detected in both samples of lucerne honey, and this could be a potential marker for this floral origin. Compound **15** (quercetin 3-*O*-rutinoside-7-*O*-rhamnoside) was the main flavonol glycoside detected in canola honey and was not detected in any other samples analyzed. However, the kaempferol triglycosides **16** and **17**, were both detected in canola and rapeseed honeys, which reflects that both honeys are of close floral origin, while **16** alone was detected in cherry blossom, taraxacum and rosemary honey samples.

The characterized flavonoid diglycosides were derivatives of quercetin, kaempferol, 8-methoxykaempferol, isorhamnetin and the flavanone hesperetin (**11**). Hesperetin 7-rutinoside (**11**) was only detected in rosemary and orange honeys (Fig. 1; Table 4). In a previous study, hesperetin had been suggested as a suitable marker for the floral origin of citrus honey, and this was due to the presence of hesperetin 7-*O*-rutinoside in citrus nectar. Orange blossom nectar was analyzed by HPLC-MSn and hesperetin 7-rutinoside (hesperidin) was the main flavonoid present (Fig. 2). Thus, it is expected that this compound could be present in orange blossom honey. However, the presence of this compound in rosemary honey is something unexpected, as previous analysis of rosemary nectar did not show the presence of flavanones suggesting that the presence of **11** in the analyzed rosemary honey samples could be due to contamination with orange nectar.

Quercetin diglycosides, such as quercetin-3-*O*-sophoroside **(2**) and quercetin-3-*O*-rutinoside **(10**), were only detected in significant quantifiable amounts in rapeseed and canola honeys, respectively. The 8-methoxykaempferol glycosides, either the 3-*O*-sophoroside or the -3-*O*-neohesperidoside (**4**, **5**), were detected in canola, cherry blossom, and in one eucalyptus sample, and in minor proportions in lucerne and rapeseed honeys. However, **5**, has only been detected in canola and in traces in rapeseed honey. These results keep showing a close relationship between canola and rapeseed honeys. The presence of **4** in only one sample of cherry blossom and eucalyptus honeys could be consistent with a contamination with pollen flavonoids, as this flavonoid was found as the main pigment in almond and other Rosaceae pollens. [25]

Kaempferol diglycoside derivatives (**3**, **6**, **8**, and **12)** have been detected in most honey samples analyzed in this study. Nevertheless, **3** and **6** have been detected in higher proportions in rapeseed honey (Table 4). Kaempferol-3-*O*-sophoroside (**6)** has also been observed in smaller amounts in all the orange and rosemary honeys analyzed, as well as in two of the linden honeys studied. Compound **8** was previously identified as a relevant constituent in rosemary nectar [11]. On the other hand, in this study kaempferol-3-*O*-neohesperidoside (**8**) was detected in cherry blossom, rhododendron and taraxacum honeys.

Isorhamnetin diglycosides, such as compounds **7**, **9** and **13**, have been detected in many of the samples analyzed. Isorhamnetin-3-*O*-sophoroside (**7**) was only identified in rapeseed, Isorhamnetin-3-*O*-gentiobioside (**13**) was identified in canola, sunflowers and rapeseed samples, in trace amounts in the last case. It is interesting that isorhamnetin 3-*O*-neohesperidoside (**9**) was present in all the tilia honey samples analyzed, and this could be an appropriate floral marker that should be further studied in the future. Moreover, this compound was also detected in cherry blossom, rapeseed, and rhododendron.

The different eucalyptus honey samples analyzed showed an inconsistent flavonoid glycoside pattern, and this was surprising as in previous studies we had demonstrated the presence of specific markers for this honey type including the flavonoids myricetin, tricetin and luteolin [19,20]. In addition, the presence of hesperetin glycosides in orange and rosemary honeys is also inconsistent with the previous suggestion of hesperetin as marker of the floral origin of citrus honey. By this reason, a study of the flavonoid glycosides present in eucalyptus and orange floral nectars using HPLC-MSn is necessary to confirm the relevance of these analyses in the determination of the floral origin of honey.

3.3. *Flavonoid glycosides in eucalyptus nectar*

The occurrence of characteristic flavonoid markers in eucalyptus honey was reported in a study of eucalyptus honeys, where myricetin, tricetin and luteolin were detected [19,20]. This flavonoid combination was not detected in any other unifloral honey analyzed [9]. However, these markers were not previously confirmed with the analysis of eucalyptus nectar, as nectar collection in this case was not an easy task, due to the location of flowers high in the trees and to the small amount of nectar produced. In the present study, eucalyptus nectar could be directly collected from eucalyptus blossom. The chromatogram at 340 nm of eucalyptus nectar showed different compounds with UV spectra characteristic of flavonols (Fig. 3). The MS/MS study showed the presence of different diglycosides, monoglycosides and aglycones of flavonoids (Table 5). The

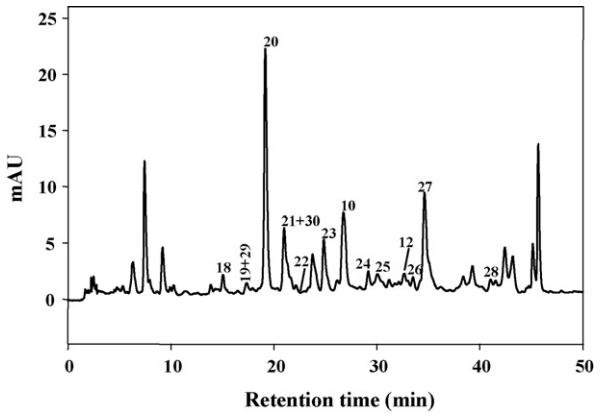

Fig. 3. HPLC-DAD chromatogram (340 nm) of eucalyptus nectar. (**18**) myricetin-3-*O*-sophoroside; (**19**) Myricetin-3,7-di-*O*-glucoside; (**20**) tricetin-7-*O*-sophoroside; (**21**) tricetin-7,4′-di-*O*-glucoside; (**22**) luteolin-7-*O*-sophoroside; (**23**) quercetin-3-*O*-glucuronide; (**10**) quercetin-3-*O*-rutinoside; (**24**) tricetin-7-*O*-glucoside; (**25**) myricetin; (**12**) kaempferol-7-*O*-rutinoside; (**26**) isorhamnetin-7-*O*-rutinoside; (**27**) tricetin; (**28**) luteolin; (**29**) myricetin-3-*O*-(pentosyl-glucoside); (**30**) tricetin-7-*O*-(pentosyl-glucoside).

Table 5
R_t, UV and -MS: $[M-H]^-$, -MS2$[M-H]^-$ data of eucalyptus nectar flavonoids.

Peak	Compounds[b]	R_t (min)	UV (nm)	$[M-H]^-$	-MS2$[M-H]^-$ m/z (%)		
					-162	-162-18	$[Aglc-H]^-$
18	Myricetin-3-*O*-soph	15.05	–	641	479 (7)	461 (1)	317 (100)
19	Myricetin-3,7-di-*O*-glc[c]	17.40	–	641	479 (100)		317 (30)
20	Tricetin-7-*O*-soph	19.17	256, 264sh, 299sh, 354	625	463 (20)	445 (20)	301 (100)
21	Tricetin-7,4′-di-*O*-glc[c]	21.44	–	625	463 (100)		301 (71)
22	Luteolin-7-*O*-soph[c]	23.30	–	609		429 (80)	285 (100)
23	Quercetin-3-*O*-gluc	26.3	258, 266sh, 300sh, 354	477			301 (100)
10	Quercetin-3-*O*-rut	27.19	259, 266sh, 300sh, 359	609			301 (100)
24	Tricetin-7-*O*-glc	29.16	–	463			301 (100)
25	Myricetin	30.59	255, 267sh, 301sh, 375	317			
12	Kaempferol-7-*O*-rut	32.10	–	593			285 (100)
26	Isorhamnetin-7-*O*-rut	33.40	254, 265sh, 305sh, 355	623			315 (100)
27	Tricetin	35.24	248, 267sh, 302sh, 351	301			
28	Luteolin[c]	40.90	254, 266sh, 292sh, 348	285			
					−132	−132-18	
29	Myricetin-3-*O*-(pent-glc)[c]	17.40	–	611			317 (100)
30	Tricetin-7-*O*-(pent-glc)[c]	21.40	–	595	463 (13)	445 (24)	301 (100)

[a] Main observed fragments. Other ions were found but they have not been included.
[b] Soph, sophoroside (glucosyl(1 → 2)glucoside); Neoh, neohesperidoside; rham, rhamnoside; rut, rutinoside; glc, glucoside; gluc, glucuronide; pent, pentoside.
[c] Compounds hidden by others or in traces. Their UV spectra have not been properly recorded.

floral markers, myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone) (**25**), tricetin (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone) (**27**), luteolin (5,7,3′,4′-tetrahydroxyflavone) (**28**) and their diglucosides (tentatively sophorosides; glucosyl(1 → 2) glucosides) were detected in the nectar chromatogram (Fig. 3). Moreover, other flavonoids such as quercetin, kaempferol, and isorhamnetin glycosides were also detected in this nectar.

The MS2$[M-H]^-$ fragmentation of compounds **18**, **20** and **22** shows in all cases a loss of a glucosyl residue (loss of 162 m.u.) and/or a glucosyl residue + H_2O (loss of 180 m.u.) (Table 4). These intermediate ions suggest an interglycosidic linkage 1 → 2 (glucose: $[M-H-162]^-$/$[M-H-180]^-$). Then these flavonoids could be tentatively identified as sophoroside derivatives (glucosyl (1 → 2) glucoside) (Table 4) [21]. In the MS–MS analyses of compounds **10**, **12** and **26** intermediate fragments were not observed suggesting an interglycosidic linkage 1 → 6 (rutinoside; rhamnosyl (1 → 6) glucoside) [27]. This indicates that **10** is quercetin-3-*O*-rutinoside, **12**, kaempferol-3-*O*-rutinoside and **26** isorhamnetin-7-*O*-rutinoside. The MS spectra of **19** and **21** show the presence of only one fragment $[M-H-162]^-$ as base peak indicating that these flavonoid diglycosides were glycosylated at two different phenolic hydroxyls of the flavonoid nucleus [23]. These compounds could be probably identified as myricetin-3,7-di-*O*-glucoside and tricetin-3,4'-di-*O*-glucoside, respectively. Compounds **29** and **30** were detected in trace amounts and they have been tentatively characterized as pentosyl-hexosides of myricetin and tricetin, respectively. Moreover, the monoglycosides **23** and **24** were identified as quercetin-3-glucuronide and tricetin-7-glucoside, respectively (Table 5).

3.4. Flavonoid compounds in eucalyptus honey

The extracts of eucalyptus honey were analyzed by HPLC-DAD-MSn (Fig. 4). The flavonoids myricetin (**25**), tricetin (**27**) and luteolin (**28**) were detected in all the eucalyptus honey samples analyzed, as describe above. The flavonoid glycosides detected in nectar, were not detected in honey. In a previous work, these flavonoid aglycones were suggested as floral markers for eucalyptus honey [19,20]. Tricetin and luteolin were the main flavonoids detected in the extracts of these eucalyptus honey samples (Table 4 and Fig. 4). Moreover, quercetin (**Q**) and kaempferol (**K**) were also detected in the eucalyptus honeys included in this study (Fig. 4). These results are fully in agreement with previous studies on European eucalyptus honeys [19]. However, quercetin and kaempferol were not detected as glucosides in the eucalyptus nectar analyzed. Therefore, these two flavonoids should not be used as markers for this floral origin, as they are common honey flavonoids found in many other unifloral honeys [9].

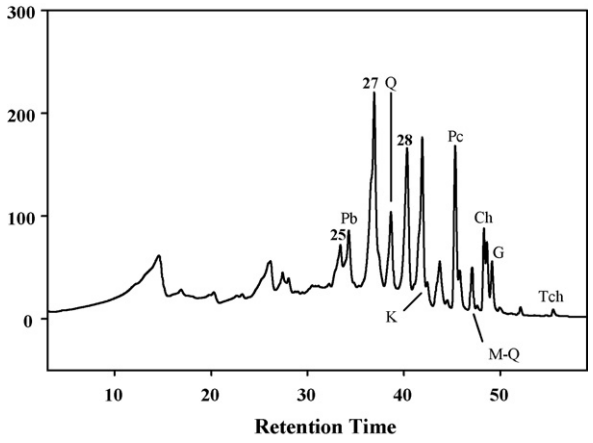

Fig. 4. HPLC-DAD chromatogram (340 nm) of eucalyptus honey. (**2**) Myricetin-3,7-di-*O*-glucoside; (**25**) myricetin; (**27**) tricetin; (**28**) luteolin (Q) quercetin; (K), kaempferol; (Pb), Pinobanksin (3,5,7-trihydroxyflavanone); (Pc), Pinocembrin (5,7-dihydroxyflavanone); (Ch), Chrysin (5,7-dihydroxyflavone); (G), Galangin (3,5,7-trihydroxyflavone; (Dm), Dimethyl-allyl-caffeate; (Fe), Phenyl-ethyl caffeate; Tch (Techtochrysin) (5-hydroxy-7-methoxyflavone).

4. Discussion and conclusion

This study reveals that flavonoid glycosides, previously found for the first time in Robinia honey [22], are common constituents in honey. They are mainly glycosides of the flavonols quercetin, kaempferol, isorhamnetin and 8-methoxykaempferol although other less common flavonoids, as is the case of flavanones in citrus honey, are also present in specific floral origins. This means that specific flavonoid markers could be found in other honeys. Honey flavonoid glycoside content is generally smaller (20–600 μg/100 g honey) than that of flavonoid aglycones (400–4000 μg/100 g honey) derived mainly from propolis contamination. The most frequent glycosidic combinations include rhamnosyl-hexosides (rutinosides and neohesperidosides) and rhamnosides, but dihexo-

sides are also very frequent, particularly hexosyl (1 → 2)hexosides. These should not be glucosides as glucose is readily removed from the flavonoid glycoside conjugates by the action of bee saliva glucosidases, as it has been shown in the case of eucalyptus honey. The presence of other flavonoid hexosides and dihexosides in honey suggests that these are the most probable combinations with other hexoses as galactose. Isolation of these minor honey constituents should be carried out in order to confirm the nature of the glycosidic residues present in the flavonoid glycosides. This is, however, a very difficult task due to the small amount of these markers in honey.

Eucalyptus honey deserves a special comment, as this honey is particularly poor in flavonoid glycosides, although the flavonoids aglycones myricetin, tricetin and luteolin were found to be good markers for this type of honey [19,20]. This can be explained after the analysis of eucalyptus blossom nectar, as this contains mainly myricetin, tricetin and luteolin sophorosides that can be readily hydrolyzed by the bee saliva glucosidases, this being the reason why the aglycones are the main metabolites occurring in honey. On the contrary, nectars rich in rhamnosyl-glucosides (rutinosides or neohesperidosides), that are not hydrolyzed by the bee saliva enzymes, lead to honeys with high amounts of flavonoid glycosides, as is the case or Robinia honey recently published [22] and is also the case of citrus honey (flavanone-rutinoside) and canola and rapeseed honeys that contain relatively high content of flavonoid glycosides.

These results also show that characteristic flavonoid glycoside patterns are found in specific floral origins, and that these findings are quite consistent within a specific floral origin. Thus, a larger number of honey samples from specific floral origins should be analyzed using HPLC-MSn in the future, in order to validate the use of this promising analytical method for the determination of the floral origin of honey.

Acknowledgements

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