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Identification and Quantification of Quercetin, Naringenin and Hesperetin by RP LC – DAD in Honey Samples from B&H

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Abstract: A large number of different products on the market comes under the name of honey, but many of them are false. Presence of flavonoids in the honey samples may be an indication of its origin. Therefore there is a need for reliable analytical methods for identification and quantification of flavonoids in the honey samples. Flavonoids as constituents of many plants, bees bring to the hive through pollen and honeydew. In this work, identification and quantification of three flavonoids: quercetin, naringenin and hesperetin from twelve honey samples of different botanical origin from Bosnia and Herzegovina were performed. The samples were collected during the period July-September, 2010. Reverse phase liquid chromatography coupled with diode array detector (RP LC-DAD) has been used to separate flavonoids and to quantify them in extractive solutions from honey samples. Results showed that the highest amount of quercentin (43.28 μg/100 g honey) and hesperetin (50.12 μg/100 g honey) was found in honey acacia (K2) and naringenin (41.40 μg/100 g honey) in the linden. The highest total content of all investigated flavonoids was 122.40 μg/100 g honey in sample of honey acacia (K2).

INTRODUCTION

Flavonoids are a group of polyphenolic compounds that are found in many plants, concentrating in seeds, fruit skin or bark, bark, leaves and flowers. As the components of fruits, vegetables, and beverages, such as wine and tea, many of the 4000 - 6400 of known different flavonoids are the part of a regular diet. In recent years, scientists have conducted extensive studies of flavonoids and determined their biological effects, such as antibacterial, antifungal, antiviral, anticancer, and others (Cushnie and Lamb, 2005; Alcerito e*t al.,* 2002; Basle e*t al*., 1999). Flavonols and flavonoids are particularly important because they posses antioxidant and free radical scavenging capacity (Kazazić, 2004). Flavonoids affect the color and flavor of food (Wen *et al*., 2010; Yuan-gang *et al*., 2006; Kazazić, 2004). Flavonoids act as antioxidant, antimicrobial, as photoreceptors and as agents for attracting attention, food rejection and protection from UV radiation (Alcerito *et al.,* 2002). Obviously, these are compounds that play an important role in maintaining and protecting the vital functions of plants. The protective role of flavonoids in biological systems is attributed to their ability to pair ("capture") free radicals electrons, to chelate metal ions (Fe^{2+}, Cu^{2+}, Zn^{2+} i Mg^{2+}), and to activate antioxidant enzymes and inhibit oxidase. Till now, research has shown, that there is a connection between the individual structural components and properties of scavenging, creating chelate complexes and antioxidant activity (Adekunle *et al.,* 2012). The mechanism of action of flavonoids at the molecular level in biological systems is not completely understood, due to large differences in chemical properties and because of their large structural heterogenity. In every organism there is a balance between oxidative stress and antioxidant reparations. Absence of antioxidant protection can cause oxidative stress in several ways (Khazai *et al.,* 2011). Free radicals are involved in the development processes of many diseases, such as asthma, cancer, cardiovascular disease, cataracts, diabetes, gastrointestinal inflammatory disease, liver disease, and other inflammatory processes (Hiran *et al.,* 2004). Free radicals can damage the lipid membrane by creating a carbon radical which reacts with oxygen

producing peroxide radical. The resulting peroxide radicals react with fatty acids creating new carbon radicals. Initiation of lipid peroxidation chain reactions, can damage many molecules by one radical. It is important that there are different mechanisms of antioxidant defense including enzymes, proteins, and antioxidants soluble in water and fats and flavonoids which react as scavengers of free radicals, because of the potentially damaging effects of free radicals in the body. (Olszewska, 2007; Kazazić, 2004). Flavonoids are the subject of research of many scientists, because of the all positive qualities (characteristics), (Stanojević *et al.*, 2009; Robbinsa, 2009). Phenolic compounds, as very important secondary metabolites of plant life, have different chemical structures and functions and generally possess an aromatic ring whit one or more hydroxyl substituents. Phenolic flavonoids, such as monomeric compounds of flavanols, flavanones, anthocianidines, flavones and flavanones have diphenylpronane ($C_6C_3C_6$) skeleton (Djilas *et al.*, 2002). So far, several thousand of phenolic compounds, which occur in free form or more often in the form of glycosides, are isolated and identified from plants. Given the great diversity of plant phenols, their classification is very complex. In the literature there are different classifications mostly by chemical structure and biosynthetic origin (Grbović, 2001). Honey is the product resulting from the processing of bee nectar and / or honeydew. Honeydew is a sweet product of aphid and whiteflies ears, and bees collect it from leaves and other parts of trees. Nectar honey is sweeter than honey of honeydew. Flavor of nectar honey, its color, viscosity and chemical structure are characteristics of the honeydew from which the nectar is collected (Dujmović and Hulina, 2007; Chang *et al.*, 2001). There is a growing interest in establishing the authenticity of food products, particularly for natural products, like honey (Nozal *et al.*, 2005). Many authors that studied phenolic compounds and flavonoids have found there is a relationship between the antimicrobial activity of honey and its geographical location, as well as botanical origin. The presence of certain flavonoids in honey primarily depends on its botanical origin, thus flavonoids are markers of botanical origin of honeys (Bertoncelj, 2008; Bogdanov and Martin, 2002). Thus, Assma and others (2009) used the flavanones hesperetin as a marker for the socalled citrus honeys. Flavonol kampferol is the marker of rosemary honey, quercetin for sunflower honey, some phenolic acids as markers of hazelnuts honey and hidroxicinnams as markers of chestnut honey (Alvarez – Suarez *et al.*, 2009; Robbinsa *et al.*, 2009; Olszewska, 2007). The main groups of flavonoids in honey are flavones, flavonols and flavanones that differ in structural formulas and in the position of substituents in rings A, B and C (Figure 1 a). Basic structural formulas of phenolic flavonoids (with different positions of the substituents R1 - R5) are shown in Figure 1. Due to poor solubility in water and oils, applications of flavonoids in foods and medicines are limited (Yue *et al.*, 2010; Alabedeen *et al.*, 2009). Until now, the flavonoids are analyzed by various instrumental techniques. Today, HPLC - DAD is largely being applied in the determination of the active ingredients in the plants as well as of the flavonoids (Yue *et al.*, 2010; Wen *et al.*, 2010; Yuan-gang et al., 2006). Most of these studies focused on the analysis of flavonoids in honey by HPLC with UV detection. Two UV absorption bands are characteristic for flavonoids, one band with maximum in the range of 240-285 nm, is believed to arise from (A) ring, and the other band with maximum in the range of 300-550 nm, probably come from the (B) ring (Figure 1). A good estimate of the concentrations of flavonoids can be obtained by comparing the data integration of target flavonoids in honey with standard chromatograms of flavonoids (Alabedeen *et al.*, 2009; AOAC Officel method, 2002).

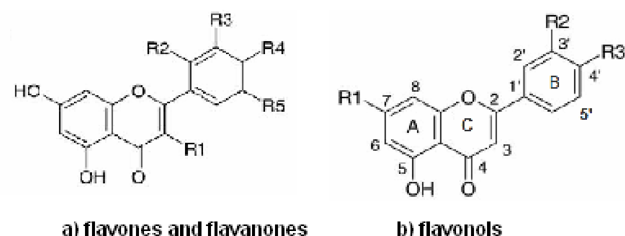

Figure 1: Basic structural formulas of flavonoids with different substituents R1 - R5.

EXPERIMENTAL

Chemicals and Reagents: Quercetin standard was purchased from Sigma - Aldrich/Gemany (Se.No. 020M1566), naringenin from SAFC/Germany (Se.No. MKAA2821), hesperetin from Fluka Analytical/Switzerland (Se.No. 059K1313). Acetonitrile and methanol HPLC grade (purity ≥ 99.6%) were obtained from the firm J & T Baker Ltd. (USA). Ethanol p.a. was obtained from the company Kemika Zagreb (Croatia). Deionized water was produced on the instrument Milli-Q Water Purification System (Millipore Corporation) - Direct Q. SPE - C18 cartridges for extraction were Resprep (6 mL, 500 mg) from Restek Corp. All prepared solutions for HPLC analysis were previously filtered through 0,45 µm pore filter of regenerated cellulose, obtained from Macherey - Nagel (Lot 8301).

Solution of standard substances: Stock solutions of flavonoids quercetin, naringenin and hesperetin (2000 µg/mL) were prepared in methanol. Working solutions of flavonoids at the concentration of 100 µg/mL, 50 µg/mL, 25 µg/mL, 5 µg/mL and 2.5 µg/mL, were prepared in methanol. All these solutions, whose 2 months stability was confirmed by HPLC analysis, were kept in the dark at + 4 ^{0}C. Prior to injection into the HPLC system, all solutions were filtered through 0.45 µm pore membrane filter.

Table 1: Relations between components of mobile phase of acetonitrile (MF) and 5 % aqueous solution of acetic acid and its flow time

Time (min)	15	10	15	30	10	10
MF–A (%)	95-85	85	85-78	78	78-75	75 -95
MF–B (%)	5-15	15	15-22	22	22-25	25-5

Apparatures and instrument conditions: The chromatographic system Agilent Technologies LC 1200 consisted of the following modules; Chemstation software, Degasera model G1322A, Autosampler model G1239A, Quat Pump model G1311A and Photodiode Array Detector model G1315D. Chromatographic separation was performed on the column, Eclipse XDB - C18 reverse phase (4.6 mm x 250 mm, Agilent Technologies, USA) with particles diameter of 5 µm, mobile phases acetonitrile (MF-A) / 5% aqueous solution of acetic acid (MF-B), gradient system, is used. Chromatographic analysis was carried out

with a constant flow rate of MF of 1mL/min, with the ratio of mobile phases A and B as shown in table 1.

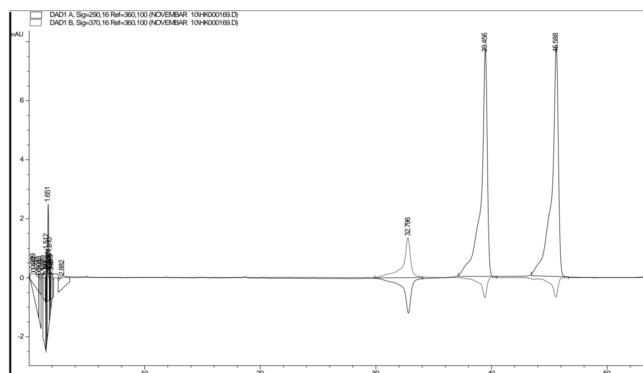

Figure 2: HPLC chromatogram of standard solution quercetin, naringenin and hesperetin in the concentration of 5 μg/mL.

Qualitative - Quantitative analysis of the flavonoids quercetin, naringenin and hesperetin were done with DAD detector at 370 nm for quercetin and 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 ^{0}C. After confirmation the retention time (Rt) and UV spectra of standard substances (Figure 2), 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.

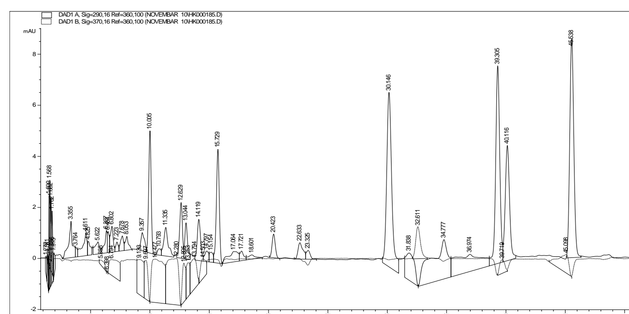

Figure 3: HPLC chromatogram of acacia honey sample from the area of Konjic / BiH (labeled K2).

Sampling and sample preparation for analysis: In the period July - September 2010, twelve samples of honey from BiH region of different botanical and geographical origin and from individual producers were collected (Table 2). Samples for analysis were prepared by dissolving 5 g of sample in 10 mL of deionized water and stirring vigorously. pH values of obtained honey solution were measured at 25 ^{0}C. Before further treatment pH of samples was adjusted to 2 with HCl solution 1 moL/dm^3. The solutions are then passed through the pre-prepared SPE - C18/500 g column with a flow rate of 1mL/min.

Preparation of SPE - C18 column: The column was washed with 9 mL of mixture acetonitrile / methanol / demineralized water (1:1:1), then with 3 mL of acidified demineralized water at pH 2 and finally with 10 mL of demineralized water. Rinsing was conducted with the constant flow rate of 1mL/min, missed fractions were discarded. Samples were extracted with 2 mL of methanol and 1 mL of acetonitrile with the same flow rate of solvent (1mL/min). Collected flavonoids are filtered through 0.45 μm pore filter and immediately analyzed on the chromatograph.
The content of flavonoids was calculated using the formula:

Content of flavonoids (μg/100g) = $c_{read\ out}$ (μg/g) x V_{final} (mL)/$m_{weighted\ sample}$ (g) x 100

RESULTS AND DISCUSSION

In this study qualitative and quantitative analysis of flavonoids quercetin (QUE), naringenin (NAR) and hesperetin (HES) in the honey samples of different biological and geographical origins from BiH, was performed (Table 2 and Table 3). Presence of flavonoids is confirmed, their content in analyzed samples of honey were significant. Obtained pH values of aqueous solutions are ranged from minimum value 3.78 to maximum value 5.29 (Table 2).

Table 2: Overview of pH values, geographical and botanical origin of 12 honey samples from BiH collected during the period July-September 2010

Label of samples	Geografic orignin	Botanical origin	pH values
O1	Olovo	Mountain honey	3.90
C1	Cazin	Linden (*Tilia* sp.)	3.82
Z1	Zenica	Meadow	3.78
Lj1	Ljubuški	Sages (*Salvia officinalis* L.)	5.29
K1	Konjic	Meadow	3.90
C2	Cazin	Chestnut (*Castanea sativa*)	3.90
K2	Konjic	Acacia (*Robinia pseudacacia*)	5.28
K3	Konjic	Acacia (*Robinia pseudacacia*)	4.45
C3	Cazin	Chestnut (*Castanea sativa*)	4.14
K4	Konjic	Meadow	4.01
G1	Gradačac	Linden (*Tilia* sp.)	4.32
C4	Cazin	Chestnut (*Castanea sativa*)	5.14
		Median value	**4.07**
		Avarage value	**4.33**
		Min value	**3.78**
		Max value	**5.29**

Average pH value for all tested samples was 4.33. Calibration curves were established through three injections of standard solutions, whereby the retention time (Rt), and the coefficient of correlation (r^2) were specified (Table 4). Extraction reproducibility R (%) was established by spiking of sample Z1 which has the lowest flavonoid content. According to the established conditions, the chromatographic analysis of the content of QUE, NAR and HES in the final extracts of honey samples were done in three parallel determinations. Different varieties of honey from 6 geographic regions; Olovo, Cazin, Zenica, Ljubuški, Konjic and Gradačac (Table 2) were analyzed. Six varieties of honey; 1 sample of mountain honey (mark O1), 2 samples of linden honey (C1 and G1), 3 samples of meadow honey (Z1, K1 and K4), 1 sample of sage honey (Lj), 3 chestnut honey samples (C2, C3 and C4) and 2 samples of acacia honey (K2 and K3) were analyzed. Contents of QUE, NAR and HES in relation to the origin of honeys is presented in Table 3.

Table 3: Contents of flavonoid QUE, NAR and HES in BiH honeys of different botanical and geographical origin

Label of samples	Individual amounts of tested flavonoids (µg / 100 g honeys)		
	Quercetin	**Naringenin**	**Hesperetin**
O1	15.98	3.98	2.88
C1	22.84	9.34	5.84
Z1	11.20	5.00	4.40
Lj1	17.20	6.44	10.08
K1	18.40	4.64	6.40
C2	27.34	5.80	13.80
K2	43.28	29.00	50.12
K3	14.74	5.40	5.80
C3	17.52	5.32	1.02
K4	16.40	7.60	0.88
G1	12.40	41.40	5.90
C4	16.80	34.00	4.40
Median value	17.00	6.12	5.82
Average value	19.51	13.16	9.29
Min value	11.20	3.98	0.88
Max value	43.28	41.40	50.12

Chromatograms of samples in Figure 3 show that beside the targeted flavonoids of QUE, NAR and HES in honey samples significant amounts of other flavonoid compounds were detected whose identity has not been established in this study. Content of investigated flavonoids QUE, NAR and HES varies depending on the origin of honey samples.

Table 4: Overviews the results of developing a method for the qualitative and quantitative targeted flavonoids analysis

	Quercetin	Naringenin	Hesperetin
Rt (min)	32.81± 6%	39.46± 6%	45.66± 6%
Coefficient of correlation (r^2)	0.99926	0.99996	0.99993
Recovery – R (%)	101.02	99.98	99.76

Among the three tested flavonoids the lowest amount of HES (0.88 µg /100 g) was found in the meadow honey sample (K4). The highest contents of QUE and HAS (Table 3) as well as the highest total content of all three investigated flavonoids was found in honey of acacia (K2) (Table 5).
The obtained results confirm the presence of significant amounts of flavonoids quercetin, naringenin and hesperetin in BiH honeys of different geographical and biological origins.

Contents of quercetin, naringenin and hesperetin in BiH honeys

Contents of quercetin, naringenin and hesperetin are given in Table 3.
Quercetin: Regardless of the variety of honey, a certain amount of QUE was detected in all samples. Average content of QUE was 19.51 µg/100 g. Content of QUE ranged from 11.20 µg/100 g of honey to 43.28 µg/100 g of honey (acacia honey). Based on the obtained results, it could be observed that there are no statistically significant differences in the content of QUE in the samples tested, regardless of their variety and geographical origin. However, higher contents were found in acacia honey of 43.28 µg/100 g (label K2), chestnut honey of 27.34 µg/100 g (label C2) and linden honey of 22.84 µg/100 g (label C1).
Naringenin: Significant amount of naringenin was found in all varieties of honeys and the highest content was in linden honey 41.40 µg/100 g of honey (label G1). The average content of NAR of 13.16 µg/100g honey differs substantially from the median value of 6.12 µg/100 g of honey, which shows that there is a statistically significant difference in the content of NAR in relation to the origin of honey. The minimum content was found in the sample of mountain honey of 3.98 µg/100 g of honey (label O1). High amounts of NAR were found in linden honey (label G1), chestnut honey (label K4) and acacia honey (label K2).
Hesperetin: Hesperetin was detected in all varieties of the honey. Its average content was 9.29 µg/100 g honey and the median value was 5.82 µg/100 g honey. The obtained results for HES content ranged from 0.88 µg/100 g of meadow honey (label K4) to 50.12 µg/100 g of honey acacia (label K2), it represents high variability of the content of this flavonoid. Considering all obtained results, it can be concluded that the amounts of HES are relatively small. However, the high content of HES of 50.12 µg/100 g of honey was found in acacia honey, then in chestnut honey (label C2) of 13.80 µg/100 g of honey and in sage honey (label Lj1 samples) of 8.10 µg/100 g of honey.

The total content of quercetin, naringenin and hesperetin in BiH honeys

The total content of quercetin, naringenin and hesperetin are given in Table 5.
The average total content of all investigated flavonoids (QUE, NAR and HES) is amounted to 41.96 µg /100 g of honey whereas the median value is 31.55 µg /100 g of honey. The minimum content of all investigated flavonoids was recorded in the sample of meadow honey of 20.60 µg /100 g (Z1) and the highest content in the sample of honey acacia of 122.40 µg/100 g (K2). According to the total content of QUE, NAR and HES, tested honey samples can be ranked by accessing the total content of all three flavonoids, in the following order,: Z1 (meadow honey) <O1 (mountain honey) <C3 (chestnut honey) <K4 (meadow honey) <K3 (acacia honey) <K1 (meadow honey) <LJ1 (sage honey) <C1 (linden honey) <C2 (chestnut honey) <C4 (chestnut honey) <G1 (linden honey) <K2 (acacia honey). The obtained results show that greater amounts of tested flavonoids were found in the examined samples of the botanical origin of honey acacia (K2), linden and chestnut, whereas smaller amounts were found in mountain and meadow honeys, while their content in
sage honey (Lj1) is near the median value of all tested samples.

Differences of QUE, NAR and HES contents in the same kind of honeys, probably occur due to variation in the vegetation period of honey collecting, elevation, scale pastures, climatic factors, the number of sunny days, types of bees, etc. (Marghitas, et . al., 2009).), which may explain the differences between the content of investigated flavonoid in the same kind and geographical origin of honey. From the foregoing, it can be concluded that the honey of acacia has the highest content of investigated flavonoids, while the honeys of meadows and mountain honey regardless of the geographical origin have almost the same content of these flavonoids.

Table 5: Total contents of flavonoid QUE, NAR and HES in BiH honeys of different botanical and geographical origin

Label of samples	Map location		Total amount of QUE, NAR i HES (μg / 100 g honey)
	Latitude	Longitude	
O1	$44^0 13'$	$18^0 60'$	22.84
C1	$44^0 97'$	$15^0 95'$	38.02
Z1	$44^0 20'$	$17^0 93'$	20.60
Lj1	$43^0 19'$	$17^0 55'$	33.66
K1	$43^0 65'$	$17^0 97'$	29.44
C2	$44^0 97'$	$15^0 95'$	46.94
K2	$43^0 65'$	$17^0 97'$	122.40
K3	$43^0 65'$	$17^0 97'$	25.94
C3	$44^0 97'$	$15^0 95'$	23.86
K4	$43^0 65'$	$17^0 97'$	24.88
G1	$44^0 87'$	$18^0 43'$	59.70
C4	$44^0 97'$	$15^0 95'$	55.20
	Median		31.55
	Avarage value		41.96
	Min value		20.60
	Max value		122.40

CONCLUSION

Twelve samples pH values of six different varieties of BiH honey, which were collected during the period July - September 2010 from 6 geographical locations (Olovo, Cazin, Zenica, Ljubuski, Konjic and Gradacac) range from 3.78 to 5.29 and it can be concluded that they are in a good correlation with last scientific knowledge (National Honey Board, 2007). This study shows that BH honeys, regardless of geographical or botanical origin contain significant amounts of quercetin, naringenin and hesperetin. Minimum amounts of tested flavonoids were found in the following samples: Quercetin in an amount of 11.20 μg / 100 in the meadow honey sample originated from the region of Zenica (Z1), naringenin of 3.98 μg / 100g sample in mountain honey (O1) from the site of Olovo and hesperetin of 0.88 μg / 100 g sample in meadow honey originating from Konjic (K4). The maximum amounts of these investigated flavonoids are found in the following honey samples: quercetin in amount of 43.28 μg / 100 g was found in a sample of acacia honey from the site of Konjic (K2), naringenin of 41.40 μg / 100g sample in linden honey originated from the site Gradačac (G1) and hesperetin of 50.12 μg / 100 g sample of acacia honey from the site of Konjic (K2). The total content of three investigated flavonoids depends on the botanical and geographical origin as well as from the other factors (vegetation period of honey collecting, elevation, scale of bee pastures, climatic factors, the number of sunny days, types of bees, etc.), which confirms the different flavonoid content in the samples from the same locality and the same botanical origin.

The honeys of linden, acacia and chestnut have higher amounts of quercetin, naringenin and hesperetin than the honeys of meadow and mountain. The maximum total amount of the three flavonoids per 100 g sample was found in honey acacia originated from Konjic (K2) of 122.40 μg / 100 while the minimum amount of 20.60 μg / 100 g honey was in meadow sample from area of Zenica (Z1). The total amount of the tested flavonoids found in 100 g of sage honey was between the values of the two mentioned varieties of honey. Considering the results, it can be concluded that BiH honeys have significant amounts of quercetin, naringenin and hesperetin which makes them powerful antioxidants.

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Summary/Sažetak

Veliki broj različitih proizvoda na tržištu dolazi pod imenom med, ali često značajan broj tih proizvoda predstavlja falsifikat. Prisustvo flavonoida u uzorcima meda, može biti znak porijekla meda. Stoga, postoji potreba za pouzdanim analitičkim metodama za identifikaciju i određivanje sadržaja flavonoida u uzorcima meda. Flavonoide kao sastojke mnogih ljekovitih biljaka pčele putem polena i medljike donose u košnice. U ovom radu provedena je identifikacija i kvantifikacija tri flavonoida: Kvercetina, naringenina i hesperetina iz 12 uzoraka meda različitog botaničkog porijekla sa područja Bosne i Hercegovine. Uzorci su sakupljeni tokom perioda juli – septembar 2010. Za separaciju flavonoida i njihovo određivanje iz ekstrakata uzoraka meda korištena je metoda tečne hromatografija sa reverznom fazom uz diodni detektor (RP LC – DAD). Rezulati pokazuju da su najveći sadržaji kvercetina (43,28 µg/ 100 g meda.) i hesperetina (50,12 µg/100 g meda) utvrđeni u medu bagrema (K2) (*Robinia pseudacacia L.*), a naringenina (41,40 µg/100 g meda) u lipovom medu (*Tilia sp.*). Najveći ukupni sadržaj sva tri ispitivana flavonoida (122,40 µg /100 g) nađen je u uzorku meda bagrema (K2).