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Determination of Rutin, Quercetin, Naringenin and Hesperetin in the Honey from Bosnia and Herzegovina (B & H) in Relation to the Composition of Pollen

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Abstract: Today, consumers and producers makes more and more requests for determining the type of honey and its variety because its technological and medicinal qualities depends on the origin. There are many factors which affect on the quality of honey, but the most important is geographical and botanical origin of which largely depends on its medicinal properties. There are several methods of dealing with the classification of honey but the most accurate is pollen analysis with some other tests, such as pH, electrical conductivity and sensory properties. Flavonoids—as one of the most important group of plant secondary metabolites, can be found in honey and its number and amount directly dependents of the origin of honey. In this study, 48 honey samples were tested, of which 29 were pollen honey, 11 were mixed and 8 were honeydew. In all samples, regardless of the type of honey, quercetin and naringenin are found, and rutin and hesperetin are found in-between 36%-50% of honey samples. The largest individual and avarages amounts of tested flavonoids are found in the pollen honey and at least in the mixed honeys.

Key words: Rutin, naringenin, hesperetin, honey, pollen, plants.

1. Introduction

The properties of honey are the subject of ongoing research in the world, but even so there is no universal method for their classification. The interests for the prove of the autencity of honey comes by consumers and producers [1, 2]. The bees (*Apis mellifera*) collect pollen and nectar at the same time due to the content of nutrients and other beneficial substances [3]. The analysis of pollen taken from the bodies of bees or honey may be able to determine plant and geographic origin as well as the season of collection [4-9]. The sensory properties of honey are direct consequences of nectar and pollen origin [9, 10]. The determination of botanical origin of honey is based on determination of the percentage of pollen and accurately determination of the origin of honey, which is still necessary to use data of sensory, physicochemical and pollen analysis of all plant elements [11, 12]. Flavonoids—the most important group of polyphenols occurs in almost all parts of the plant and the largest amounts contain fruit and vegetables, green and black tee, beries, honey and propolis [13]. The presence of flavonoids in honey primarly depends on the botanical origin, therefore, some of them are labeled as markers of botanical origin [14-17]. It was found that the healing properties of honey significantly associated with flavonoids but total antioxidant activity of honey depens all antioxidant substances in it [18-20].

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2. Materials and Methods

2.1 Materials

Standard substances: rutin purity ≥ 94%, quercetin purity ≥ 94%, naringenin purity ≥ 98% and hesperetin purity ≥ 95% (Sigma-Aldrich).

Chemicals: acetonitrile and methanol, HPLC grade; acetic acid 96% p.a. (Kemika Zagreb); deionized water produced in laboratory (Millipore—Direct Q);

SPE-C18 cartridges tubes 6 mL/500 mg (Resprep); gelatin Ph. Eur. (Fluka); phenol puris p.a.; glicerol puris p.a. and basic fucsin for microscopy (Sigma—Aldrich).

Equipment: pH 510 series; analytical balance Sartorius BP 110 S; vortex—2 genie, scientific industries; SPE extractor supelco visiprep with 12 places; HPLC 1200 series, Agilent technologies with chemstation software, degaser G1322A, autosampler G1239A, quat pumpe G1311A and photodiode array detector G1315D; centrifuge sigma—sartorius 10-16; conductometer eutech instruments oakton con 11/110; light microscope leica DM LS2.

2.2 Methods

2.2.1 Sampling

In the season May-August 2011, 48 samples of honey were collected from different areas and from different honey producers of Bosnia and Herzegovina (B & H) in quantities ≥ 200 g (Table 1). Samples were collected by the method of random selection. Laboratory analyses were carried out in three parallel determinations of the same of honey sample.

2.2.2 Sample Preparation, Determination of pH and Electrical Conductivity

About 5 g honey was dissolved in 10 mL demineralized water, and the pH values of the solution were measured at 25 °C, then it was adjusted to pH 2 using 1 mol/L HCl. Thereafter, the samples were flowed through a previously prepared column (SPE-C18, 6 mL/500 g) with a flow rate of 1 mL/min. A method of preparing the SPE column was: firstly, rinsing done with 9 mL of acetonitrile/methanol/demineralized water (1:1:1); secondly, wash the column with 3 mL

Table 1 Localities of honey samples from the study area.

Oznaka uzorka			Geografsko Područje		
Geographical area		Sign of sample	Geographical area		Sign of sample
The wider locality	The narrow locality		The wider locality	The narrow locality	
Trnovo	Brda	TB1	Zenica	Smetovi	ZS3
Trnovo	Čuhovići	TČ1	Hadzićе	Miševići	HM1
Sokolac	Romanija	SR1	Olovo	O. Luke	OOL1
Kupres	Begovo selo	KBS1	Sanski Most	Gornja Sanica	SMGS1
Fojnica	Živčići	FŽ1	Foča	Dunići	FD1
Konjic	Bradina	KBr1	Rogatica	Vragolovi	RV1
Han Pijesak	Pjenovac	HPP1	Sanski Most	Gornja Sanica	SMGS2
Stolac	Žegulja	SŽ1	Goražde	Petibor	GP1
Rogatica	Han Stjenice	RHS1	Goražde	Petibor	GP2
Zenica	Smetovi	ZS1	Konjic	Podorašac	KP1
Sokolac	Kaljina	SK1	Konjic	Borci	KB1
Sokolac	Novoseoce	SNS1	Žepče	Novi Šeher	ŽNŠ1
Livno	Suhača	LS1	Cazin	Stijena	CS1
Ilijaš	romanijsko područje	IRP1	Tešanj	Potočani	TP1
Vogošća	Srednje	VS1	Bratunac	bliža okolina	Bbo1
Olovo	Dugandžići	OD1	Bosanska Kostajnica	Čitluk	BKČ1
Foča	Tjentište-Mrkalji	FTM1	Ljubuški	Vitina	LJV1
Žepče	Željezno Polje	ŽŽP1	Velika Kladuša	bliža okolina	VKbo1
Zenica	Smetovi	ZS2	Doboj	Kotorsko	DK1
Foča	Jeleč	FJ1	Doboj	Kotorsko	DK2
Bratunac	Cerska	BC1	Čelić	Koraj	ČK1
Žepa	Požeplje	ŽP1	Čapljina	Počitelj	ČP1
Stolac	Donja Bitunja	SDB1	Čapljina	bliža okolina	Čbo1
Tuzla	Majevica	TM1	Ljubuški	Trebižet	LJT1

HCl solution adjusted to pH 2 and thirdly, elute 10 mL of demineralized water. After the failure of samples, SPE columns were washed in the following order. Firstly, wash with 2 mL of diluted HCl acid to pH 2 and secondly, wash with 10 mL of demineralized water. Missed fractions were discarded. Thereafter, in the labeled vials, it collected performed flavonoids fraction, added to 2 mL of methanol and 1 mL of acetonitrile with the same flow rate of solvent (1 mL/min). Collected fractions are filtered through 0.45 μm pore filter of regenerate cellulose and immediately analyzed on the chromatograph. Thus, prepared samples were chromatographed immediately or after 24 hours when they were standing in the refrigerator. For the determination of the electrical conductivity, about 20 g of honey were dissolved in 40 mL of water, and then measured at 20 °C by a conductivity meter with a constant cell of the 1 mS/cm.

2.2.3 Pollen Analysis—Botanical Origin

A sample of 10 g honey was dissolved in 20 mL water at 45 °C. The solution was centrifuged for 15 minutes at 3,500 rpm. The liquide portion was decanted and sediment was transfered to the glass slide and smeared evently on the surface of 15 mm × 20 mm. The sample was dried in an oven at ≤ 45 °C and then embedded in glicerol gelatin. After that preparation, it was collored by adding drops of 0.1 % (w/v) of base fucsin in glicerol gelatin. The sample was covered with a cover glass and returned to the oven at drying. Microscopy was done with magnification from 200 times to 600 times changed visual fields until it counted pollen grains, which are classified to the plant species. Plant species are determined based on pollen grains form, grain size, texture wall of grains and according to the type, shape and number of holls of germination. Pollen grains are compared with reference preparation and image grains in the atlas [21-25]. To characterize the presence of pollen in honey, it has been used adopted international criteria [26, 27].

2.2.4 HPLC Analysis

Chromatographic separation was performed on the column, Eclipse XDB-C18 reverse phase (4.6 mm × 250 mm) with particles diameter of 5 μm, mobile phase A (MF A)—5% aqueous solution of acetic acid/mobile phase B (MF B)—99.6% of methanol. Mobile phases were in relation to 65% MF A/35% MF B isocratic separation. Chromatographic analysis was carried out with a constant flow rate of 1 mL/min. Analysis of the flavonoids rutin, quercetin, naringenin and hesperetin were done with diode array detector, at 370 nm for rutin and quercetin, and at 290 nm for naringenin and hesperetin. Flow rate of mobile phase was 1 mL/min, injection volume 20 μL and the temperature of column was 35 °C. After confirmation the Retention time (Rt) and UV spectra of standard substances (Fig. 1), calibration curve with 5 points in the concentration range from 2.5 μg/mL to 100 μg/mL was established. Coefficients of correlation for all target analytes were $R^2 \geq 0.999$. In such conditions, quantification of the target flavonoids was performed using an external standard. In the same conditions, the method set out Limits of Detection (LOD ≥ 0.01 g/mL) and Limits of Quantification (LOQ ≥ 0.03 mg/mL). Calculation of the results is done according to Eq. (1):

$$Content\ of\ flavonoids\ (\frac{\mu g}{100g}) = \frac{Vfinal(mL)}{m_{weighted\ \ sample}\ (g)} \times 100 \quad (1)$$

The results were statistically analyzed using the softwares Microsoft Excel 2007 and SPSS statistics.

3. Results and Discussion

The richness and the dynamics of bee pastures in the areas of collecting honey samples directly determined the weather, so it could not predict exactly time when extracting a precondition for harvesting the honey, and the honeycomb cells must be closed and riped [28]. Samples were collected with a 43 narrower

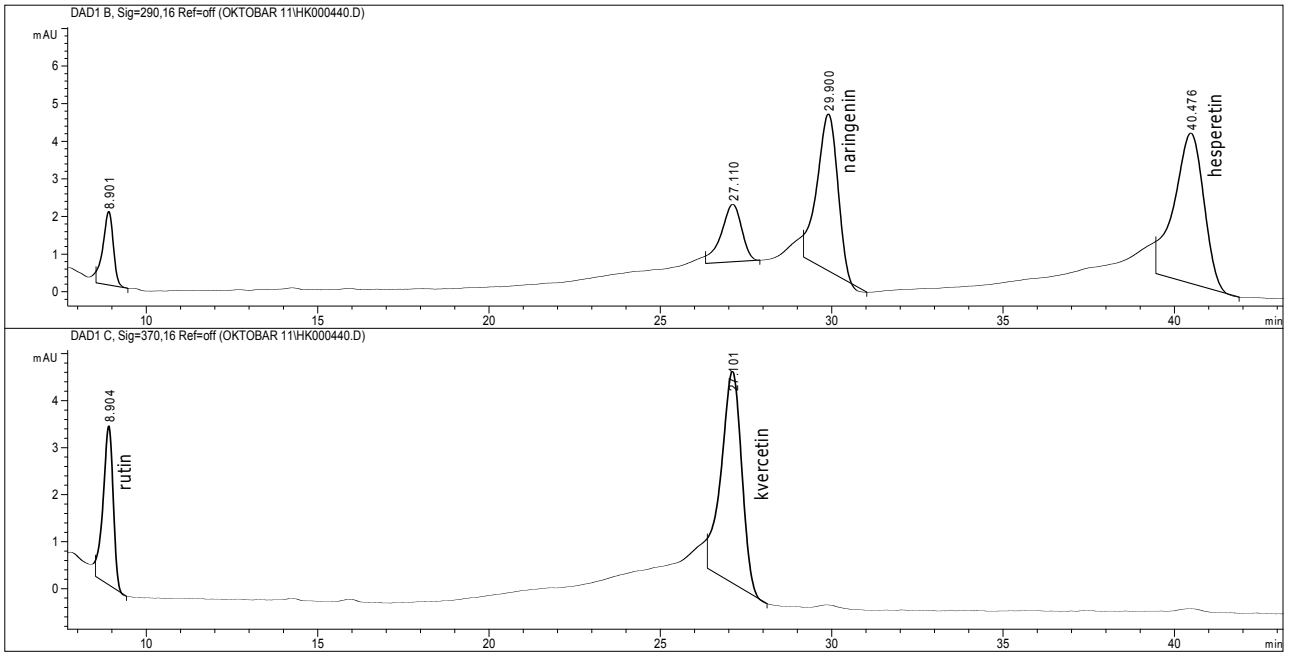

Fig. 1 PLC chromatogram of standard solution rutin, quercetin, naringenin and hesperetin in the concentration of 5 µg/mL.

geographic areas and 29 larger geographic areas of B & H as set out in Table 1. Sensory testing of honey were done with the help of the authors own senses, immediately after sampling [28]. In the pollen analysis, pollen of various families of plants were found: *Apiaceae*, *Asteraceae*, *Betulaceae*, *Rosaceae*, *Brassicaceae*, *Oleaceae*, *Ulmaceae*, *Caryophylaceae*, *Fagaceae*, *Cornaceae*, *Convolvuluceae*, *Liliaceae*, *Fabaceae*, *Cupressaceae*, *Ericaceae*, *Rubiaceae*, *Junglandaceae*, *Malvaceae*, *Laminaceae Poaceae*, *Plantagiaceae*, *Polygonaceae* and *Sumbucaceae*.

In a significant number of samples, it was found the pollen whose plant origin was not determined, and its larger quantities were in honey FJ1. Some honey samples with labels Bbo1, CS1, ZS3 and Vkbo1 were characterized as monofloral honeys of chestnuts because they contained pollen of the family *Fagaceae* with exact origin of plant species of the *Castanea s*., with its pollen content > 85%. In the sample, DK1 was dominant pollen from family *Fabaceae* with content ≥ 75% (*Robinia ps*.) and it was determined as monofloral honey of locust. As the prevailing amount of pollen grains (> 45%) of pollen honey, it was found the presence of pollen from five of the following families of plant: *Asteraceae* (FŽ1, BKČ1), *Brassicaceae* (SNS1, TP1), *Fagaceae* (Bbo1, CS1, ZS3 and Vkbo1), *Fabaceae* (TM1, VS1, ŽP1, RV1, GP2, KP1, DK1, SR1, KBS1 and KBr1) and *Ulmuacea* (IRP1).

Out of 48 honey samples tested, 43 samples were found pollen from 8 familie of plants which total pollen content excess 20% in relation of total pollen content. Property of honey is extricably connected with its origins and a number of environmental factors, so, it is very rare to get unifloral honeys. This study has confirmed that type of honey (pollen honey, mixed and honeydew) is substantially determined by vegetation and environmental condition, such as the state of climate and time of collection [29, 30]. The criteria used to classify the testing honey was as following: (a) for the pollen (nectar) honey, the number of pollen grains ≥ 300, pH > 3.70, electrical conductivity < 0.8 mS/cm except for chesnut honey, then organoleptic properties; (b) for the mixed honey, the number of pollen grains ≥ 100 and ≤ 300, value of electical conductivity, organoleptic properties, then the honeydew elements presence (a great presence of spores and fungi, strach grains, etc.); (c) for honeydew,

the number of pollen grains < 100, conductivity > 0.8 mS/cm, pH > 4.24, then the huge presence of spores and fungi, starch grains and other elements of honeydew [31]. Using results obtained by mentioned criteria, it was found that the 29 honey samples were pollen honey, 8 were mixed honey and 11 were honeydew of the 48 samples tested.

3.1 Content of the Detrmined Flavonoids in Pollen (nectarean) Honey in Relation to the Pollen Composition

In the pollen honeys, pollen from 1 (sample CS1) to 13 (KB1, RHS1 and ŽP1) families of plants (Table 2) were found. In the sample SŽ1, it was found the highest content of all investigated flavonoids and pollen of 9 families of plants. In this sample, the highest pollen content had plant families *Asteraceae, Brassicaceae*, *Fagaceae* and *Fabaceae*. In the sample CS1, pollen grain from only one family (*Fagaceae*) and one plant species *Castanea sat*. was presented which was the highest content of rutin of all sample tested. The sample RV1 had pollen 8 families of plants, quercetin was found in the highest content but rutin and hesperetin were not detected. In the sample RHS1, pollen from 13 families of plants were found. The followings have had a major presence: *Brassicaceae*, *Lamiacea*, *Caryophylaceae* and *Fabaceae*. Of all tested flavonoids, the highest content had naringenin

Table 2 The content of flavonoid in pollen honey in relation to the number of different families of plants in the pollen.

Sign of sample	Total number of families	Content of flavonoids (μg/100 g)					Total number of grains
		Total	Rutin	Quercetin	Naringenin	Hesperetin	
CS1	1	579.7	401.8	151.3	26.6	n.d.	300
Bbo1	2	675.2	209.1	159.6	96.2	210.3	300
VKbo1	4	429.7	n.d.	150.4	67.8	215.5	304
IRP1	6	433.5	67.3	157.4	42.6	166.3	301
ZS2	6	810.8	312.2	172.8	38.0	287.8	327
KP1	6	363.9	n.d.	156.0	29.3	178.7	307
FJ1	7	191.4	n.d.	172.1	19.3	n.d.	350
SDB1	7	457.1	n.d.	184.2	20.0	252.9	309
ZS3	7	489.7	58.3	166.9	61.6	202.9	315
TP1	7	563.4	53.9	172.9	33.0	303.6	325
BKČ1	7	624.8	185.7	164.7	42.1	232.3	303
SR1	8	194.2	n.d.	155.5	38.7	n.d.	300
RV1	8	306.7	n.d.	239.4	67.2	n.d.	321
Čbo1	8	398.7	n.d.	171.8	24.0	202.9	352
SŽ1	9	840.4	286.8	193.2	149.2	211.2	345
GP2	9	542.1	100.8	156.3	128.5	156.4	313
KB1	9	189.7	n.d.	165.4	23.7	n.d.	328
DK1	9	315.6	116.6	158.1	41.6	n.d.	322
LjT1	9	438.5	42.8	165.5	23.1	207.1	302
SNS1	10	757.0	273.4	0.9	476.8	5.9	394
VS1	10	235.7	6.1	211.4	18.3	n.d.	384
TM1	10	110.6	65.8	10.7	11.2	22.9	323
FD1	10	229.1	n.d.	192.4	36.7	n.d.	344
ČK1	11	267.1	88.4	156.9	21.8	n.d.	386
ZS1	11	768.9	97.9	190.3	57.1	423.6	303
KBS1	12	377.1	n.d.	156.5	26.2	194.9	344
KBr1	13	182.8	n.d.	154.0	28.8	n.d.	300
RHS1	13	659.4	17.9	48.2	574.5	18.8	325
ŽP1	13	217.6	n.d.	196.1	21.6	n.d.	349
Avarage values		436.2	82.2	156.2	77.4	120.5	

in this sample. In the sample ZS1, pollen of 11 different families of plants which the highest presence of pollen had *Asteraceae*, *Fabaceae*, *Lamiaceae* and *Rosaceae* were found. Of all tested flavonoids, the highest amount had hesperetin in this sample. All other results are presented in Table 2, showing that quercetin and naringenin are found in all samples, while rutin and hesperetin were not detected in a number of samples.

3.2 Content of the Detrmined Flavonoids in Mixed Honey in Relation to the Pollen Composition

In the mixed honey, pollen from 4 (LS1) to 12 (SMGS1) families of plants (Table 3) were found. As per total and individual content were tested, flavonoids stood out the samples of LS1 and HM1. In the sample, LS1 were found pollen grains of 4 next families of plants *Fagaceae*, *Fabaceae*, *Plantagiaceae* and *Ulmuceae*. Pollen grains of 9 different families of plants, which *Fabaceae (Robinia ps*.) was dominant were found in the sample HM1. In all mixed honeys, rutin, quercetin and naringenin were found, whose amounts could be said were not dependent on the number of families of plants or total pollen grains. Hesperetin was not found in the most of mixed honeys, but a significant amount was found in SMGS1 and LS1 samples (Table 3). In the mixed honeys, the largest number of pollen grain was found in families *Fabaceae* and *Fagaceae*, and significantly fewer grains of other families that were determined in this work.

3.3 Content of the Detrmined Flavonoids in Honeydew Honey in Relation to the Pollen Composition

In honeydew, pollen from 1 (ŽNŠ1) to 9 (SMGS1) families of plants (Table 4) were found. In a number

Table 3 The content of flavonoid in mixed honey in relation to the number of different families of plants in the pollen.

Sign of sample	Total number of families	Content of flavonoids (μg/100 g)					Total number of grains
		Total	Rutin	Quercetin	Naringenin	Hesperetin	
LS1	4	556.3	151.9	161.5	73.4	169.6	110
OD1	4	29.1	9.3	11.0	6.3	2.5	180
FŽ1	7	54.7	27.8	10.8	6.1	9.9	227
OOL1	7	94.3	82.6	9.3	2.5	n.d.	280
GP1	7	233.6	35.4	163.3	34.9	n.d.	107
HM1	9	516.2	295.6	170.7	49.9	n.d.	170
SK1	12	154.2	31.1	98.0	25.2	n.d.	245
SMGS2	12	416.0	2.3	152.8	31.6	229.4	280
Acvarage values		256.8	79.5	97.2	28.7	51.4	

Table 4 The content of flavonoid in honeydew in relation to the number of different families of plants in the pollen.

Sign of sample	Total number of families	Content of flavonoids (μg/100 g)					Total number of grains
		Total	Rutin	Quercetin	Naringenin	Hesperetin	
ŽNŠ1	1	414.9	n.d.	161.0	32.8	221.0	37
TČ1	4	447.0	n.d.	164.5	111.5	171.0	69
ŽŽP1	4	233.3	15.2	179.2	39.0	n.d.	68
DK2	4	281.6	68.7	162.5	50.4	n.d.	83
HPP1	5	203.9	n.d.	161.1	42.9	n.d.	27
TB1	7	398.6	n.d.	165.6	94.7	138.3	43
BC1	7	234.6	19.3	186.6	28.8	n.d.	99
FTM1	8	464.9	60.4	166.8	36.7	201.0	85
LJV1	8	399.4	33.3	179.1	21.2	165.8	53
ČP1	8	452.3	85.2	158.5	34.1	174.4	33
SMGS1	10	272.3	9.9	156.0	106.4	n.d.	68
Avarage values		345.7	26.5	167.4	54.4	97.4	

of samples, rutin and hesperetin were not detected but quercetin and naringenin were found in all honeydew samples. The quantities of quercetin are very consistent except in samples OD1 and OOL1 where relatively small amounts were found. In the TČ1 and SMGS1, significant amounts of naringenin were found. In the sample TČ1, pollen grains of 4 different families of plants were found. In the SMGS1 sample, pollen grains of 10 different families of plants were found. In both samples the largest number of pollen grains was from familly of *Fabaceae*.

4. Conclusion

In all tested samples, at least 2 of the 4 studied flavonoids were found regardless of the pollen composition or type of honey. Their avarage content in B & H samples of honey are different and the largest in the pollen honeys is in amount of 436.2 μg/100 g, higher than honeydew of 345.7 μg/100 g, and the lowest amount in the mixed honey is 256.8 μg/100 g. Avaliable reference shows that the content of flavonoids could be up to 6,000 μg/kg [31]. However, in these studies, it was found that the sum of flavonoids rutin, quercetin, naringenin and hesperetin can reach to over 8,000 μg/kg per honey sample. In comparation to previous studies, it can be said that the avarage content of investigated flavonoids in B & H honeys obtained in this research are correlated with the results obtaind in the world [32-37].

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