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Changes of Antioxidant Activity and Phenolic Content in Acacia and Multifloral Honey During Storage

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Summary

Total flavonoid and total phenolic content were studied in acacia and multifloral honey for 12 months in 6-month intervals. DPPH (1,1-diphenyl-2-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) methods were used to determine total antioxidant activity in honey samples during the same period of time. Samples were stored in transparent glass containers at room temperature, on shelves exposed to natural light during daytime and in the dark during nighttime. Two types of honey from four different regions in Varaždin county, Croatia, were investigated: monofloral – acacia (*Robinia pseudoacacia* L.) and multifloral. Of the total of 40 samples, there were 20 of each type of honey (5 from each region). The goal of this study is primarily to demonstrate the changes in the antioxidant activity of the two investigated types of honey during one year of storage, and not to make comparisons between them. According to the obtained data, the rate of decrease in the content of total flavonoids and phenolics was determined and changes in the antioxidant activity in honey samples were measured. After one year of storage, total phenolic content decreased by 91.8 % in acacia honey, and by 88.6 % in multifloral honey. Total flavonoid content also decreased in both types of honey, by 45.6 % in acacia honey and by 43.8 % in multifloral honey. During the same period, an increase from 12.20 to 16.73 mg/mL (*i.e.* by 37.1 %) was recorded in the IC_{50} values in multifloral honey, while in acacia honey this increase was from 44.64 to 407.01 mg/mL (*i.e.* by 811.7 %). Decrease in the antioxidant activity measured by FRAP method was also bigger in acacia honey than in multifloral honey (by 428.0 and 72.5 %, respectively), which corresponds well with the results obtained by DPPH method. Simple correlations were made to determine how each of the investigated parameters affects the others. The analysis of variance was used in order to determine the influence of the region, honey type and storage time on different parameters of antioxidant activity as well as on the total phenolic and total flavonoid content in honey samples.

Key words: antioxidant activity, phenolic compounds, honey, storage

Introduction

Antioxidant activity has recently been determined in various foodstuffs by many scientists and research groups around the world. It has been proven that numerous natural compounds show different antioxidant activity that depends on their origin, chemical structure, bioavailability, *etc.*, and that some of these compounds work together in protecting against various degenerative disorders including cancer, stroke, cardiovascular, Alzheimer's and Parkinson's diseases (*1–4*). There are several different levels of protection mechanisms which function by

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inhibiting the formation of free radical species, intercepting radical chain reactions, converting the existing free radicals into less harmful molecules and by repairing oxidative damage (*5*). All plants produce numerous secondary metabolites, among which phenolics are one of the most important groups. They are characterized by at least one aromatic ring (C6), to which one or more hydroxyl groups are attached. Polyphenols are synthesized from cinnamic acid, which is formed from phenylalanine (*6*).

From ancient times honey has been used as a natural sweetener and as a remedy. It has quite complex chemical composition, so there are basically no two samples alike. Its highly various sensorial and physicochemical characteristics are due to diverse climatic and environmental conditions and various origins of plants from which it is harvested. It is essentially a concentrated aqueous solution of inverted sugar, but it also contains around 200 substances which form a mixture of other saccharides, enzymes, amino and organic acids, polyphenols, carotenoid-like substances, Maillard reaction products, vitamins and minerals (*7*). Some of these compounds (phenolics, vitamin C, Maillard reaction products, some amino acids, *etc*.) give the honey its bioactive properties (*8*). This makes it more than just a nourishment of high value, but a valuable dietary source of antioxidants. Besides that, honey contains phenolic acids, flavonoid aglycones (pinobanksin, chrysin, galangin, luteolin, kaempferol, myricetin, quercetin, *etc*.) and flavonoid glycosides, an antioxidant pool that by acting synergistically can explain many of the biological or therapeutic properties of honey (*9,10*).

Because of its diverse and complex composition, even honey samples from the same botanical origin can show different antioxidant activity. Results from several studies show that honey types that are light in colour (acacia or lime) show lower values for some parameters of antioxidant activity than honey types that are darker (forest, chestnut, spruce or fir) (*11,12*). Increased browning of honey during heating correlates very well with its antioxidant activity (*13*).

Melisopalynological analysis is at the moment the only recognized method for determination of botanical origin of honey. It is easy to comprehend that majority of analytical efforts until now have focused on the characterization of some potential markers of botanical, geographical and seasonal origin, for example: amino acids, proteins, minerals, volatiles, sugar composition and recently phenolics. In the last years, more and more research has been done on determining the antioxidant properties of various types of honey from around the world. On the other hand, almost nothing has been done regarding monitoring the changes in the antioxidant activity during storage. This is an important topic because honey is mostly not consumed immediately after production, especially if it is bought in a supermarket and not directly from a beekeeper. Having this in mind, the main objective of this study is to determine the changes in the antioxidant activity and in total flavonoid and phenolic content in acacia and multifloral honey during one year of storage.

Materials and Methods

Samples

Twenty acacia and twenty multifloral honey samples from Varaždin county in Croatia were used in this study. Five 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 light. These conditions were chosen because they correspond very well to the average conditions and storage period of honey in households. To confirm their botanical origin, all of the samples were subjected to melisopalynological analysis.

Chemicals

Fructose (ultra pure; ≥99 %), glucose (ultra pure; ≥99.5 %), maltose (≥99 %), sucrose (ultra pure; ≥99.5 %) and ferric sulphate were acquired from Fluka, Steinheim, Germany. Folin-Ciocalteu phenol reagent, gallic acid, polyvinylpyrrolidone (PVPP), 1,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma-Aldrich, Steinheim, Germany. Sodium nitrite, aluminium chloride, sodium hydroxide, sodium acetate trihydrate and acetic acid were obtained from Kemika, Zagreb, Croatia. Di-sodium-hydrogenphosphate-heptahydrate and ferric chloride were purchased from Merck, Darmstadt, Germany, and sodium dihydrogen phosphate monohydrate was purchased from J.T. Baker, Deventer, The Netherlands.

Methods for determination of physicochemical parameters

In all samples, nine physicochemical parameters were determined. Water mass fraction (moisture) was measured by refractometer using the AOAC Official Methods (*14*) and total reducing sugar, sucrose, ash mass fractions and acidity were also measured conformant to the same methods. Electrical conductivity, measured by Mettler conductivity meter, diastase and invertase activities and proline mass fraction were determined according to the methods proposed by the International Honey Commission (IHC) (*15*).

Total phenolic content analysis

Total phenolic content in aqueous honey solutions was determined according to Beretta *et al.* (*8*) and Bertoncelj *et al.* (*11*). The method is based on the coloured reaction of phenolics with Folin-Ciocalteu reagent. Upon the reaction with phenols, Folin-Ciocalteu reagent is reduced to a blue coloured oxide. The intensity of the resulting colour was measured in a spectrophotometer at 750 nm.

A mass of 5 g of honey was diluted in 50 mL of distilled water. A volume of 300 μL of honey solution was pipetted into a test tube and 3 mL of 10 % Folin-Ciocalteu reagent were added. This was mixed on a vortex for 2 min and after 20 min the absorbance was measured in a spectrophotometer (Pye Unicam SP6-500, Cambridge, UK) at 750 nm. Honey analogue (saturated aqueous solution of sugars (in %): fructose 40, glucose 30, maltose 8 and sucrose 2) was made to prepare the blind control samples. The preparation and measuring procedure was

the same as the one used for honey samples. Gallic acid was used as the standard for the construction of a calibration curve for total phenolic content determination. The concentration of total phenolics is expressed as mg of gallic acid equivalents (GAE) per kg of honey.

Total flavonoid content analysis

Total flavonoid content in honey samples was determined according to Blasa *et al.* (*12*) and Kim *et al.* (*16*). A blank was used to eliminate the interference of reducing sugars, and it was prepared according to Gheldof *et al.* (*7*). Briefly, 0.1 g of insoluble polyvinylpyrrolidone (PVPP) was added to 5 mL of 75 mM phosphate buffer (sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate heptahydrate), pH=7.0, and moisturized at 4 °C for 24 h. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. A volume of 5 mL of a honey solution (5 g of honey in 25 mL of phosphate buffer, pH=7) was added to the residual sediment, stirred for 30 min at 30 °C and then filtered. This solution was used as a blank.

The determination of total flavonoids in honey samples starts by mixing 1 mL of sample solution with 0.3 mL of 5 % $NaNO_2$ in a test tube. After 5 min, 0.3 mL of 10 % $AlCl_3$ were added to the solution by mixing in a vortex. After 6 min of the reaction, the solution was neutralized with 2 mL of 1 M NaOH. This solution was once more mixed in a vortex and transferred to a glass cuvette. The absorbance was measured in a spectrophotometer at 510 nm. Three replications were made for each sample. For the calibration curve, quercetin solutions were used, so the results are given in mg of quercetin equivalents (QE) per 100 g of honey.

DPPH radical scavenging assay

Radical scavenging activity in honey samples was determined according to Beretta *et al.* (*8*) and Brand-Williams *et al.* (*17*). Stable DPPH radical reaches the absorbance maximum at 517 nm and its colour is purple. The change of this colour into yellow is a result of pairing of an unpaired electron of a DPPH radical with the hydrogen of the antioxidant, thus generating reduced DPPH-H. Adding an antioxidant results in the decrease of absorbance, which is proportional to the concentration and antioxidant activity of the compound.

Stock honey solution was prepared by diluting 15 g of honey in 25 mL of distilled water. Eleven different concentrations of honey solutions were made (3 to 60 mg/mL). A volume of 0.3 mL of each of these solutions was mixed with 0.8 mL of acetate buffer (pH=5.5) in 11 test tubes. Because of the low content of antioxidants in acacia honey, three times higher volumes were used. In each of the test tubes, 1.9 mL of 130 μM DPPH reagent were added, briefly mixed on a vortex and left in the dark at room temperature for 90 min. After that, the absorbance was measured in spectrophotometer at 517 nm. Besides that, the absorbance of blank and control samples was measured. Percentage of the remaining DPPH was calculated from the following equation:

$$\text{DPPH}=(A_{\text{sample}}-A_{\text{blank}})/A_{\text{control}}\cdot 100 \qquad /1/$$

Results are shown as IC_{50} values, *i.e.* the concentration of antioxidant (honey concentration) that causes 50 % inhibition of DPPH. IC_{50} is calculated from the equation of the curve for each individual sample.

The final results were calculated using the equation of the curve where the X-axis represented concentrations and Y-axis the values calculated with the above-mentioned Eq. 1. Each of the calculated values corresponds to its concentration and for each sample the corresponding curve was made. The concentrations for acacia honey ranged from 3 to 60 mg/mL (in 6 mg/mL increments) and for multifloral honey from 1 to 20 mg/mL (in 2 mg/mL increments). During the development of the method, concentration range of honey solutions was determined for each type of honey. For the calculation of the final results it was important that, at this range, the relation between the concentration of honey solutions and the inhibition of DPPH reagent was linear.

FRAP (ferric reducing antioxidant power) assay

Antioxidant activity of honey was determined according to Bertoncelj *et al.* (*11*) and Benzie and Strain (*18*). The method is based on the ability of the honey sample to reduce the ferri form of 2,4,6-tri(2-pyridyl)-1,3,5-triazine complex (Fe^{3+}-TPTZ) to ferro, coloured form (Fe^{2+}-TPTZ) at acidic pH. Reduction is monitored by measuring the changes of absorbance at 593 nm.

Briefly, aliquots of 400 μL of the sample (5 g of honey sample was diluted in 50 mL of distilled water) were mixed with 3.6 mL of fresh FRAP reagent solution (2.5 mL of 10 mM TPTZ reagent, plus 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH=3.6) and after incubation for 10 min at 37 °C, the absorbance was measured at 593 nm. A calibration curve was constructed using $FeSO_4$ solutions (concentrations from 0.2 to 1 M, in 0.2 mM increments) and the absorbance was measured at the same wavelength. The results were expressed as μM of Fe(II) in 10 % honey solution.

Except for the physicochemical parameters, total phenolic and flavonoid contents and radical scavenging and antioxidant activities were determined three times during one year of storage – at the beginning of the study, after six, and after twelve months of storage.

Measurements for all methods used in this research were done in three replications for each sample.

Results and Discussion

Physicochemical parameters

To confirm the botanical origin provided by manufacturers, all of the honey samples were subjected to melisopalynological analysis, and all of them satisfied the conditions given by the Croatian regulation on quality of monofloral honey (*19*).

In the first phase of this research, to ensure that all of the samples comply to the Croatian regulations on honey (*20*), nine physicochemical parameters (water content, acidity, electrical conductivity, total reducing sugars, sucrose, ash and proline content, as well as invertase and diastase activities) were determined. With 20.52 %, one multifloral honey sample failed to meet the de-

mands of the regulation on water content. The same sample did not satisfy the regulations on diastase activity, with the value of 4.1 DN (diastase number). The results for all other parameters for all the remaining samples met the demands given by the Croatian regulation. Because none of these parameters affected considerably the antioxidant activity or total phenolic and flavonoid content, the study was conducted on all samples regardless of whether they satisfied the demands of the regulation or not. Average values of all parameters are shown in Table 1.

Total phenolic and flavonoid content

As phenolics are present in all plants, they are also found in honey. Fruit and beverages such as tea and red wine are the main sources of polyphenols. Certain polyphenols such as quercetin are found in all plant products (fruit, vegetables, cereals, leguminous plants, fruit juices, tea, wine, infusions, *etc.*), whereas others are specific to particular foods (flavanones in citrus fruit, isoflavones in soya, phlorizin in apples). In most cases, foods contain complex mixtures of polyphenols, which are often poorly characterized (*21*). Compared to some other foodstuffs (some berries, tea, red wine, apples, *etc.*), honey is not considered to be a good and rich source of these compounds. Their content depends on the geographical and botanical origin of honey; also, darker types have been found to have a higher content than the light coloured honey types (*11,12*).

Initial values of total phenolic content in acacia honey samples ranged from 69.71 to 112.57 mg of GAE per kg of honey, with the average value of 86.26 mg of GAE per kg of honey. The average values for individual geographic regions ranged from 76.76 to 101.71 mg of GAE per kg of honey (Table 2).

These results are somewhat higher than those obtained in other studies. The average value for total phenolic content in Italian acacia honey obtained by Beretta *et al.* (*8*) was (55.2±2.8) mg of GAE per kg of honey. Bertoncelj *et al.* (*11*) obtained similar results, from 25.7 to 67.9 mg of GAE per kg of honey, with average value of 44.8 mg of GAE per kg of honey. The lowest values were obtained by Al *et al.* (*22*), who determined the total phenolic content in Romanian acacia honey to be in the range from 2.0 to 39.0 mg of GAE per kg of honey. The study from 2009 on Croatian acacia honey also showed slightly lower values than those obtained in this study, with the range from 31.72 to 80.11 mg of GAE per kg of honey (average value of 43.66 mg of GAE per kg of honey) (*23*).

Initial values for total phenolic content in multifloral honey ranged from 141.14 to 247.81 mg of GAE per kg of honey, with the average value of 201.14 mg of GAE per kg of honey. The average values according to individual geographic regions ranged from 163.62 to 236.38 mg of GAE per kg of honey (Table 2). These results are also a bit higher than those obtained in Slovenian and Italian research on the same type of honey (*8,11*). The average value obtained by Lachman *et al.* (*24*) in the Czech multifloral honey was 112.07 mg of GAE per kg of honey, which is almost half of the average value obtained in this study.

Honey samples used in the first phase of this research were freshly produced. As it has been demonstrated here that total phenolic content in both honey types was

Table 2. Average values of total phenolic content in acacia and multifloral honey during one year of storage

Honey type	Region	*w*(TP as GAE)/(mg/kg)		
		September–October 2009	March–April 2010	September–October 2010
acacia	Bednja	101.71±7.87	25.06±3.53	8.23±2.34
	Ivanec	76.76±8.37	37.46±16.33	7.01±1.77
	Novi Marof	85.14±6.00	35.63±19.50	5.61±0.75
	Ludbreg	81.33±8.34	17.62±10.62	7.56±3.35
	average±S.D.	86.26±10.87	28.94±9.32	7.10±1.11
multifloral	Bednja	195.81±5.78	175.54±41.73	27.58±1.98
	Ivanec	208.76±15.94	129.21±33.60	23.32±5.49
	Novi Marof	236.38±8.05	173.58±32.97	23.90±1.16
	Ludbreg	163.62±13.22	127.77±31.93	16.77±1.98
	average±S.D.	201.14±30.20	151.53±26.62	22.89±4.50

TP=total phenols, GAE=gallic acid equivalent

Table 1. Physicochemical parameters of acacia and multifloral honey samples (average value±standard deviation)

Honey type	Region	*w*(water)	*b*(acidity)	Electrical conductivity	*w*(total reducing sugars)	*w*(sucrose)	*w*(ash)	*w*(proline)	Invertase activity	Diastase activity
		%	mmol/kg	mS/cm	%	%	%	mg/kg	IN	DN
acacia	Bednja	15.80±0.29	12.62±1.97	0.16±0.02	70.26±1.88	0.51±0.46	0.05±0.03	213.67±98.77	6.0±1.93	9.5±2.77
	Ivanec	17.03±0.96	14.27±4.37	0.17±0.01	69.54±2.29	0.61±0.36	0.04±0.00	279.74±69.35	8.1±1.04	11.3±3.30
	Novi Marof	16.20±1.13	10.79±1.87	0.16±0.01	69.15±1.83	0.54±0.45	0.05±0.01	250.57±98.07	9.9±3.96	7.6±1.54
	Ludbreg	18.07±1.01	14.56±8.66	0.13±0.01	69.83±1.18	0.52±0.53	0.04±0.03	340.41±59.55	9.1±2.24	8.8±1.35
multifloral	Bednja	16.81±1.53	24.11±4.07	0.68±0.11	68.47±3.36	1.47±1.58	0.33±0.09	750.23±52.89	22.9±6.69	24.5±10.02
	Ivanec	18.62±1.92	25.76±2.13	0.53±0.05	69.63±1.76	0.54±0.37	0.20±0.03	737.95±34.67	10.4±6.37	16.0±7.84
	Novi Marof	16.67±1.25	28.04±5.36	0.62±0.09	70.34±1.23	0.58±1.04	0.41±0.17	746.17±56.40	16.9±7.95	25.6±3.88
	Ludbreg	17.54±1.19	17.71±4.51	0.30±0.04	69.09±1.97	1.20±1.99	0.14±0.04	686.03±43.58	11.2±7.83	10.9±5.26

IN=invertase number, DN=diastase number

decreasing during storage, a possible explanation for the higher results obtained in the first phase of this study is that samples used in the cited studies might not have been fresh but stored for some time, thus their total phenolic content was lower. From Table 2 it is clearly visible that the results of this research obtained after six months of storage correspond much better to those obtained in the above cited studies. After one year of storage, total phenolic content decreased by 91.8 % in acacia honey, and by 88.6 % in multifloral honey. The changes in total phenolic content during one year of storage are presented in Fig. 1. Wang *et al.* (*25*) demonstrated that after six months of storage total phenolic content decreased by 25 % in clover honey and by 17 % in buckwheat honey. Storage of buckwheat honey at 4 °C resulted in a decrease of total phenolic content by 31 %.

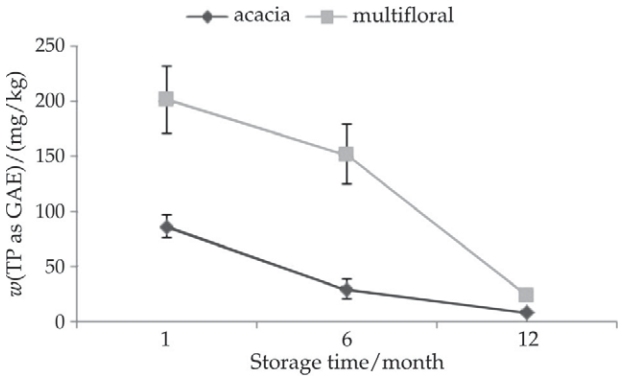

Fig. 1. Changes in total phenolic (TP) content in acacia and multifloral honey during one year of storage; GAE=gallic acid equivalent

As in total phenolic content, similar decrease pattern was determined in total flavonoid content. This was expected because flavonoids are one of the compounds that fall into the group of phenolics or polyphenols. The decrease of total flavonoid content was not as high as that of total phenolic content. After one year of storage, the measured values decreased by 45.6 % in acacia honey, and by 43.8 % in multifloral honey (Fig. 2). Initial values of total flavonoid content in acacia honey ranged from 8.29 to 29.65 mg of QE per 100 g of honey, with the average value of 15.26 mg of QE per 100 g of honey. The average values for individual geographic regions ranged from 11.50 to 18.05 mg of QE per 100 g of honey. Initial values in multifloral honey ranged from 19.92 to 28.65 mg of QE per 100 g of honey, with the average value of 25.37 mg of QE per 100 g of honey. The average values for individual geographic regions ranged from 22.12 to 28.05 mg of QE per 100 g of honey (Table 3). Meda *et al.* (*26*) determined total flavonoid content in various Burkina Fasan honey types in the range from 0.17 to 8.35 mg of QE per 100 g of honey. Al *et al.* (*22*) obtained similar results in acacia honey in the range from 0.91 to 2.42 mg of QE per 100 g of honey. These results are significantly lower than those obtained in our study. High average values for total flavonoid content were also obtained by Polish researchers who determined 28.5 mg of QE per 100 g of raspberry honey, 24.3 mg of QE per 100 g of hawthorn honey and 21.2 mg of QE per 100 g of thyme honey (*27*).

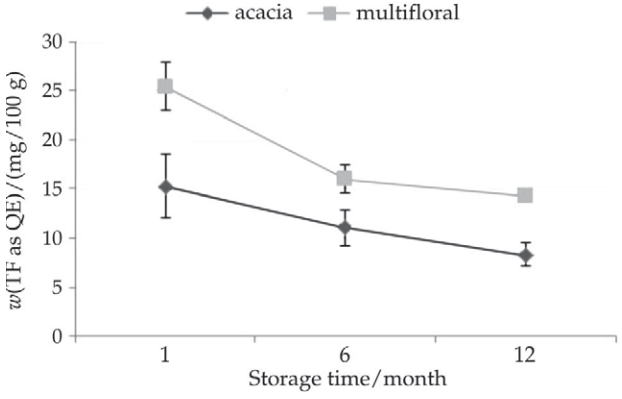

Fig. 2. Changes in total flavonoid (TF) content in acacia and multifloral honey during one year of storage; QE=quercetin equivalent

Table 3. Average values of total flavonoid content in acacia and multifloral honey during one year of storage

Honey type	Region	*w*(TF as QE)/(mg/100 g)		
		September–October 2009	March–April 2010	September–October 2010
acacia	Bednja	13.68±2.62	11.21±0.66	8.51±0.45
	Ivanec	17.82±5.18	12.62±0.83	9.39±0.57
	Novi Marof	18.05±7.65	11.96±1.34	8.69±0.78
	Ludbreg	11.50±1.03	8.51±0.59	6.61±2.29
	average±S.D.	15.26±3.21	11.08±1.80	8.30±1.19
multifloral	Bednja	28.05±0.47	16.25±8.94	14.93±1.11
	Ivanec	25.65±1.75	15.34±0.69	14.01±6.59
	Novi Marof	25.67±1.94	14.39±1.06	14.38±2.58
	Ludbreg	22.12±1.42	17.73±8.80	13.73±1.46
	average±S.D.	25.37±2.44	15.93±1.42	14.26±0.52

TF=total flavonoids, QE=quercetin equivalent

Antioxidant activity of honey

Radical scavenging activity measurement by DPPH method was one of the two methods used to determine the antioxidant activity of honey. This method is specific because lower absorbance value means higher antioxidant activity and *vice versa*. The addition of an antioxidant results in the decrease of the absorbance, which is proportional to the concentration and antioxidant activity of the compound itself. Results obtained in our study showed an increase in IC_{50} values after one year of storage in comparison with the starting values, which means that total antioxidant activity decreased. Initial average IC_{50} value measured in acacia honey was 44.64 mg/mL, while multifloral honey showed lower average value of 12.20 mg/mL. This was to be expected, so was an increase of this parameter during one year of storage. An increase from 12.20 to 16.73 mg/mL (*i.e.* by 37.1 %) was recorded in the IC_{50} values in multifloral honey during one year of storage. In acacia honey, the IC_{50} values in-

creased from 44.64 to 407.01 mg/mL (*i.e.* by 811.7 %) (Fig. 3). As seen in Table 4, the average IC_{50} values for acacia honey samples from Novi Marof and Ludbreg

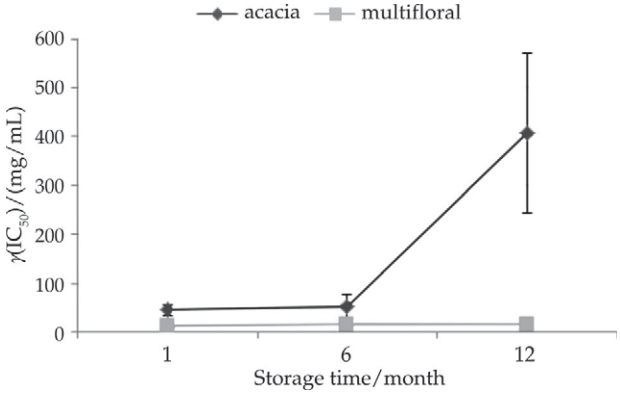

Fig. 3. Changes in IC_{50} values in acacia and multifloral honey during one year of storage

Table 4. Average IC_{50} values in acacia and multifloral honey during one year of storage

Honey type	Region	γ(IC50)/(mg/mL)		
		September–October 2009	March–April 2010	September–October 2010
acacia	Bednja	45.73±10.94	49.65±16.35	219.23±34.64
	Ivanec	33.15±12.36	34.75±4.67	323.05±31.83
	Novi Marof	41.37±4.90	42.40±6.17	559.76±180.86
	Ludbreg	58.31±9.31	87.41±28.95	525.99±188.63
	average±S.D.	44.64±10.50	53.55±23.38	407.01±163.09
multifloral	Bednja	11.94±0.71	12.41±1.60	14.10±1.32
	Ivanec	11.86±1.99	14.24±2.45	15.31±4.77
	Novi Marof	11.26±1.06	11.64±0.72	12.71±1.45
	Ludbreg	13.73±6.95	16.62±1.88	24.77±2.20
	average±S.D.	12.19±1.06	13.73±2.22	16.73±5.47

regions are the highest. If we exclude these results, and take into account only the ones from Bednja and Ivanec regions, this increase is a little bit smaller, *i.e.* 507.4 %. As acacia honey is one of the honey types that has the lowest antioxidant activity (*28*), it is possible that it decreases to almost non-existant after one year of storage. Darker honey types probably contain certain substances which prevent so severe decrease of antioxidant activity.

Beretta *et al.* (*8*) and Bertoncelj *et al.* (*11*) also determined the antioxidant activity in acacia and multifloral honey samples, and obtained similar results to our starting value. IC_{50} values obtained by Buratti *et al.* (*29*) were quite low and ranged from 8.0 to 12.0 mg/mL, which corresponds better to the ones measured in multifloral honey in our research and in previously cited studies. The average IC_{50} value of 111.05 mg/mL in acacia honey obtained by Krpan *et al.* (*23*) is somewhat higher than the one obtained in this study. The reason for this is that samples used in that study were not fresh, but had been stored for a certain period of time.

The second method used to determine the antioxidant activity in our samples was ferric reducing antioxidant power (FRAP) method. Compared to the results obtained by DPPH method, FRAP values were in the expected range. Thus, the initial FRAP values determined in acacia honey ranged from 39.53 to 173.46 μM of Fe(II) in 10 % honey solution (average value of 99.23 μM of Fe(II) in 10 % honey solution), and in multifloral honey from 106.32 to 453.90 μM of Fe(II) in 10 % honey solution (average value of 319.41 μM of Fe(II) in 10 % honey solution). The average values for acacia honey in individual geographic regions ranged from 73.45 to 126.92 μM Fe(II) in 10 % honey solution, and for multifloral honey from 244.55 to 398.02 μM of Fe(II) in 10 % honey solution (Table 5). These results are very similar to the ones obtained in an Italian study where the determined average FRAP values in acacia honey were (79.5±3.7) μM of Fe(II)

Table 5. Average FRAP values in acacia and multifloral honey during one year of storage

Honey type	Region	FRAP μM of Fe(II) in 10 % honey solution		
		September–October 2009	March–April 2010	September–October 2010
acacia	Bednja	75.76±12.10	48.35±15.03	16.10±7.92
	Ivanec	120.80±26.26	52.89±10.75	23.36±9.54
	Novi Marof	126.92±46.07	40.28±13.72	20.40±6.12
	Ludbreg	73.45±19.66	25.45±15.34	15.47±7.31
	average±S.D.	99.23±28.56	41.74±12.05	18.83±3.73
multifloral	Bednja	398.02±36.87	334.93±26.53	240.81±13.08
	Ivanec	283.98±30.23	249.34±80.57	183.30±26.11
	Novi Marof	351.10±86.09	289.69±17.12	191.85±12.86
	Ludbreg	244.55±32.93	215.98±28.19	124.68±10.32
	average±S.D.	319.41±68.42	272.49±51.39	185.16±47.62

and in multifloral honey (361.9±10.8) μM of Fe(II) both in 10 % honey solution (*8*). Very similar range of FRAP values was obtained in a Slovenian study in both honey types (*11*). During one year of storage, FRAP values also decreased in both honey types (Fig. 4). Antioxidant activity measured by FRAP method decreased by 428.0 %

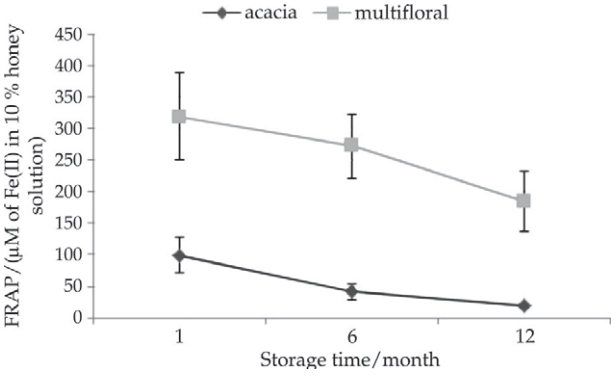

Fig. 4. Changes in FRAP values in acacia and multifloral honey during one year of storage

Table 6. Correlation coefficients (R)

	Total flavonoid content (A)	Total flavonoid content (MF)	FRAP (A)	FRAP (MF)	IC_{50} (A)	IC_{50} (MF)
Total phenolic content (A)	0.8113		0.8722		0.4243	
Total phenolic content (MF)		0.7618		0.8325		0.5791
Total flavonoid content (A)			0.9633		0.4477	
Total flavonoid content (MF)				0.7062		0.4272
FRAP (A)					0.3811	
FRAP (MF)						0.7363

A=acacia honey, MF=multifloral honey

in acacia honey and by 72.5 % in multifloral honey. These results are in very good correlation with the ones obtained for IC_{50} values.

Statistical analysis

To determine the significance of the impact of region (geographical origin), honey type (botanical origin) and storage time on total phenolic and flavonoid content, FRAP and IC_{50} values, analysis of variance was conducted using STATISTICA v. 9.1 (*30*) software. This analysis showed that the region which a honey sample comes from does not have any impact on the IC_{50} values and total phenolic and flavonoid content, while its impact is statistically significant on FRAP values in both types of honey. Storage time and honey type were shown as factors which have statistically significant impact on all investigated parameters.

The following qualitative and quantitative correlations were conducted: between IC_{50} value and total phenolic content, IC_{50} value and total flavonoid content, FRAP value and total phenolic content, FRAP value and total flavonoid content, total phenolic and total flavonoid contents, and IC_{50} and FRAP values. All of the correlations were described by linear functions and the obtained correlation coefficients are shown in Table 6. High correlation coefficients obtained by correlating FRAP values with total phenolic and total flavonoid content in both types of honey show that both phenolics and flavonoids are responsible for the antioxidant activity of honey measured by FRAP method. High correlation coefficients were also obtained by correlating total phenolic content with total flavonoid content. Correlation coefficients obtained by correlating other methods were lower and not very significant. Blasa *et al.* (*12*) used exponential function to describe the functional correlation between FRAP values and total phenolic content (R=0.9685), and linear function to describe the functional correlation between FRAP values and total flavonoid content (R= 0.9914).

Conclusion

It has been demonstrated in this study that total phenolic and flavonoid content of honey, as well as its antioxidant activity, measured by two methods, decrease during one year of storage. Antioxidant activity decreases in much bigger extent in acacia honey than in multifloral honey. Honey type and storage time have been shown to have statistically significant impact on all investigated parameters.

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