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Flavonoids in Croatian Chestnut (*Castanea sativa*) honey

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Summary

All honey types can be generally described as supersaturated sugar solutions, but at the same time they differ in components, such as flavonoids, which are present in small amounts but are responsible for many of their specific properties. The aim of this study was to determine the content of flavonoids in Croatian unifloral chestnut (*Castanea sativa*) honey. For that purpose 9 chestnut honey samples, for which characterisation was achieved by the combination of physicochemical properties and pollen analysis, have been analysed. Flavonoid fraction was extracted from honey and then analysed using reversed-phase high performance liquid chromatography (RP-HPLC) method. Flavonoids myricetin, quercetin, luteolin, kaempferol, apigenin, isorhamnetin, chrysin and galangin were identified and quantified in each sample. Total amount of identified flavonoids varied from 149 μg/100 g of honey to 313 μg/100 g of honey, with the average of 231 μg/100 g of honey. All analysed samples showed common flavonoid profile.

Keywords: Croatian unifloral chestnut honey, flavonoids, RP-HPLC analysis

Introduction

Honey is supersaturated sugar solution which, besides the sugars, contains many other compounds e.g. organic acids, proteins, amino acids, minerals, vitamins and phytochemicals.

While the gross composition of honey is of concern to the regulatory authorities who are attempting to ensure that the public does not purchase adulterated products, it is important to remember that honey also contains a wide range of trace substances which may well endow the product with special therapeutic properties. The identification of minor compounds from honey is in the most cases a demanding but worthwhile task, because many of those compounds may well contribute to its reputation as a "health food" (Al-Qassemi and Robinson, 2003).

At the top of the list of the biologically active components present in honey are flavonoids, class of natural compounds that recently has been the subject of considerable scientific and therapeutic interest.

Flavonoids are major functional compounds which are proven to have antioxidative (Alan and Miller, 1996), antibacterial (Weston, 2000), antitumor

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and anti-HIV (Wang et al., 1998; Ren et al., 2003) properties, but their usage for medicinal purposes is still in many developed countries allowed only in the form of natural flavonoid mixtures (herbal and insect preparations, e.g., propolis or honey) which are considered as supplements, while their marketing as drugs is very limited (Havsteen, 2002).
Amounts of the total flavonoids found in honey are about 6000 μg/kg (Anklam, 1998), and the presence of particular flavonoids depends on the botanical origin of honey.
The aim of this work was to determine the flavonoids present in the Croatian unifloral *Castanea* honey.

Materials and Methods

Honey samples

9 samples of *Castanea* honey were provided by the beekeepers from different parts of the Republic Croatia.
Though beekeepers, based on the position of the hives and flowering season, declared samples as unifloral *Castanea* honey, all the samples were subjected to pollen analysis with the aim of confirming honey type. Additional characterisation of the samples was achieved by the physicochemical attributes analysis in compliance with Croatian Regulation (Ministry of Agriculture and Forestry, 2000) and Harmonised methods of the European Commission (Bogdanov et al., 1997). Afterwards, samples were stored till the flavonoids analysis. Since flavonoids are relatively stable compounds, resistant to heat, oxygen and moderate degrees of acidity honey samples were prior to analysis stored in dark place but at room temperature (Peterson and Dwyer, 1998).

Pollen analysis

Analysis was conducted according to the method of Croatian Regulation (Ministry of Agriculture and Forestry, 2000), and identification of pollen grains was made by reference to the literature data (Von der Ohe and Von der Ohe, 2003) and personal comparative preparations.

Physicochemical analysis

Physicochemical parameters were determined according the methods prescribed by the Croatian Regulation (Ministry of Agriculture and Forestry, 2000) and Harmonised methods of the European Honey Commission (Bogdanov et al., 1997). Moisture content was determined using refractometric method, free acidity by titration of honey sample solution with 0.1M sodium hydroxide to pH

8.30, and electrical conductivity of the 20 % (w/v) water solution of honey (dry matter basis) was measured at 20.0 °C.

Flavonoids isolation

Flavonoids were isolated according to the method previously developed by Ferreres et al. (1994). Honey sample (*ca.* 50 g) was diluted with five parts of acidified water (pH adjusted on 2-3 with HCl). Solution was than passed through a glass column (25×2 cm) filled with Amberlite XAD-2 resins (pore size 9 nm, particle size 0.3-1.2 mm, Supelco, Bellefonte). During this passing the various phenolic compounds remained in the column, while sugars as well as other polar compounds were eluted with the aqueous solvent. Further, the column was washed with 100 mL of acidified water, and 300 ml of distilled water. The whole phenolic fraction was eluted with *ca.* 300 ml of methanol and taken to dryness under the reduced pressure. The dry residue was redisolved in 5 mL of distilled water and partitioned with ethyl ether (3×5 mL). The ether extracts were combined and ether removed under the reduced pressure. At the end of the extraction procedure, dry residue containing flavonoid fraction was redisolved in 0.5 mL of methanol and analysed by HPLC.

RP-HPLC analysis of honey flavonoids

For this purpose Varian ProStar liquid chromatographic system consisting of Solvent Delivery Module, Column Valve Module, UV/Vis Detector was used. Data were collected and analysed by ProStar 5.5 Star Chromatography Workstation and PolyView 2000 Ver. 6.0 Software. LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany, 12.5×0.4 cm I.D., 5μm particle size) was used for separation of flavonoids. The mobile phase consisted of a mixture of water and formic acid (95:5) (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min. To achieve better separation gradient elution was used starting with 30 % of methanol which remained isocratic for the first 15 minutes, and than followed by gradient to obtain 40 % of methanol at 20 min, 45 % of methanol at 30 min, 60 % of methanol at 50 min, 80 % of methanol at 52 min, and which than again become isocratic until the end of analysis in the 60 minutes. Chromatograms were recorded at 340 nm. The injection volume was 10 μL. The flavonoids identification was achieved through comparison of chromatographic data with authentic markers, while quantification was performed through external calibration data with the same compounds. Authentic markers were used for chromatographic comparison of data. Quercetin (3,3′,4′,5,7-Pentahydroxyflavone), luteolin (3′,4′,5,7-Tetrahydroxyflavone) and myricetin (3,3′,4′,5,5′,7-Hexahydroxyflavone) were supplied by Sigma, while chrysin (5,7-Dihydroxyflavone), apigenin (4′,5,7-Trihydroxyflavone), kaempferol (3,4′,5,7-Tetrahydroxyflavone) galangin (3,5,7-Trihydroxyflavone) and isorhamnetin (3′-

Methoxy-3,4´,5,7-tetrahydroxyflavone) were by Fluka (Buchs/Schweiz, Switzerland). Formic acid (Fluka) and methanol (Merck) were HPLC grade.

Data analysis

Mean values and standard deviations (SD) were calculated using computer programme Microsoft Excel 2000 (Microsoft Corp.).

Results and Discussion

Results of pollen analysis (Table 1) conducted with the aim of confirming the botanical origin showed that all samples, besides the sample M-72 which contains 84 % of *Castanea sativa* pollen grains, are in agreement with Croatian Regulation (Ministry of Agriculture and Forestry, 2000) which, due to strong over-representation of *Castanea sativa* pollen, prescribes minimum of 85 % for the declaration of honey as unifloral *Castanea* honey. Still, sample M-72 was included in further analyses due to the fact that it complied with prescribed values in all other parameters, and pollen analysis is known to have some problems (Anklam, 1998).

Table 1. Specific pollen content (%) and flavonoid content (μg/100 g of honey) of Croatian unifloral *Castanea* honey samples

Sample code	% of *Castanea* sativa pollen grains	Flavonoids (μg/100 g of honey)								
		MYR	QUE	LUT	KAE	API	ISH	CHR	GAL	Total
M-09	94	43.6	39.4	12.1	31.3	12.1	-	72.4	76.9	287.9
M-14	90	44.3	36.5	0.0	13.8	28.4	-	35.6	59.9	218.5
M-27	95	52.8	17.6	4.9	8.6	24.7	-	20.9	19.1	148.5
M-43	90	113.3	25.8	5.2	20.3	6.7	-	24.3	27.9	223.5
M-45	96	72.9	32.8	7.7	33.9	10.6	-	72.3	82.8	313.0
M-49	94	27.2	27.7	6.6	48.1	58.1	-	53.0	51.9	272.5
M-72	84	23.7	22.0	6.5	52.5	13.6	-	35.8	45.0	199.1
M-88	87	32.9	31.6	5.9	37.0	12.5	-	34.5	45.4	199.8
M-94	94	43.5	42.7	4.9	20.2	10.5	-	52.8	43.9	218.3
							-			
Mean	92	50.4	30.7	6.0	29.5	19.7	-	44.6	50.3	231.2
SD	4	27.7	8.2	3.2	15.0	16.0		19.1	20.7	51.1
MYR - myricetin, QUE - quercetin, LUT - luteolin, KAE - kaempferol, API - apigenin, ISH - isorhamnetin, CHR - chrysin, GAL - galangin; - flavonoid not detected										

Analysis of selected physicochemical parameters showed that all samples comply with the Croatian Regulation in the respect of water content and free acidity (Ministry of Agriculture and Forestry, 2000). Considering the electrical conductivity, two samples had lower values from the minimum (0.8 mS/cm) prescribed by the European Community Directive (The Council of the European Union, 2002), but since this parameter was not obligatory by the national Regulation, and they complied in other prescribed parameters, they were also included in further analysis.

The RP-HPLC analysis of isolated *Castanea* honey fraction revealed that all the samples have common flavonoid profile, which is shown on the Fig. 1. The largest chromatographic area, and therefore assumable the largest amounts of particular compounds, belong to the compounds that elute during the first ten minutes of the analysis. These are the unidentified phenolic acids. Identified flavonoids, which eluted during the next 50 minutes, were present in much smaller amounts than phenolic acids in all samples.

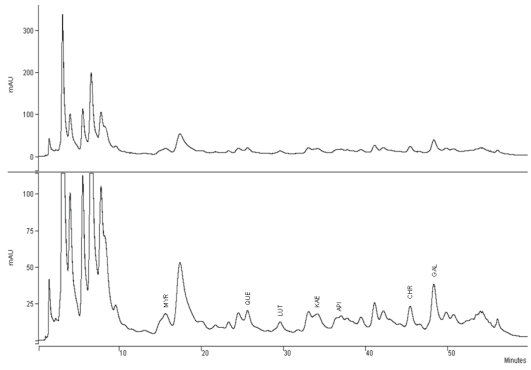

Fig. 1. Typical RP-HPLC chromatogram of flavonoids present in Croatian unifloral *Castanea* honey recorded at 340 nm. Up: whole area of absorbance. Down: Zoomed area of lower absorbances. Peaks: MYR- myricetin, QUE-quercetin, LUT-luteolin, KAE-kaempferol, API-apigenin, CHR-chrysin and GAL-galangin

Results of the flavonoid quantification conducted at 340 nm are shown in the Table 1. Average value of total identified flavonoids was 231.2±51.1 μg/100 g of honey. Tomás-Barberán et al (2001) have reported 169-1300 μg of total flavonoids/100 g of honey for the Italian, Spanish, French and German *Castanea* honeys. In all analysed samples flavonols myricetin, quercetin and kaempferol, and flavones luteolin, apigenin, chrysin and galangin were found. Flavonol isorhamnetin, which was previously detected in rosemary (Gil et al., 1995),

heather (Ferreres et al., 1994), and different types of French unifloral honeys (Soler et al., 1995), was not found in any of the samples.
Flavonoids originating from pollen-nectar contributed in average 58.9 % of total identified flavonoids, while propolis derived flavonoids chrysin an galangin contributed 41.1% of total flavonoid content (Fig 2.). Among pollen-nectar derived flavonoids, the most represented was myricetin (21.8 %, 50.4 µg/100 g of honey), followed by quercetin (13.3 %, 30.7 µg/100 g of honey), and kaempferol (12.8 %, 29.5 µg/100 g of honey). Flavone luteolin was present in the smallest amounts (2.6 %, 6.0 µg/100 g of honey) from all identified flavonoids.

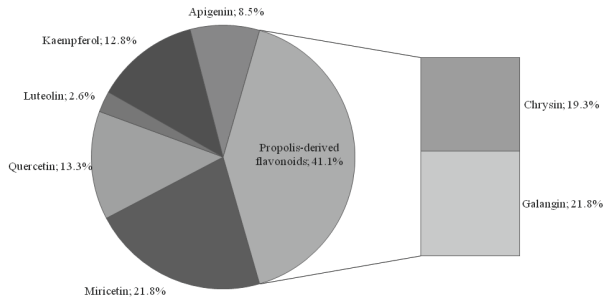

Fig. 2. Average share (%) of individual flavonoid compounds in total identified flavonoids

Castanea honey contained in average more total flavonoids than *Robinia* (208.4±111.8 µg/100 g of honey) (Kenjerić et al., 2007) and less than *Salvia* (288.5±114.3 µg/100 g of honey) (Kenjerić et al., 2008) honeys which were also produced in Croatia. Flavonol myricetin, which was the most represented from all identified pollen-nectar derived flavonoids in *Castanea* honey samples, was not present neither in *Robinia* (Kenjerić et al., 2007) nor in *Salvia* (Kenjerić et al., 2008) honey produced in Croatia. Additionally, it is noticeable that the share of pollen-nectar derived flavonoids in total identified flavonoids is higher in *Castanea* than in both previously reported honey types.

Conclusions

Content of total flavonoids in *Castanea* honeys varied from 148.5 µg/100 g of honey to 313.0 µg/100 g of honey. Pollen-nectar flavonoids dominated over propolis derived flavonoids.

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