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The Changes of Flavonoids in Honey during Storage

Goran Šarić [1,*], Nada Vahčić [2], Danijela Bursać Kovačević [2] and Predrag Putnik [2,*]

[1] Department of Food Technology, Karlovac University of Applied Sciences, Trg J. J. Strossmayera 9, 47000 Karlovac, Croatia

[2] Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia; nvahcic@pbf.hr (N.V.); dbursac@pbf.hr (D.B.K.)

* Correspondence: gsaric@vuka.hr (G.Š.); pputnik@alumni.uconn.edu (P.P.)

Received: 3 July 2020; Accepted: 31 July 2020; Published: 6 August 2020

Abstract: The purpose of this study was to determine the changes in the contents of flavonoids that were the most prevalent in acacia and multifloral honey during one year of storage. Samples were stored in transparent glass containers, at room temperature, on open shelves exposed to light during daytime. Eight individual flavonoids identified and quantified using HPLC-Diode Array Detector (DAD) belongs to three subgroups: flavonols (quercetin, luteolin, kaempferol and galangin), total flavanons (hesperetin and pinocembrin) and total flavones (apigenin and chrysin). Obtained results revealed that multifloral honey had more total flavonoids than acacia samples did. On average from all of the samples, multifloral honey had more of quercetin, hesperetin, luteolin, kaempferol and apigenin than acacia honey did. Content of flavonoids increased in samples between the 1st and 6th month of storage and then started to decrease until the 9th month, when they remained relatively constant all the way until the 12th month of storage. In conclusion, acacia and multifloral honey after one-year of storage still can be a valuable source of flavonoids.

Keywords: honey; flavonoids; storage; HPLC; marker; floral origin

1. Introduction

Chemically, honey is quite a complex mixture of more than 70 different compounds. Some of them are added by honeybees, some come from the nectar producing plants, while some during ripening of honey in honeycombs [1]. Despite the rapid development of various analytical methods, composition of honey is still not completely elucidated [2–4]. In some ways, this is not an issue as not knowing all the ingredients prevents artificial industrial production and adulterations. That way honey maintains it properties of a natural product, produced solely by the honeybees. Perhaps the most important property by which the chemical composition of honey can be described is variability hence there are virtually no two exactly the same honey samples. Different honey types, as well as honeys within those varieties, differ in their chemical composition depending on their botanical and geographical origins, climate conditions, honeybee breed and skillfulness of the beekeepers [5,6]. The most represented compounds in honey are water and carbohydrates, mostly fructose and glucose, which together make 99% of the honey. The rest includes proteins (including enzymes), minerals, vitamins, organic acids, phenolic compounds, volatile compounds (aroma substances) and various chlorophyll derivatives. Although the share of those compounds is very small, they are greatly responsible for sensory and nutritive properties of honey [7,8].

It was proven that honey has medicinal benefits and for centuries, or even millenniums, it was used as a sweetener (food) and a medicine. Until 1800s, honey was the only easily available sweetener used for human nutrition, when it was replaced with industrial made sugar. Over the last 20 years, a new branch of medicine was developed, called "apitherapy" that relies on medicines based on honey

and other bee products for different diseases. Today, for medicinal purposes, honey is mainly used to treat wounds, burns and infections [9].

Phenolic compounds are one of the numerous groups of compounds from honey where a large portion falls to flavonoids, mainly in the form of glycosides [10]. Usually detected flavonoids from honey originate from propolis, while pollen is not a good source of such compounds, as their content is quite low in there. Characteristic flavonoids found in propolis are chrysin, galangin, tektorizin, pinocembrin and pinobanksin. As flavonoids and other phenolic compounds come exclusively from plants, it is clear that their content will be defined by botanical origins of honey [9], however storage conditions also have an important effect [11].

There are several reports, which confirmed that phenolic and flavonoid profiles determined in investigated honey samples, as well as their antioxidant activities, could be used as markers for honey identification, mainly due to the prevalence of some specific compounds [12–14]. For instance, 52 Spanish honey samples from different botanical origins were analyzed with the aim to determine their botanical origins according to the flavonoid and phenolic acid contents. Additional to the other honey types, authors also analyzed seven acacia samples where they found 0.06–5.11 mg kg^{-1} of quercetin, 2.30–18.69 mg kg^{-1} of pinocembrin and 0.39–4.36 mg kg^{-1} of chrysin, while luteolin (0.06–0.32 mg kg^{-1}) and kaempferol (0.35–0.37 mg kg^{-1}) were detected only in two of the samples [15]. Results from this study also showed that phenolic compounds are potential markers for the floral origins for some honey types (e.g., useful for the heather, chestnut, eucalyptus, rapeseed and lime-tree); while they cannot be used for others due to the absence of their floral specificity (e.g., lavender and acacia). Furthermore, Akbari et al. analyzed a total of 60 samples from 6 different types of honey with respect to phenolic acids and flavonoids. Here, the flavonol quercetin, p-coumaric acid, caffeic acid, flavone chrysin and flavanone hesperetin were detected in all honey samples. In acacia honey, authors found 22.54 ± 7.1 µg/100 g of chrysin and 8.04 ± 4.37 µg/100 g honey of hesperetin, while quercetin was not detected. Among all investigated types, jujube and thyme had the highest total flavonoid aglycone contents [14].

Although there are various papers associating the contents of different phenolic compounds in the honey samples with the floral origin [11,16,17], with over addressing the health benefits of phenolics [18], still there is scarcity of reports that deal with the stability and/or changes of the single flavonoids during prolonged storage. Honey is quite a stable food with the ability to stay unspoiled over longer time due to its high sugar and low water contents, however native flavonoid content may be degraded by the storage. Therefore, phenolics markers have limited usefulness as indicators for determining botanical origins of the samples, as honey is often stored for longer periods before consumption. Consequently, this study aimed to highlight the quality of the samples in terms of individual flavonoids during one year of storage.

2. Materials and Methods

2.1. Honey Samples

Twenty acacia and twenty multifloral honey samples (n = 40) from Varaždin county in Croatia were used in this study. Five different samples of each honey type were collected from each of the four regions of Varaždin County. They were stored for one year in transparent glass containers, at room temperature, on shelves exposed to indirect sunlight. These conditions were chosen because they correspond very well to the average conditions and storage period of honey in households and food markets.

To confirm their botanical origin, all of the samples were subjected to melisopalynological analysis, which was done by counting the pollen grains by a microscope. Briefly, honey samples were diluted in distilled water (5 g in 10 mL), and after thorough mixing they were centrifuged at 3000 rpm for 5 min. The supernatant was discarded while the residue was mixed with glacial acetic acid and centrifuged again. The obtained residue was then mixed with acetolysis mixture (1 mL sulfuric acid mixed with

9 mL acetic anhydride), boiled in a water bath at 85 °C for 3 min, again centrifuged and decanted. The residue was thoroughly washed with distilled water, centrifuged, decanted and mixed with liquid glycerin. The obtained sample was then used for microscopic analysis, which was done by an optical microscope at 400×. Pollen grains were identified using the relevant literature [19].

2.2. Chemicals

Methanol HPLC and p.a. grade (Panreac, Barcelona, Spain); Amberlite XAD-2 (Supelco, Sigma-Aldrich, Steinheim, Germany); formic acid 98–100%, p.a. grade (Sigma-Aldrich, Steinheim, Germany); hydrochloric acid 37% analytical reagent grade (Fisher Scientific, Loughborough, UK) and diethyl-ether Reag. Ph. Eur. (Panreac, Barcelona, Spain). For quantification and identification of individual flavonoids in honey samples, pure compounds of analytical grade were used as internal standards: quercetin (3,3′,4′,5,7-pentahydroxyflavone), hesperetin (3′,5,7-Trihydroxy-4′-methoxyflava none), luteolin (3′,4′,5,7-tetrahydroxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), apigenin (4′,5,7-trihydroxyflavone), pinocembrin (5,7-dihydroxyflavanone), chrysin (5,7-dihydroxyflavone) and galangin (3,5,7-trihydroxyflavone) were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

2.3. Sample Preparation

The mass of 50 g of honey was weighed in a 400 mL glass and was dissolved in 250 mL of acidified distilled water (pH = 2, using HCl, 37% p.a.; total of 350 mL of acidified water was prepared per sample), which was then flown through a glass column filled with Amberlite XAD-2 filler. Flow speed was 9.5–11 mL/min, so there was no loss of phenolic compounds due to leakage through a layer of absorbent. In this phase, various phenolic compounds were bind to filler particles, while sugars and other polar compounds were washed away from the column by water solvent. After that, the same flow rate was maintained, the column was rinsed with an additional 100 mL of previously prepared acidified distilled water, and 300 mL of distilled water until the pH value of the eluate was 7. Using a specially designed pump, residual water was forced out of the column to shorten the time of the following evaporation process.

The total phenolic fraction was rinsed away from the filler by 300 mL of methanol at an elution speed of 2.5 mL/min. Methanol was than evaporated in a low-pressure atmosphere at 40 °C. Dry residue was diluted in 5 mL of distilled water and extracted three times per 5 mL of diethyl-ether. All three etheric extracts were combined together and again evaporated until dry in a low-pressure atmosphere at 40 °C. Before HPLC analysis, dry residue was diluted in 0.5 mL of methanol and filtered through the syringe filter with the pore size of 0.45 μm.

Flavonoid analyses were conducted on six occasions during one year, after one, three, six, nine and twelve months of storage. Each honey sample was analyzed in two parallels.

2.4. HPLC-DAD Analysis

Prior injection to HPLC apparatus, dry flavonoid fraction was prepared and dissolved in 0.5 mL of methanol and filtered through the syringe filter (Cameo, 17F Syringe filter, Teflon, 0.45 μm pore size, 17 mm diameter). The detector used for the HPLC (Shimadzu, Kyoto, Japan) analysis was DAD (Diode Array Detector) and wavelengths were between 290 and 340 nm, depending on the specific flavonoid. The LiChrospher 100 RP-18 (Merck; 12.5 cm × 0.4 cm; 5 μm particle size) column was used for the separation of the compounds. Acidified demineralized water-3% formic acid (mobile phase A) and methanol-HPLC grade (mobile phase B) were used in gradient flow mode. Gradient program was: from 0 to 15 min—30% methanol; at 20 min—40% methanol; at 30 min—45% methanol; at 50 min—60% methanol and at 52 min 80% methanol until the end of 60 min. The operating conditions included an injection volume of V = 20 μL and the flow rate of the 1 mL/min [20]. Individual flavonoids were identified by comparing the obtained chromatographic data (the retention times and the UV spectra)

with the data obtained from the analysis of internal standards. Quantification was performed through external calibration data with the same compounds.

2.5. Statistical Analysis

Descriptive statistic was used to assess the basic information about the experimental dataset (e.g., to obtain a sample basic metrics, check for the normality of distribution). As recommended by the literature [21], continuous variables were tested by the multivariate analysis of variance (two-way ANOVA). The significance levels for all tests were at $p \leq 0.05$. Dependent variables were grouped and individual flavonoids, while independent variables included types of honey and lengths of storage in months with their interactions. Statistical analyses were performed with IBM SPSS Statistics (v.20), and by Statgraphics Centurion (StatPoint Technologies, Inc., Warrenton, VA, USA).

3. Results and Discussion

Results of the quantitative analysis of individual phenolic compounds regarded as the sum of total flavonols (quercetin, luteolin, kaempferol and galangin), total flavanons (hesperetin and pinocembrin) and total flavones (apigenin and chrysin) are presented in Table 1.

Table 1. The changes in total flavonols, flavanones, flavones and flavonoids in acacia and multifloral honey samples during 1, 3, 6, 9 and 12 months of storage.

Source of Variation		*n*	Total Flavonols	Total Flavanons	Total Flavones	Σ Total Flavonoid Content (TFC)
Honey type			$p \leq 0.01$ †	$p = 0.07$ ‡	$p \leq 0.01$ †	$p \leq 0.01$ †
Acacia Honey		20	1.07 ± 0.14 ^{b}	0.70 ± 0.10 ^{a}	0.73 ± 0.09 ^{b}	2.50 ± 0.28 ^{b}
Multifloral Honey		20	2.60 ± 0.14 ^{a}	0.97 ± 0.10 ^{a}	1.58 ± 0.09 ^{a}	5.15 ± 0.28 ^{a}
Shelf life			$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
1 month		8	1.58 ± 0.22 ^{b}	0.42 ± 0.16 ^{b}	0.66 ± 0.15 ^{b}	2.65 ± 0.45 ^{c}
3 months		8	2.38 ± 0.22 ^{a}	0.79 ± 0.16 ^{b}	1.18 ± 0.15 ^{c}	4.34 ± 0.45 ^{b}
6 months		8	2.57 ± 0.22 ^{a}	1.43 ± 0.16 ^{a}	1.64 ± 0.15 ^{a}	5.63 ± 0.45 ^{a}
9 months		8	1.14 ± 0.22 ^{b}	0.84 ± 0.16 ^{b}	0.95 ± 0.15 ^{b,c}	2.92 ± 0.45 ^{c}
12 months		8	1.52 ± 0.22 ^{b}	0.72 ± 0.16 ^{b}	1.36 ± 0.15 ^{a,c}	3.59 ± 0.45 ^{b,c}
Honey type by Shelf life			$p = 0.28$ ‡	$p = 0.30$ ‡	$p = 0.66$ ‡	$p = 0.52$ ‡
Acacia Honey	1 month	4	0.98 ± 0.31 ^{a}	0.13 ± 0.23 ^{a}	0.24 ± 0.21 ^{a}	1.34 ± 0.63 ^{a}
	3 months	4	1.37 ± 0.31 ^{a}	0.70 ± 0.23 ^{a}	0.70 ± 0.21 ^{a}	2.76 ± 0.63 ^{a}
	6 months	4	1.56 ± 0.31 ^{a}	1.44 ± 0.23 ^{a}	1.21 ± 0.21 ^{a}	4.21 ± 0.63 ^{a}
	9 months	4	0.70 ± 0.31 ^{a}	0.88 ± 0.23 ^{a}	0.71 ± 0.21 ^{a}	2.29 ± 0.63 ^{a}
	12 months	4	0.77 ± 0.31 ^{a}	0.35 ± 0.23 ^{a}	0.81 ± 0.21 ^{a}	1.92 ± 0.63 ^{a}
Multifloral Honey	1 month	4	2.18 ± 0.31 ^{a}	0.71 ± 0.23 ^{a}	1.08 ± 0.21 ^{a}	3.97 ± 0.63 ^{a}
	3 months	4	3.38 ± 0.31 ^{a}	0.87 ± 0.23 ^{a}	1.67 ± 0.21 ^{a}	5.92 ± 0.63 ^{a}
	6 months	4	3.58 ± 0.31 ^{a}	1.41 ± 0.23 ^{a}	2.06 ± 0.21 ^{a}	7.04 ± 0.63 ^{a}
	9 months	4	1.58 ± 0.31 ^{a}	0.79 ± 0.23 ^{a}	1.20 ± 0.21 ^{a}	3.56 ± 0.63 ^{a}
	12 months	4	2.27 ± 0.31^{a}	1.09 ± 0.23^{a}	1.91 ± 0.21^{a}	5.26 ± 0.63^{a}
SAMPLE MEAN		40	1.83 ± 0.10	0.84 ± 0.07	1.16 ± 0.07	3.83 ± 0.20

* Results are expressed as mean ± standard error in mg kg^{-1} of honey. † Values represented with different letters are statistically different at $p \leq 0.05$; ‡ not statistically significant at $p \leq 0.05$.

Eight flavonoid aglycone compounds were identified and quantified and total flavonoid aglycone content (TFC) was calculated as the sum of all individual flavonoids. In general, multifloral honey had twice more total flavonoids than acacia honey samples (5.15 vs. 2.50 mg kg^{-1}). Moreover, multifloral honey contained significantly higher amounts of total flavonols and total flavones as compared to acacia honey samples. Furthermore, total flavanones were not significantly different with respect to investigated honey samples.

The content of TFC increased in the samples between the 1st and the 6th month of the storage and then started to decline at the 9th month, when it remained relatively constant until the 12th month. In other words, they returned to the values from the start of the study or to the 1st month of the storage.

This way flavonoids lost 48% and 36%, respectively from the maximum measured value obtained in the 6th month as compared to the contents measured after the 9th and 12th months of storage (see Table 1). The largest losses from the maximum measured values were obtained in the 6th month for flavonols (56% and 41% for the 9th and the 12th month, respectively) and flavanones (41% and 50% for the same respective storage times). The contents of flavones also declined by the 42% and 17% for the 9th and 12th month, respectively. This trend was the same for both honey types. Our results showed that storage had significant impacts on flavonoids and botanical origin of honey that is contrary to the findings from Maurya et al., who stated that storage had only minor influences on phenolics in the honey [22]. Authors also claimed that the botanical origin played predominant roles in changes of antioxidants and phenolics in honey. On the other hand, Brudzynski and Kim (2011) concluded that storage had adverse effects on the antibacterial activity of honeys. Dietary phenolics are known antimicrobial agents [23], and similar to our results the antibacterial activity of the honey samples was rapidly reduced during the storage. For instance, the 50% decreased activity was observed in the first 3–6 months of storage and further reduction was gradually identified over the period of 12–36 months. These findings implicated that the compounds responsible for the microbial inhibition were chemically unstable during the storage [24]. Furthermore, Sousa et al. concluded that the honey samples showed high antibacterial activities due to unique phenolic profile, including flavonoids [25]. Hence, it can be concluded that determination of instability of honey flavonoids during storage is of great importance for the evaluation of the botanical origins, antioxidant and antibacterial activities that change during the storage.

On average in all of the samples, multifloral honey had higher contents of quercetin, hesperetin, luteolin, kaempferol and apigenin than acacia honey did. However, at the beginning of the storage multifloral honey had only hesperetin, luteolin and apigenin in higher concentrations than acacia. No differences in contents of pinocembrin, chrysin and galangin among different types of honey samples were observed. Stability of polyphenols during the length of shelf life followed various patterns for different compounds. Hesperetin content increased in the samples by 1.5 times from the initial measurements in the 1st month and until the 6th month of storage, and then stabilized thorough the end of the study. Pinocembrin, chrysin and galangin increased in content by the 6th month of storage and then started to decline by the end of the study. Quercetin and kaempferol contents remained more or less the same during storage of honey over 12 months, while luteolin and apigenin followed a trend similar for hesperetin with a difference for the 9th month of storage when their contents experienced sharp drop, only to be back to a constant value in the 12th month of storage. The type of honey did not have an influence on stability of the most polyphenols. Hesperetin content only increased in multifloral honey during storage but remained constant in acacia samples. Data strongly indicated that in order to retain the most of the polyphenols as markers for botanical origins, honey should not be tested later than 6 months from the initial harvest and flavonoids content will be equally well preserved in both types of honey samples.

The average values of individual flavonoids obtained after one month of storage in acacia honey were somewhat lower than the ones determined in multifloral honey samples (Table 2). Figures 1 and 2 show the typical HPLC chromatograms of eight identified and quantified flavonoids detected in both honey samples. Considering that eight identified flavonoids represent a smaller part of the total flavonoid content in honey samples, the trends in changes of individual flavonoids might be compared with the trends in changes of total flavonoids during a prolonged storage. Similarly, our previous research reported that total flavonoids, total phenolic content and corresponding antioxidant activity decreased during one year of storage [26].

Table 2. The changes in individual flavonoids in acacia and multifloral honey samples during 1, 3, 6, 9 and 12 months of storage.

Source of Variation			Total Flavonols				Total Flavanons		Total Flavones	
		n	Quercetin	Galangin	Luteolin	Kaempferol	Hesperetin	Pinocembrin	Chrysin	Apigenin
Honey type			$p \leq 0.01$ †	$p = 0.05$ ‡	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p = 0.97$ ‡	$p = 0.51$ ‡	$p \leq 0.01$ †
Acacia Honey		20	0.20 ± 0.02 ^{b}	0.55 ± 0.10 ^{a}	0.10 ± 0.03 ^{b}	0.22 ± 0.06 ^{b}	0.06 ± 0.02 ^{b}	0.64 ± 0.10 ^{a}	0.44 ± 0.06 ^{a}	0.29 ± 0.05 ^{b}
Multifloral Honey		20	0.52 ± 0.02 ^{a}	0.82 ± 0.10 ^{a}	0.39 ± 0.03 ^{a}	0.86 ± 0.06 ^{a}	0.34 ± 0.02 ^{a}	0.64 ± 0.01 ^{a}	0.50 ± 0.06 ^{a}	1.08 ± 0.05 ^{a}
Shelf life			$p = 0.06$ ‡	$p \leq 0.01$ †	$p \leq 0.01$ †	$p = 0.49$ ‡	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †	$p \leq 0.01$ †
1 month		8	0.37 ± 0.04 ^{a}	0.60 ± 0.15 ^{a,b}	0.18 ± 0.04 ^{a,b}	0.42 ± 0.09 ^{a}	0.14 ± 0.02 ^{b}	0.28 ± 0.16 ^{b}	0.18 ± 0.10 ^{b}	0.47 ± 0.08 ^{c}
3 months		8	0.45 ± 0.04 ^{a}	0.98 ± 0.15 ^{a}	0.33 ± 0.04 ^{a}	0.62 ± 0.09 ^{a}	0.16 ± 0.02 ^{b}	0.62 ± 0.16 ^{a,b}	0.44 ± 0.10 ^{b}	0.74 ± 0.08 ^{b}
6 months		8	0.49 ± 0.04 ^{a}	1.15 ± 0.15 ^{a}	0.29 ± 0.04 ^{a}	0.63 ± 0.09 ^{a}	0.21 ± 0.02 ^{a}	1.22 ± 0.16 ^{a}	0.84 ± 0.10 ^{a}	0.80 ± 0.08 ^{b}
9 months		8	0.18 ± 0.04 ^{a}	0.35 ± 0.15 ^{b}	0.10 ± 0.04 ^{b}	0.51 ± 0.09 ^{a}	0.27 ± 0.02 ^{a}	0.56 ± 0.16 ^{a,b}	0.52 ± 0.10 ^{a,b}	0.43 ± 0.08 ^{c}
12 months		8	0.33 ± 0.04 ^{a}	0.34 ± 0.15 ^{b}	0.33 ± 0.04 ^{a}	0.51 ± 0.09 ^{a}	0.21 ± 0.02 ^{a}	0.51 ± 0.16 ^{b}	0.37 ± 0.10 ^{b}	0.99 ± 0.08 ^{a}
Honey type by Shelf life			$p = 0.73$ ‡	$p = 0.29$ ‡	$p = 0.06$ ‡	$p = 0.97$ ‡	$p = 0.04$ †	$p = 0.19$ ‡	$p = 0.76$ ‡	$p = 0.08$ ‡
Acacia Honey	1 month	4	0.24 ± 0.05 ^{a}	0.51 ± 0.21 ^{a}	0.07 ± 0.06 ^{a}	0.16 ± 0.13 ^{a}	0.06 ± 0.03 ^{a}	0.07 ± 0.23 ^{a}	0.06 ± 0.14 ^{a}	0.17 ± 0.11 ^{a}
	3 months	4	0.27 ± 0.05 ^{a}	0.68 ± 0.21 ^{a}	0.15 ± 0.06 ^{a}	0.27 ± 0.13 ^{a}	0.06 ± 0.03 ^{a}	0.64 ± 0.23 ^{a}	0.42 ± 0.14 ^{a}	0.27 ± 0.11 ^{a}
	6 months	4	0.29 ± 0.05 ^{a}	0.82 ± 0.21 ^{a}	0.15 ± 0.06 ^{a}	0.29 ± 0.13 ^{a}	0.06 ± 0.03 ^{a}	1.39 ± 0.23 ^{a}	0.90 ± 0.14 ^{a}	0.31 ± 0.11 ^{a}
	9 months	4	0.11 ± 0.05 ^{a}	0.34 ± 0.21 ^{a}	0.06 ± 0.06 ^{a}	0.18 ± 0.13 ^{a}	0.08 ± 0.03 ^{a}	0.80 ± 0.23 ^{a}	0.51 ± 0.14 ^{a}	0.20 ± 0.11 ^{a}
	12 months	4	0.12 ± 0.05 ^{a}	0.40 ± 0.21 ^{a}	0.07 ± 0.06 ^{a}	0.18 ± 0.13 ^{a}	0.05 ± 0.03 ^{a}	0.30 ± 0.23 ^{a}	0.31 ± 0.14 ^{a}	0.49 ± 0.11 ^{a}
Multifloral Honey	1 month	4	0.51 ± 0.05 ^{a}	0.69 ± 0.21 ^{a}	0.30 ± 0.06 ^{a}	0.68 ± 0.13 ^{a}	0.23 ± 0.05 ^{b}	0.48 ± 0.23 ^{a}	0.30 ± 0.14 ^{a}	0.78 ± 0.11 ^{a}
	3 months	4	0.63 ± 0.05 ^{a}	1.29 ± 0.21 ^{a}	0.50 ± 0.06 ^{a}	0.97 ± 0.13 ^{a}	0.27 ± 0.05 ^{b}	0.61 ± 0.23 ^{a}	0.46 ± 0.14 ^{a}	1.20 ± 0.11 ^{a}
	6 months	4	0.69 ± 0.05 ^{a}	1.48 ± 0.21 ^{a}	0.44 ± 0.06 ^{a}	0.97 ± 0.13 ^{a}	0.36 ± 0.05 ^{a}	1.05 ± 0.23 ^{a}	0.78 ± 0.14 ^{a}	1.28 ± 0.11 ^{a}
	9 months	4	0.24 ± 0.05 ^{a}	0.36 ± 0.21 ^{a}	0.14 ± 0.06 ^{a}	0.83 ± 0.13 ^{a}	0.46 ± 0.05 ^{a}	0.33 ± 0.23 ^{a}	0.53 ± 0.14 ^{a}	0.66 ± 0.11 ^{a}
	12 months	4	0.54 ± 0.05 ^{a}	0.29 ± 0.21 ^{a}	0.59 ± 0.06 ^{a}	0.84 ± 0.13 ^{a}	0.36 ± 0.05 ^{a}	0.73 ± 0.23 ^{a}	0.42 ± 0.14 ^{a}	1.48 ± 0.11 ^{a}
SAMPLE MEAN		40	0.36 ± 0.02	0.69 ± 0.07	0.25 ± 0.02	0.54 ± 0.04	0.20 ± 0.01	0.64 ± 0.07	0.47 ± 0.04	0.69 ± 0.04

* Results are expressed as mean ± standard error in mg kg^{-1} of honey. † Values represented with different letters are statistically different at $p \leq 0.05$; ‡ not statistically significant at $p \leq 0.05$.

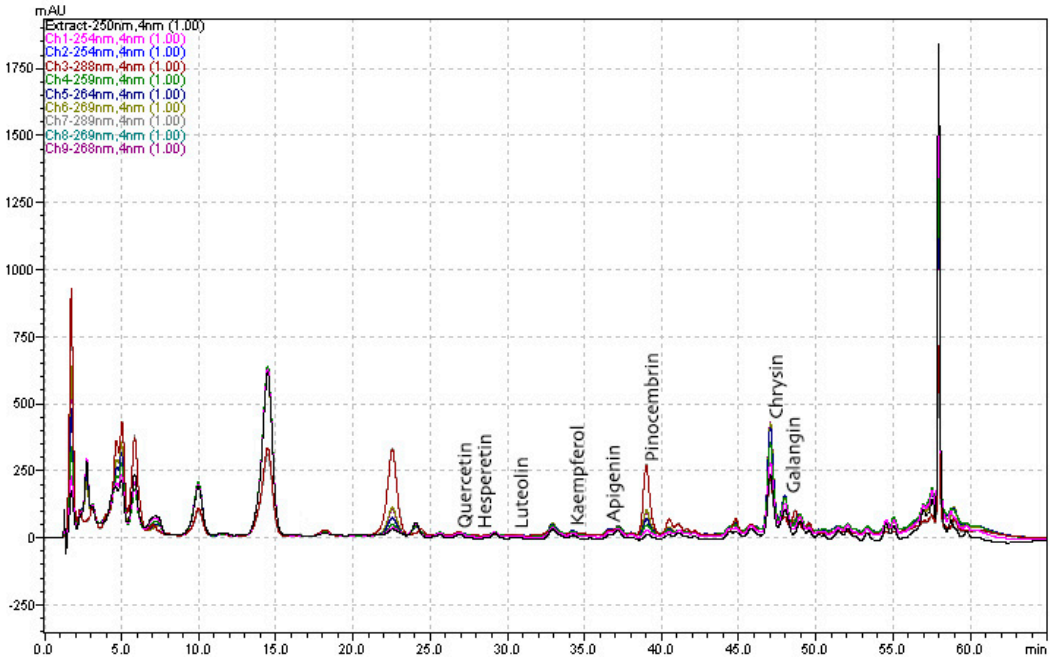

Figure 1. A typical chromatogram of acacia honey.

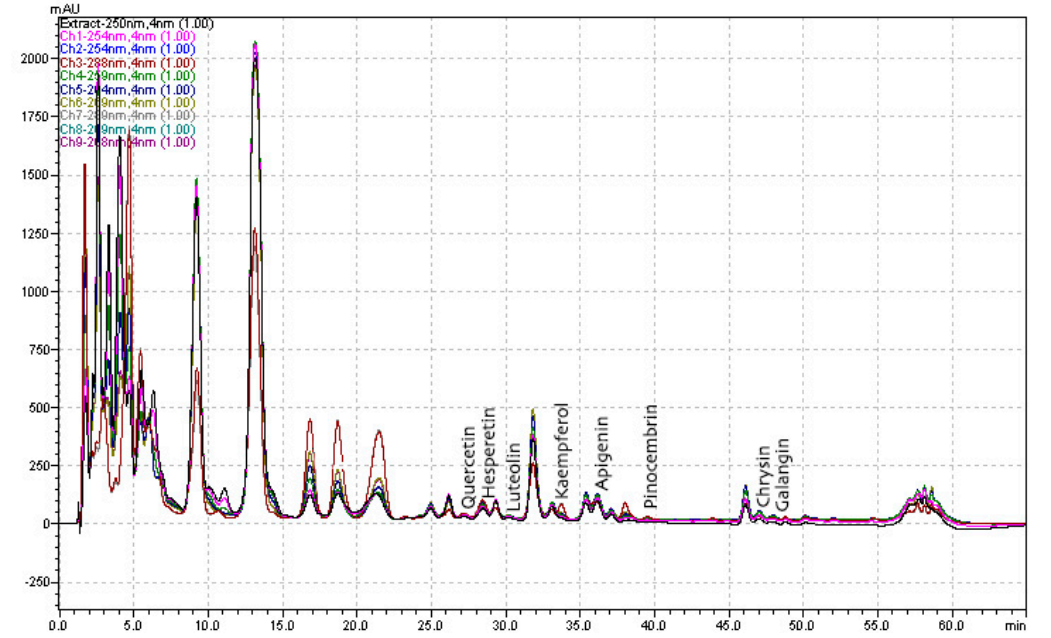

Figure 2. A typical chromatogram of multifloral honey.

The results for acacia samples from the 1st month of our study had fifteen times lower contents of hesperetin (0.004 vs. 0.06 mg kg^{-1}), eight times less apigenin (0.021 vs. 0.17 mg kg^{-1}), three times lower content of pinocembrin (0.027 vs. 0.07 mg kg^{-1}) and quercetin (0.077 vs. 0.24 mg kg^{-1}), while contents of luteolin, chrysin and kaempferol were very similar (0.07 vs. 0.04 mg/kg, 0.06 vs. 0.04 mg kg^{-1} and 0.17 vs. 0.16 mg kg^{-1}, respectively) in comparison to those found in literature [27].

A research on honey samples harvested from three different geographic regions in Argentina was conducted with the aim to evaluate the changes in the flavonoids content. Contents of three individual flavonoids were determined with the average values for all three regions: myricetin (1.9–5.8 mg kg^{-1}), quercetin (3.8–10.2 mg kg^{-1}) and luteolin (1.8–4.3 mg kg^{-1}) [28]. These values were slightly higher than the ones obtained in our research and there are two possible reasons for this discrepancy. The most

likely is that the authors used different types of honey that originated from different geographical and climatic conditions, and the second reason is possibly due to the use of different sample preparation and flavonoid determination procedures.

Results from another study revealed in acacia honey 0.19 mg kg^{-1} of apigenin, 1.56 mg kg^{-1} of chrysin, 0.42 mg kg^{-1} of hesperetin, 0.38 mg kg^{-1} of kaempferol, 6.00 mg kg^{-1} of pinocembrin and 2.95 mg kg^{-1} of quercetin. These results were similar to the ones that were obtained in our research, although there were some significant differences regarding the contents of several flavonoids namely chrysin (0.064 mg kg^{-1} vs. 1.56 mg kg^{-1}) and pinocembrin (0.073 mg kg^{-1} vs. 6.00 mg kg^{-1}) [29].

Due to considerably larger varieties of herbal nectars from the nature that bees use to make floral honeys commonly, its chemical compositions and corresponding flavonoid contents are typically very different from that in acacia honey. Additionally, one has to bear in mind that these differences are strongly dependent on the percentage of different plant nectars that are present in multifloral samples. In line with this, by using a pollen analysis, it was found that the samples of acacia honey had 34.0% of pollen grains of acacia (*Robinia pseudoacacia* L.), 29.0% of pollen from rose family plants (*Rosaceae*), 16.0% from legumes family (*Fabaceae*), 11.0% from the crucifers (*Brasicaceae*) and 1.5% from plant species of the *Asteraceae* family. On the other hand, the mean percentage values of the most represented pollen grains in multifloral honey samples used in this research were: 45.2% of pollen from rose family plants (*Rosaceae*); 16.3% from the crucifers (*Brasicaceae*); 9.8% from willow (*Sallix* sp.); 7.1% from acacia (*Robinia pseudoacacia*); 6.8% from sweet chestnut (*Castanea sativa*); 5.3% from legumes family (*Fabaceae*) and 2.7% from amorpha (*Amorpha fruticosa*). Other research suggested that the variation in the pollen composition during different times of acacia honey harvest plays a significant role in the total flavonoid contents [27].

All of the various compounds potentially have different influences on each other, which might be a possible reason for differences in flavonoid stability and changes in their content during storage [30]. Scripca et al. [31] determined the contents of twelve phenolic compounds in various honey types, including acacia and floral samples. The contents of total polyphenols measured in acacia honey (0.08–0.06 mg 100 g) against the floral honey (9.13–1.84 mg/100 g) showed quite a large difference. Interestingly, authors did not find any amounts of myricetin, quercetin, luteolin or kaempferol in acacia honey samples, which differ from the results obtained in our current research.

Content of the most investigated flavonoids for the both honey types was the highest in the middle of a storage period (i.e., 6th–9th month of storage). To the best of our knowledge, there are no available scientific papers that explained what exactly influenced flavonoids or similar molecules in honey during prolonged periods of storage, but it is possible to speculate several possibilities. For instance, the results from a recent study pointed out that chemical reactions in honey could spontaneously produce new compounds from the existing substrates with no impact of the botanical source during the extended storage under the UV absorbance [24]. Moreover, it is possible that enzymatic activity caused changes in molecular structure of flavonoids or they were resynthesized after initial degradation, or storage temperature might affect their stability (as it was not controlled). Moreover, flavonoids occur mainly as *O*-glycosides in plants, thus, most flavonoid aglycones in plants originate from flavonoid glycosides produced by enzymatic hydrolysis by enzymes in honey or microorganisms [10]. In fact, flavonoid glycosides are widespread in honey and it is therefore likely that the honey samples in this investigation also contained flavonoid glycosides.

Connection of temperature and antioxidant activity was investigated by Turkmen et al., who studied the effects of elevated heating on antioxidant activity on the color of honey. They discovered that the biggest changes for both investigated parameters occurred at 70 °C, while they were less pronounced at 50 and 60 °C. Authors demonstrated that there is a strong correlation between antioxidant activities and increased browning of honey. So, even though, Turkish honey was exposed to much higher temperatures that the samples from our study, still the influence of temperature on our samples might be similar but more spread over the longer period of storage.

Wang et al. analyzed the effects of processing and storage on antioxidant capacity of honey by determining the contents of some flavonoids among other parameters. The obtained results after six months of storage showed that contents of all investigated flavonoids remained virtually constant. Another study confirmed that thermal treatment of honey does not have a significant impact on flavonoid profiles in investigated honey samples [13] or antioxidant activity after short thermal treatment of honey [26].

Finally, analyzed data suggested that honey samples should be stored no longer than 6 months to retain most of the flavonoids contents while they will be equally well preserved regardless of the honey type. Meaning that botanical origin will not have large influence on the stability of flavonoids, which is a good characteristic for potential markers. Hence, determining the changes of individual flavonoids in honey during storage has great potential, aligned with the recent attempts to use these individual compounds as floral markers. This is additional to the possibility to securely and accurately determine and predict the antioxidative activity of honey and potential health benefits.

4. Conclusions

In general, it was found that multifloral honey samples had higher contents of total aglycone flavonoids as compared to acacia honey samples. On the other hand, the changes among their contents during storage were very similar. The content of flavonoids increased in the samples between the 1st and the 6th month of storage and then started to decline at the 9th month, when it remained almost constant up until the 12th month of storage. The reasons behind an initial increase of the flavonoids and the subsequent drop to initial values were not fully clear. However, flavonoids showed the potential to be used as markers for botanical origin of honey, but they should be best used before the end of the six months of storage. Anyhow, more research should be done on this topic to further elucidate the changes that might be occurring in the honey during the storage and/or processing, as honey is an important commodity on food markets.

Author Contributions: Conceptualization, G.Š., and N.V.; methodology, G.Š., and N.V.; formal analysis, G.Š.; investigation, G.Š., N.V., P.P., D.B.K.; data curation, P.P.; writing—original draft preparation, G.Š., P.P., D.B.K.; writing—review and editing, G.Š., P.P., D.B.K. All authors have read and agreed to the published version of the manuscript.

Funding: This paper was produced as part of a project co-financed by the European Union from the European Regional Development Fund and the Operational Program Competitiveness and Cohesion 2014–2020.

Conflicts of Interest: The authors declare no conflict of interest.

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