Contents of Major Phenolic and Flavonoid Antioxidants in Selected Czech Honey

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Abstract

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The chemical constitution of antioxidants contained in honey is derived from its origin. Forty honey samples (harvest 2006), which came from various locations of the Czech Republic and varied in their origins, were evaluated spectrophotometrically for their total polyphenol content, total flavonoids and 3',4'-dihydroxyflavones and flavonols, and major antioxidants were identified by HPLC-DAD and GC-MS. The kind of honey, location, and date of the honey harvest were shown to have a significant effect on the contents of phenolic antioxidants (average content 11.02 mg gallic acid equivalents/100 g), total flavonoids (0.66 mg quercetin equivalents/100 g), and 3',4'-dihydroxyflavones and flavonols (4.32 μg quercetin equivalents/100 g). In the Czech honey, ferulic acid (0.11 mg/100 g) and chrysin (0.06 mg/100 g) and other minority phenolics and flavonoids were identified and quantified as honey phenolic antioxidants contained. The results obtained support and extend complete knowledge on the contents of bioactive phenolics in the Czech honey, which could serve as a good source of natural antioxidants effective in reducing the risk of the occurrence of heart disease, cancer, cataracts, different inflammatory processes and immuno-system decline.

Keywords: antioxidants; kinds of honey; total polyphenols; phenolic acids; flavonoids; 3',4'-dihydroxyflavones and flavonols; chrysin; ferulic acid; GC-MS; HPLC-DAD

Among natural food antioxidants, polyphenols are ubiquitously distributed in the vegetable kingdom as plant secondary metabolites. Also various kinds of antioxidant components in honey may play important roles in a combinative or synergistic contribution to its total antioxidant activity (Gheldof & Engeseth 2002). Antioxidants, which act as preservatives because of their antioxidative activity, include both enzymatic (e.g. catalase and glucose oxidase) and non-enzymatic (e.g., organic acids, Maillard reaction products, amino acids, proteins, flavonoids, phenolics, α-tocopherol, ascorbic acid and carotenoids) substances (National Honey Board 2003). The flavonoid content reaches about 0.5% in pollen, 10% in propolis and about 6 mg/kg in honey (Anklam 1998). Many

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authors have studied the phenolic and flavonoid contents of honey to determine if a correlation exists with floral origins (Tomás-Barberán *et al.* 2001; Meda *et al.* 2005) and also to determine the presence of antimicrobial activity (National Honey Board 2003). The content of phenolic antioxidants showed a good correlation with the characteristic antioxidant activities (R^2 = 0.96 for propolis and 0.90 for honey) (Buratti *et al.* 2007). Flavonoids pinobanksin, pinocembrin, quercetin, chrysin, galangin, luteolin and kaempferol were reported to be present in honey (Gheldof *et al.* 2002), while pinocembrin, pinobanksin and chrysin are characteristic flavonoids of propolis (Gardana *et al.* 2007); these flavonoids were determined in most of the previously analysed European honey samples (Yao *et al.* 2004). The screening of honey phenolic extracts by HPLC resulted in the identification of *p*-coumaric acid, chrysin, kaempferol, and apigenin in all samples tested. Honey with pine, birch, and stinging nettle extracts was richer in apigenin than other natural honey samples (Baltrušaitytė *et al.* 2007). It was reported that the composition and antioxidant capacity of honey depend on the floral source used to collect nectar; seasonal and environmental factors as well as processing may also have an effect on the honey composition and antioxidant activity (Chen *et al.* 2000; Al-Mamary *et al.* 2002; Gheldof & Engeseth 2002; Gheldof *et al.* 2002; Yao *et al.* 2003).

Phenolic content expressed as gallic acid equivalent ranged from 44.8 mg/kg in acacia honey to 241.4 mg/kg in fir honey (average 83.7 mg/kg) (Bertoncelj *et al.* 2007). The antioxidant activity was the lowest in the brightest acacia and lime honey kinds and the highest in darker kinds of honey, namely fir, spruce, and forest honey kinds. Flavonoid contents in ether and water fractions, were 2.57 mg and 1.64 mg catechin equivalents in 100 g honey, respectively (Blasa *et al.* 2007). The comparison of the contents of flavonoids in Italian *Acacia* and *Millefiori* kinds of honey recently revealed that *Millefiori* samples showed the highest contents of flavonoids and antioxidant activity and also demonstrated that these parameters are dependent upon the honey origin (Blasa *et al.* 2007). In Burkina Fasan honey, total phenolic content (mg gallic acid equivalents GAE/100 g of honey) varied from 32.59 mg to 114.75 mg with a mean of 74.38 ± 20.54 mg using the standard curve of gallic acid (Meda *et al.* 2005). The total phenolic content varied from 32.59 mg in multifloral honey to 93.66 mg in honeydew honey. Using the standard curve generated by quercetin, the total flavonoid content of honey samples (mg QE/100 g) varied from 0.17 mg to 8.35 mg with a mean value of 2.57 ± 2.09 mg, with the highest and the lowest levels observed in multifloral kinds of honey. Only a low correlation (R^2 = 0.11) was shown between total phenolic and total flavonoid contents. The comparison of the phenolic contens of several Chilean kinds of honey showed great variations in flavonoid concentration among the samples analysed (Muñoz *et al.* 2007). The major flavonoids detected were pinobanksin, chrysin, hesperetin, luteolin, 3-methylquercetin, isorhamnetin, pinocembrin, 3,7,4',5'-tetramethylmyricetin, galangin, 3-methylgalangin, tectochrysin, 8-methoxykaempferol, apigenin, quercetin, kaempferol, pinobanksin-3-acetate, ellagic acid, and esters of caffeic acid (dimethyl-, ethylphenyl- and dimethylallyl-). The results obtained from the partial identification of honey phenolic compounds by high-performance liquid chromatography with a diode array detector showed that *p*-hydroxybenzoic acid, cinnamic acid, naringenin, pinocembrin, and chrysin were the phenolic compounds present in most of the analysed samples of Northeast Portugal honey (Estevinho *et al.* 2008). However, in another study only chrysin, pinocembrin, kaempferol, ferulic acid, and *p*-coumaric acid could be identified in methanol-water extract of rosemary honey using UV-VIS coupled to capillary electrophoresis, though chrysin and pinocembrin overlapped (Gomez-Caravaca *et al.* 2006). Because of this, only kaempferol, ferulic acid, and *p*-coumaric acid were quantified.

The purpose of this study was to determine and evaluate the contents of total phenolic, flavonoid, and 3',4'-dihydroxyflavones and flavonols of several Czech honey samples of different origins, locations and dates of honey harvest as well as to identify and quantify major phenolic and flavonoid antioxidants in the Czech honey.

MATERIAL AND METHODS

Chemicals and instruments. For the determination of total polyphenol content (TP), total flavonoid content (TF), and 3',4'-dihydroxyflavones and flavonols content (DHF) the following equipment and chemicals were used: UV-VIS spectrophotometer Heλios γ (Spectronic Unicam, Garforth, UK),

Table 1. Characteristics of analysed honey samples from the harvest in the year 2006

Location	Nr. of sample	Date of harvest	Harvest sequence	Type of honey, main plant sources	Sensory characteristics (colour, consistency)
Brumovice	1	June, 24	1	multifloral (fruit trees)	extra light amber, granulated, solid
	2	July, 22	2	multifloral (lime, ornament. wood)	extra light amber, granulated, solid
	3	June, 8	1	rape	extra white, very light, granulated, solid
	4	June, 27	2	mixture (forest)	amber, dark, fine granulated, viscous
	5	June, 13	1	multifloral (rape, fruit trees)	light amber, medium dark and solid, granulated
	6	July, 20	3	mixture (lime, poppy, forest)	extra white, very light, granulated, solid
Pocheň	7	July, 7	2	mixture (rape, forest)	amber, dark, granulated, very solid
	8	July, 18	3	mixture (lime, forest)	extra light amber, granulated, solid
	9	June, 20	1	rape	extra white, very light, fine granulated, viscous
	10	July, 2	2	mixture (rape, forest)	light amber, medium dark, fine granulated, viscous
	11	July, 25	3	mixture (lime, forest)	extra white, very light and solid, granulated
	12	June, 25	1	mixture (rape, forest)	light amber, medium dark, granulated, solid
	13	July, 7	2	mixture (forest)	light amber, medium dark, granulated, viscous
	14	July, 31	3	lime	extra white, very light and solid, granulated
Lichnov	15	July, 8	1	mixture (forest)	light amber, medium dark, granulated, very solid
	16	Aug, 2	2	lime	extra white, very light, granulated, solid
Býkov	17	May, 27	1	rape	extra white, very light, granulated, medium solid
	18	June, 17	2	raspberry	extra light amber, granulated, solid
	19	July, 12	3	lime	extra light amber, granulated, solid
Široká Niva	20	July, 5	1	mixture (forest, fruit trees)	light amber, medium dark, granulated, solid
	21	July, 28	2	mixture (forest)	amber, dark, granulated, medium solid
Budišov	22	July, 6	1	mixture	amber, dark, granulated, medium solid
Podvihov	23	July, 11	1	mixture	amber, dark, granulated, solid
Žimrovice	24	July, 15	1	mixture	amber, dark, granulated, medium solid
Kružberk	25	June, 20	1	floral (fruit trees)	extra light amber, granulated, solid
	26	July, 8	2	raspberry	amber, very dark and solid, granulated
Úvalno	27	June, 17	2	multifloral (rape, raspberry)	extra white, very light and solid, granulated
	28	June, 28	3	mixture (rape, raspberry, spruce)	extra white, very light, granulated, solid
	29	June, 19	1	multifloral (rape, raspberry)	extra light amber, light, granulated, solid
	30	July, 8	2	mixture (raspberry, forest)	light amber, medium dark, granulated, very solid
	31	July, 20	3	mixture (lime, forest)	extra light amber, granulated, viscous
Nasavrky	32	July, 6	2	mixture (forest)	amber, dark, granulated, solid
Rymice	33	July, 27	2	mixture (forest)	light amber, medium dark, bright, viscous
	34	July, 20	3	lime	extra white, very light, granulated, solid
Říčany u Prahy	35	Aug, 18	1	honeydew	light amber, medium dark, fine granulated, viscous
Tehov	36	July, 8	1	honeydew	light amber, medium dark, bright, very viscous
Kamenice nad Lipou	37	July, 14	2	honeydew	amber, dark, bright, very viscous
Kašava	38	July, 19	2	honeydew	dark amber, very dark, bright, viscous
Zbiroh	39	July, 9	1	honeydew	light amber, medium dark, fine granulated, viscous
Nová Včelnice	40	July, 10	1	honeydew	dark amber, very dark, granulated, solid

magnetic stirrer Fisher Scientific, analytical weigh Kern, pH-meter Schott, micropipettes (0.5–5 ml; 100–1000 µl) Socorex, rotary vacuum evaporator Büchi Rotavapor R-200, membrane filters Spartan, Schleicher & Schuell 0.45 µm, Amberlite® XAD® 2 Catex (Supelco, St. Louis, USA); the chemicals used were anhydrous sodium carbonate p.a, Folin-Ciocalteau reagent, monohydrate of gallic acid, quercetin, 99% acetic acid, 38% hydrochloric acid, sulphuric acid 96%, p.a., methanol p.a., diethyl-ether, anhydrous sodium sulphate p.a., sodium nitrite p.a., anhydrous sodium hydroxide p.a., sodium acetate crystalline, p.a., diphenyl boric acid 2-amino-ethyl ester p.a. > 97.

For HPLC-DAD were used an analytical Waters™ High Performance Liquid Chromatograph (HPLC) with linear gradient elution (Waters™ 600S pump, Waters™ 717 plus autosampler, Waters™ PDA 996 – UV-VIS detector, Nova-Pack® C18 150 × 3.9 mm, 4 µm column), methanol HPLC Gradient Grade, Baker HPLC analysed, demineralised water, and formic acid (HCOOH, 99% p.a., Aros Organics, Thermo Fisher Scientific Inc., New Jersey, USA).

Preparative HPLC was performed using Delta-Chrom™ (Watrex) equipped with DeltaChrom™ Prep 100 Chromatography Pump. The system is coupled to Thermo Finningan UV6000LP Photodiode Array Detector.

GC-MS analyses were performed on an Agilent 6890 gas chromatograph coupled to Agilent 5973 mass spectrometer.

Samples. All 40 honey samples were collected and characterised directly from beekeepers in different locations in the Czech Republic (Table 1). The honey samples were harvested in the period from May to August 2006. The samples were sampled directly by beekeepers using the extraction method (by virtue of centrifugal force on honey in combs) into sterilised 250 ml glass sample bottles with glass caps and then stored in a dry and dark place at a temperature of 20°C. The origin of each particular honey sample was characterised directly by the beekeeper in relation to the location where the beehives were situated and accessibility of plant food sources. The purity of honey was carefully checked according to the international rules of the International Honey Committee and Harmonised Methods of the European Honey Commission (Bogdanov *et al.* 1997), and the honey samples were also characterised by means of some selected sensory characteristics, such as colour and honey consistency, and their origins were verified by qualitative microscopic pollen analysis (melissopalynology). On this basis, the honey samples were classified into six categories: multifloral, lime, rape, raspberry, mixture, and honeydew kinds of honey. The dates, sequence of harvest, main plant sources, and some sensory characteristics are given in Table 1.

Extraction of phenolics. For the extraction of phenolics from the honey samples, a modified method (Yao *et al.* 2004) using column chromatography was applied. A 100 g honey sample was totally dissolved in 500 ml acidified distilled water, using a magnetic stirrer the pH value was adjusted with HCl to pH 2.0 at laboratory temperature. The solution obtained was filtered through a lump of cotton wool in a funnel to remove the solid particles. The filtrate was mixed with 150 g of Amberlite® XAD® 2 (pore size 9 nm, particle size 0.3–1.2 mm) and stirred for 10 min with a magnetic stirrer. This mixture was transferred into a glass column (35 × 3.4 cm) and eluted with 250 ml of acidified distilled water (pH 2.0 adjusted with HCl) followed by 300 ml of distilled water for removing all saccharides. Phenolics adsorbed on the solid phase were eluted with 400 ml methanol and the methanolic extract was subsequently evaporated to dryness in a rotary vacuum evaporator at 40°C. The solids were dissolved in 5 ml of distilled water and extracted three times with 5 ml of diethyl ether. Subsequently, the diethyl etheric extracts were combined, dried with anhydrous sodium sulphate, and diethyl ether was removed using a nitrogen flow. The dry extract obtained was stored in a refrigerator (4°C) for the analyses.

Total phenolic content (TP) determination with Folin-Ciocalteau method. For the determination of the total polyphenols (TP) content, a modified spectrophotometrical method (Lachman *et al.* 2006) with Folin-Ciocalteau reagent was used. Dry extract of phenolics was dissolved in 5 ml of methanol. For the determination, a 0.5 ml of the sample solution was pipetted into a 10 ml volumetric flask and diluted with distilled water. Subsequently, 0.5 ml Folin-Ciocalteau reagent was added to the solution and after stirring 1.2 ml 20% sodium carbonate solution was added. After refilling with distilled water to the mark and thorough agitation, the reaction mixture was left standing for 20 min and then was measured on the spectrophotometer at λ = 765 nm against the blank. TP was expressed as mg gallic acid equivalents in 100 g of honey (mg GAE 100/g) as the average of three

parallel determinations. The calibration curve was linear in the range of 0.02–0.45 mg GAE.

Spectrophotometrical total flavonoid content (TF) determination. For the determination of total flavonoid (TF) content, a modified spectrophotometrical method (Spilková *et al*. 1996) with $NaNO_2$ was used. Dry extract of phenolics was dissolved in 5 ml of methanol. For the determination, 0.4 ml of the sample solution was pipetted into a 10 ml volumetric flask and diluted with distilled water. Subsequently, 1.2 ml of 0.2 mol/l H_2SO_4, 1.2 ml of 3 mol/l $NaNO_2$, and 1.2 ml of 10% NaOH were added to the solution. After refilling with distilled water to the mark and thorough agitation, the reaction mixture was left standing for 15 min and then measured on the spectrophotometer at λ = 395 nm against the blank. TF was expressed as mg quercetin equivalents in 100 g of honey (mg QE/100 g) as the average of three parallel determinations. The calibration curve was linear in the range of 0.10–3.00 μg QE.

Spectrophotometrical of determination of 3',4'-dihydroxyflavones and flavonols content (DHF). For the determination of 3',4'-dihydroxyflavones and flavonols content (DHF), a modified spectrophotometrical method (Spilková *et al*. 1996) with diphenyl boric acid 2-amino-ethyl ester was used. Dry extract of phenolics was dissolved in 5 ml of methanol. For the determination, 1 ml of the sample solution was pipetted into 10 ml volumetric flask and diluted with distilled water. 0.4 ml of 2 mol/l CH_3COONa and 0.4 ml of 1% diphenyl boric acid 2-amino-ethyl ester were added. After refilling with distilled water to the mark and thorough agitation, the reaction mixture was left standing for 15 min and then measured on the spectrophotometer at λ = 395 nm against the blank. DHF was expressed as mg quercetin equivalents in 100 g of honey (mg QE/100 g) as the average of three parallel determinations. The calibration curve was linear in the range of 0.005–0.50 μg QE.

HPLC-DAD of phenolics. Dry extracts of phenolics were dissolved in 5 ml of methanol each and filtered through a membrane filter Spartan 0.45 μm. Chromatographic conditions: column Nova-Pack® C18 (150 mm × 3.9 mm, particle size 4 μm), mobile phase: A – 5% v/v HCOOH, B – CH_3OH, flow rate volume 1 ml/min, temperature 35°C, sample injection volume 10 μl, detection in the wave length range 200–400 nm. The chromatograms were evaluated at λ = 290 nm and λ = 340 nm. Chromatographic separation was performed with gradient elution:

70% mobile phase A + 30% mobile phase B, isocratic elution time 0–15 min,
60% mobile phase A + 40% mobile phase B, linear increase time 16–20 min,
55% mobile phase A + 45% mobile phase B, linear increase time 21– 30 min,
40% mobile phase A + 60% mobile phase B, linear increase time 31–50 min,
20% mobile phase A + 80% mobile phase B, linear increase time 51–52 min,
10% mobile phase A + 90% mobile phase B, linear increase time 52–60 min,
10% mobile phase A + 90% mobile phase B, isocratic elution time 61–63 min,
70% mobile phase A + 30% mobile phase B, linear increase time 64–73 min,
70% mobile phase A + 30% mobile phase B, isocratic elution time 74–75 min.

The major phenolics in the individual honey samples were compared with authentic standards of ferulic acid ($C_{10}H_{10}O_4$, Fluka Chemie, Buchs, Switzerland), chrysin ($C_{15}H_{10}O_4$, Carl Roth, KG-D 75, Karlsruhe, Germany), and apigenin ($C_{15}H_{10}O_5$, Fluka Chemie, Buchs, Switzerland). Linear ranges of the calibration curves for the individual standards were: ferulic acid 10–300 μg/ml, apigenin 10–200 μg/ml, and chrysin 10–200 μg/ml.

Preparative HPLC-DAD. The separation of combined forty honey samples was performed using preparative Nucleosil C18 column (120-5, 250 × 21 mm) and H_2O:acetonitrile gradient elution (from 50:50 at the start to 0:100 in 50 min, followed with isocratic elution of 100% acetonitrile for 10 min); the flow rate was 10 ml/min and 290 nm wave length was used for the detection. Five fractions were collected during approximate 10 min intervals, starting at the fifth minute of the elution. The five samples were then evaporated, dissolved in methanol and submitted to GC-MS analysis.

GC-MS of phenolics. 1 μL of the sample for GC/MS analyses was injected in split mode 1/10; injector temperature was 250°C. DB-5MS column (30 m × 0.25 mm × 0.25 μm) was used with He as a carrier gas in the constant flow mode 1 ml/minute. The temperature programme started at 5°C, was held for 3 min, and then continued to 290°C at 10°C/minute. The mass spectrometer operated in 70 eV ionization mode, m/z = 35–450, ion source temperature was 230°C. The individual peaks were identified by comparison of their mass spectra with those given in the NIST mass spectra database, and by comparison of their retention

times and mass spectra with those of the authentic compounds.

Statistical analysis. Statistical analyses were performed using the software Statistica 8.0 (StatSoft) on the basis of parametrical and non parametrical tests at the significance level $\alpha = 0.05$ and cluster analysis.

RESULTS AND DISCUSSION

The types, sensory characteristics, TP, TF and DHF of the honey samples analysed are described in Tables 1 and 2. The honey kinds differed significantly from one another in colour, granulation, and viscosity (Table 1). On average, the highest TP and DHF were found in raspberry and honeydew honey kinds and the lowest ones in multifloral honey samples (Tables 2 and 3, Figure 1). Cluster analysis on the basis of TP and TF revealed the greatest difference between floral and honeydew kinds of honey while the most similar were floral and mixture kinds of honey. Also in relation to DHF the floral and honeydew honey samples differed significantly, however, in this case common cluster between the mixture and honeydew honey samples could be determined.

The colour and consistency of honey depend on the contents of water, saccharides, minerals, pollens, and polyphenolic compounds (Baltrušaitytė *et al.* 2007). By means of sensory evaluation of the Czech honey, we found that floral honey samples have a predominant light colour, high crystallisation ability and a high viscosity, whereas monofloral honey samples showed considerably lighter colour than the multifloral kinds of honey. This corresponds to the results obtained with sensory evaluation of the Italian honey colour (Blasa *et al.* 2006) or to the objective determination of the colour of honey from Slovenia (Bertoncelj *et al.* 2007). On the contrary, honeydew kinds of honey were dark, bright, with a low viscosity. Our results confirmed the fact that dark honey types have higher TP contents as compared to the light ones. In our dark coloured honey samples, we also found higher TF (Table 2).

TP ranged from 3.92 mg GAE/100 g multifloral honey (fruit trees sample No. 1) to 16.71 mg GAE/100 g honeydew honey (sample No. 36) with the mean value of 11.02 mg GAE/100 g honey. The values obtained are in accordance with the previously reported results (Gheldof & Engeseth 2002; Beretta *et al.* 2005; Meda *et al.* 2005) though TP contents were determined directly in the honey samples and not in the polyphenol compounds extracts and were lower than those reported recently in the floral (115.03 mg GAE/100 g) and honeydew (129.03 mg GAE/100 g) Czech honey (Vit *et al.* 2008). The most similar to Czech honey was shown to be Italian honey (Blasa *et al.* 2006), in which TP content ranged from 3.00 mg GAE/100 g to 17.50 mg GAE/100 g, while the most different proved to be honey from South Africa (Meda *et al.* 2005) with the high values of 32.59 mg to 114.75 mg GAE/100 g. The distinctive differences between the Czech and South African kinds of honey are caused by different locations, especially the climatic and vegetation conditions. However, differences could be found even in the honey samples from different locations of the Czech Republic (Table 2). Among floral honey samples, the highest average TP was found in raspberry honey (15.48 mg GAE/100 g) and the lowest one in multifloral honey (8.50 mg GAE/100 g). However, in some samples of monofloral honey low TP levels were reported, e.g. in acacia honey from Italy (Beretta *et al.* 2005) or coconut honey from Malaysia (Aljadi & Kamaruddin 2004). Higher TP were found in the honey samples harvested in the period from May 16 to July 15 as compared to the honey collected in the first half of June, which is related to the flowering period of nectariferous plants.

TF content ranged from 0.53 mg QE/100 g raspberry honey (sample No. 18) to 1.23 mg QE/100 g honeydew honey (sample No. 36), the average value being 0.66 mg QE/100 g honey. The measured values are lower than the results found in Burkina Faso honey (Meda *et al.* 2005) or sage unifloral honey (Kenjerić *et al.* 2008); the cause may be the different natural conditions in the Czech Republic and the method based on the reaction of analytes with sodium nitrite, which is generally used, but has not yet been used for honey samples. The most similar to the Czech honey was Italian honey (Blasa *et al.* 2006) that ranged from 0.45 mg QE/100 g to 1.01 mg QE/100 g, and the most different South African honey (Meda *et al.* 2005) with high values of 0.17–7.13 mg QE/100 g. TF also varied in the honey samples from different locations of the Czech Republic. The highest average TF content was found in honeydew honey samples (0.83 mg QE/100 g) and lower contents in multifloral honey samples (0.57 mg QE/100 g).

Table 2. Content of total polyphenols (TP), total flavonoids (TF) and 3',4'-dihydroxyflavones and flavonols (DHF) in individual samples of the Czech honey

Sample number	Bee forage plants	TP (mg GAE[a]/100 g ± SD)	TF (mg QE[b]/100 g ± SD)	DHF (μg QE[b]/100 g ± SD)
Multifloral honey				
1	FT	3.92 ± 0.13	0.56 ± 0.01	3.63 ± 0.32
2	L, OS	9.54 ± 0.12	0.59 ± 0.01	3.06 ± 0.22
5	FT, R	8.16 ± 0.09	0.57 ± 0.01	1.87 ± 0.22
25	FT	7.37 ± 0.24	0.62 ± 0.01	3.32 ± 0.24
Rape honey				
3	R	9.43 ± 0.06	0.59 ± 0.02	4.85 ± 0.14
9	R	9.87 ± 0.09	0.58 ± 0.00	4.25 ± 0.16
17	R	11.92 ± 0.16	0.61 ± 0.03	5.67 ± 0.18
Lime honey				
14	L	8.07 ± 0.14	0.63 ± 0.09	3.69 ± 0.02
16	L	11.19 ± 0.19	0.65 ± 0.05	3.56 ± 0.14
19	L	14.51 ± 0.32	0.69 ± 0.01	6.16± 0.06
34	L	7.47 ± 0.02	0.55 ± 0.01	3.07 ± 0.23
Raspberry honey				
18	RB	14.73 ± 0.38	0.53 ± 0.05	4.77 ± 0.14
26	RB	16.23 ± 0.06	0.73 ± 0.02	6.39 ± 0.30
Forest honey				
4	F	8.85 ± 0.07	0.57 ± 0.00	3.33 ± 0.07
13	F	11.93 ± 0.08	0.62 ± 0.02	4.50 ± 0.02
15	F	8.07 ± 0.15	0.56 ± 0.03	2.76 ± 0.10
21	F	15.56 ± 0.19	0.90 ± 0.12	4.32 ± 0.09
22	F	8.73 ± 0.35	0.53 ± 0.08	5.38 ± 0.07
23	F	11.44 ± 0.31	0.71 ± 0.04	4.14 ± 0.17
32	F	11.16 ± 0.24	0.58 ± 0.02	3.77 ± 0.31
33	F	13.45 ± 0.59	0.68 ± 0.04	6.48 ± 0.23
Mixture honey (rape, forest)				
7	R, F	9.44 ± 0.06	0.58 ± 0.03	3.92 ± 0.11
12	R, F	16.70 ± 0.03	0.93 ± 0.05	4.94 ± 0.02
30	R, F	9.12 ± 0.15	0.58 ± 0.00	3.44 ± 0.26
Mixture honey (lime, forest)				
8	L, F	10.16 ± 0.24	0.60 ± 0.03	3.34 ± 0.30
11	L, F	13.93 ± 0.10	0.67 ± 0.03	5.87 ± 0.07
31	L, F	10.72 ± 0.26	0.65 ± 0.09	4.92 ± 0.19
Other mixture honey				
6	L, P, F	11.19 ± 0.25	0.61 ± 0.01	6.61 ± 0.22
10	R, L	8.85 ± 0.15	0.55 ± 0.02	2.52 ± 0.15
20	FT, F	9.55 ± 0.24	0.63 ± 0.03	2.37 ± 0.06
24	PH	11.60 ± 0.66	0.63 ± 0.03	3.51 ± 0.06
27	R, RB	8.70 ± 0.14	0.58 ± 0.01	3.73 ± 0.30
28	R, RB, S	10.29 ± 0.52	0.66 ± 0.05	5.18 ± 0.22
29	R, RB	9.07 ± 0.15	0.55 ± 0.02	4.14 ± 0.02
Honeydew honey				
35	HDH	10.68 ± 0.19	0.666 ± 0.003	3.380 ± 0.101
36	HDH	16.71 ± 0.26	1.23 ± 0.01	6.56 ± 0.41
37	HDH	12.03 ± 0.25	0.82 ± 0.01	4.38 ± 0.13
38	HDH	9.38 ± 0.02	0.54 ± 0.01	2.15 ± 0.17
39	HDH	13.63 ± 0.11	0.87 ± 0.02	4.64 ± 0.36
40	HDH	13.23 ± 0.45	0.86 ± 0.01	6.00 ± 0.22

[a]expressed in gallic acid equivalents; [b]expressed in quercetin equivalents; FT – fruit trees; L – lime; OS – ornamental species; R – rape; RB – raspberry; F – forest; P – poppy; PH – phacelia; S – spruce; HDH – honeydew honeys

Table 3. Statistical evaluation of TP, TF and DHF in floral, mixture and honeydew kinds of honey

Kind of honey	Floral honey					Mixture honey					Honeydew honey
	lime	rape	raspberry	multifloral	floral total	forest	rape, forest	lime, forest	other mixture	mixture total	
TP (mg GAE[a]/100 g)											
Average	10.31	10.41	15.48	8.50	10.57	11.15	11.77	11.30	9.89	10.88	12.61
SD	3.24	1.33	1.07	0.94	2.95	2.57	4.31	2.04	1.16	2.36	2.56
Median	9.63	9.87	15.48	8.54	9.54	11.30	9.44	10.72	9.55	10.29	12.63
Minimum	7.47	9.43	14.72	7.37	7.37	8.07	9.12	10.16	8.70	8.07	9.38
Maximum	14.51	11.92	16.23	9.54	16.23	15.55	16.70	13.93	11.60	16.70	16.71
n[c]	4	3	2	4	13	8	3	3	7	21	6
TF (mg QE[b]/100 g)											
Average	0.63	0.59	0.63	0.57	0.61	0.65	0.70	0.64	0.60	0.64	0.83
SD	0.06	0.02	0.04	0.03	0.06	0.12	0.20	0.04	0.04	0.10	0.24
Median	0.64	0.59	0.63	0.58	0.59	0.60	0.58	0.65	0.61	0.61	0.84
Minimum	0.55	0.58	0.53	0.56	0.53	0.53	0.58	0.60	0.55	0.53	0.54
Maximum	0.69	0.61	0.73	0.62	0.73	0.90	0.93	0.67	0.66	0.93	1.23
n[c]	4	3	2	4	13	8	3	3	7	21	6
DHF (μg QE[b]/100 g)											
Average	4.12	4.92	5.58	2.97	4.17	4.36	4.10	4.71	4.01	4.25	4.52
SD	1.39	0.72	1.15	0.77	1.33	1.17	0.77	1.28	1.49	1.19	1.63
Median	3.62	4.85	5.58	3.19	3.69	4.32	3.92	4.92	3.73	4.14	4.51
Minimum	3.07	4.25	4.77	1.87	1.87	2.76	3.44	3.34	2.37	2.37	2.15
Maximum	6.16	5.67	6.39	3.63	6.39	6.48	4.94	5.87	6.61	6.61	6.56
n[c]	4	3	2	4	13	8	3	3	7	21	6

[a]expressed in gallic acid equivalents; [b]expressed in quercetin equivalents; [c] number of samples

Table 4. Content of ferulic acid, chrysin and apigenin (mg/100 g) in individual samples of the Czech honeys

Sample number	Bee forage plants	Ferulic acid	Chrysin	Apigenin
Multifloral honeys				
1	FT	0.09	0.05	n.d.
2	L, OS	0.13	0.07	n.d.
5	FT, R	0.14	0.04	n.d.
25	FT	0.04	0.05	n.d.
Rape honeys				
3	R	0.18	0.04	0.011
9	R	0.09	0.05	n.d.
17	R	0.12	0.05	n.d.
Lime honeys				
14	L	0.10	0.03	n.d.
16	L	0.16	0.05	n.d.
19	L	0.13	0.04	n.d.
34	L	0.02	0.05	n.d.
Raspberry honeys				
18	RB	0.13	0.06	n.d.
26	RB	0.07	0.06	n.d.
Forest honeys				
4	F	0.08	0.53	n.d.
13	F	0.12	0.06	n.d.
15	F	0.06	0.01	n.d.
21	F	0.18	0.02	n.d.
22	F	0.02	0.04	n.d.
23	F	0.16	0.02	n.d.
32	F	0.04	0.06	n.d.
33	F	0.08	0.10	n.d.
Mixture honeys (rape, forest)				
7	R, F	0.07	0.04	n.d.
12	R, F	0.13	0.04	n.d.
30	R, F	0.12	0.03	n.d.
Mixture honeys (lime, forest)				
8	L, F	0.11	0.04	n.d.
11	L, F	0.21	0.07	n.d.
31	L, F	0.11	0.05	n.d.
Mixture honeys				
6	L, P, F	0.21	0.13	0.03
10	R, L	0.12	0.04	n.d.
20	FT, F	0.06	0.02	n.d.
24	PH	0.13	0.01	n.d.
27	R, RB	0.08	0.05	n.d.
28	R, RB, S	0.13	0.07	n.d.
29	R, RB	0.08	0.04	n.d.
Honeydew honeys				
35	HDH	0.15	0.03	n.d.
36	HDH	0.82	0.03	n.d.
37	HDH	0.11	0.03	n.d.
38	HDH	0.05	0.01	n.d.
39	HDH	0.28	0.04	n.d.
40	HDH	0.07	0.04	n.d.

FT – fruit trees; L – lime; OS – ornamental species; R – rape; RB – raspberry; F – forest; P – poppy; PH – phacelia; S – spruce; HDH – honeydew honeys; n.d. – non determined

Table 5. Statistical evaluation of the content of ferulic acid and chrysin in the floral, mixture and honeydew Czech honey kinds

Kind of honey	Floral honey					Mixture honey					Honeydew honey
	lime	rape	raspberry	multifloral	floral total	forest	rape, forest	lime, forest	other mixture	mixture total	
Ferulic acid (mg/100 g)											
Average	0.10	0.13	0.10	0.11	0.11	0.09	0.11	0.15	0.12	0.11	0.25
SD	0.06	0.05	0.04	0.06	0.04	0.05	0.03	0.06	0.05	0.05	0.29
Median	0.11	0.12	0.10	0.11	0.12	0.08	0.12	0.12	0.12	0.12	0.13
Minimum	0.02	0.09	0.07	0.04	0.02	0.02	0.07	0.11	0.06	0.02	0.05
Maximum	0.16	0.18	0.13	0.14	0.18	0.18	0.13	0.21	0.21	0.21	0.82
n^c	4	3	2	4	13	8	3	3	7	21	6
Chrysin (mg/100 g)											
Average	0.04	0.05	0.06	0.05	0.05	0.11	0.04	0.05	0.05	0.07	0.03
SD	0.01	0.01	0.00	0.01	0.01	0.17	0.01	0.01	0.04	0.11	0.01
Median	0.05	0.05	0.06	0.05	0.05	0.05	0.04	0.05	0.04	0.04	0.03
Minimum	0.03	0.04	0.06	0.04	0.03	0.01	0.03	0.04	0.00	0.00	0.01
Maximum	0.05	0.05	0.06	0.07	0.07	0.53	0.04	0.07	0.13	0.53	0.04
n^c	4	3	2	4	13	8	3	3	7	21	6

[c]number of samples; SD – standatd deviation

However, higher TF contents (7.13 mg QE/100 g) were found in multifloral honey samples of South Africa (Meda *et al.* 2005) – the differences could be caused by different natural conditions (Czech Republic × South Africa) and methods used for TF determination (sodium nitrite x aluminium chloride). In monofloral honey, low TF concentrations were reported, e.g. heathery Portuguese honey (Ferres *et al.* 1992, 1994) (0.06 mg QE/100 g) or Italian acacia honey (Beretta *et al.* 2005) (0.45 mg QE/100 g); a relatively high TF content was found in Spanish rosemary honey (2.35 mg QE/100 g) (Tomás-Barberán *et al.* 1993). Regarding the date of honey harvest, high TF were found in the honey harvested in the first half of July and low ones in the honey harvested in the first half of June. The flavonoid content of *Trigona carbonaria* honey (Oddo *et al.* 2008) from Australia (10.00 ± 1.59 mg QE/100 g) was evaluated as higher than those of Czech floral and honeydew honey samples reported previously by Vit *et al.* (2008) as 6.59 in floral honey and 7.25 mg QE/100 g in honeydew honey; however, these flavonoid contents as compared with our data (0.61 mg QE/100 g in floral honey and 0.83 mg QE/100 g in honeydew honey) are also higher due to using different methods.

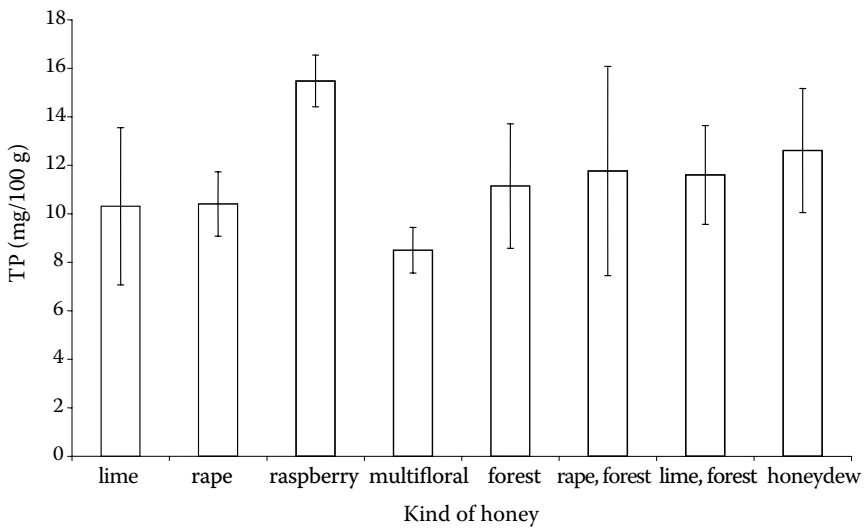

Figure 1. Content of total polyphenols (TP) in different kinds of honey (average value±standard deviation)

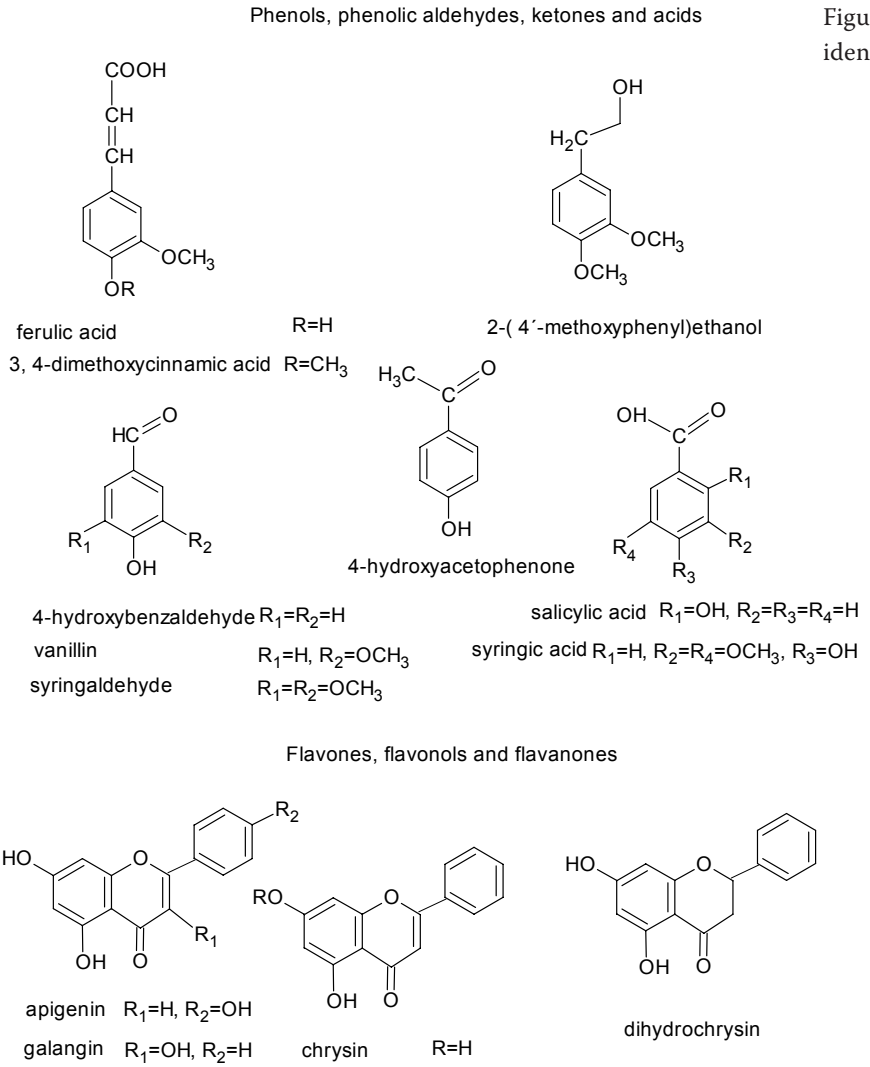

Figure 2. Structure of major phenolics identified in honey samples

DHF was very low (1.87 μg QE/100 g–6.61 μg QE/100 g, average 4.32 μg QE/100 g). The effects of location, kind of honey and date of honey harvest were similar as in the TP and TF cases. A relatively high DHF content was found in raspberry honey (average 5.58 μg QE/100 g) and rape honey (4.92 μg QE/100 g) and a low one in multifloral honey (2.97 μg QE/100 g).

HPLC-DAD revealed that out of the phenolic acids identified, ferulic acid (R_t = 3.2–3.6 min min) and flavones chrysin (R_t = 39.3–39.7 min) were present in all honey samples as major compounds (Tables 4 and 5, Figures 2 and 3). Ferulic acid and apigenin contents were determined at the wave length λ = 340 nm, chrysin at λ = 290 nm. Apigenin was found in two samples only (rape and mixed honey). Ferulic acid concentration ranged from 0.02 to 0.82 mg/ 100 g, with the average of 0.11 mg/100 g (Table 4). Similar levels of ferulic acid were reported previously in Australian flower honey (Yao *et al.* 2004) in the range of 0.04–1.08 mg/100 g. The highest average value was found in honeydew honey (0.25 mg/100 g) and the lowest one in the forest honey (0.09 mg/100 g). Similarly as found with TP, TF, and DHF, the kind of honey, location, and date of honey collection also affected ferulic acid content. Chrysin content ranged in the analysed samples between 0.01–0.53 mg/100 g; the average was 0.06 mg/100 g (Table 4). Similar values were determined in Australian floral honey (Yao *et al.* 2004) (0.00–0.38 mg/100 g) or salvia honey originating in Croatia (Kenjerić *et al.* 2008) (0.03–0.25 mg/100 g). High amounts of chrysin were determined in forest honey (0.11 mg/100 g) and low ones in honeydew honey (0.03 mg/100 g) (Table 5). Apigenin was found only in two honey samples (rape honey 0.01 mg/100 g and lime, poppy, and forest plants 0.03 mg/100 g). Both samples originated from the same location (Brumovice), where rosemary and wild chamomile also flowered,

Table 6. Compounds found in honey fractions by GC-MS

Fraction	Compound	Retention time (min)	Fraction	Compound	Retention time (min)
1	3-phenyl propan-1-ol	10.365	1	3,7-dimethyl-1,5,7-octatrien-3-ol	10.178
	2-(4´-methoxyphenyl)ethan-1-ol	14.190		decanedioic acid	18.405
	3-phenyl propanal	9.189		abscinic acid	22.860
	dihydrocinnamic acid	10.365		3-oxo-6-hydroxy-α-ionone	19.227
	ferulic acid isomers	18.859, 20.269	2	benzoic acid	11.155
	3,4-dimethoxycinnamic acid isomers	19.571, 20.365		dihydrocinnamic acid	13.488
	2-phenyl ethan-1-ol	8.981	3	dihydrochrysin	25.561
	4-hydroxybenzaldehyde	14.003	4	chrysin	27.085
	vanillin	14.564		tectochtysin	26.422
	syringaldehyde	17.737		dihydrochrysin	25.562
	benzoic acid	11.332		galangin	27.484
	salicylic acid	13.062	5	decanoic acid – methyl ester	13.510
	syringic acid	19.377		dodecanoic acid – methyl ester	16.138
	4-hydroxyacetophenone	15.066		tetradecanoic acid	18.852
	4-acetyl benzoic acid	16.428		palmitic acid – methyl ester	20.614
	3-methyl-1-isopropyl benzene	8.848		oleic acid – methyl ester	22.314
	2,3-dihydro-1,4-benzoquinone	8.691		stearic acid – methyl ester	22.544
	caprolactone	10.808		1-methylene indene	11.170

which corresponds to the fact that apigenin is a marker for rosemary honey (Tomás-Barberán *et al.* 1993).

Combined extracts of all forty samples were separated by preparative HPLC into five fractions; individual fractions were concentrated in the vacuum

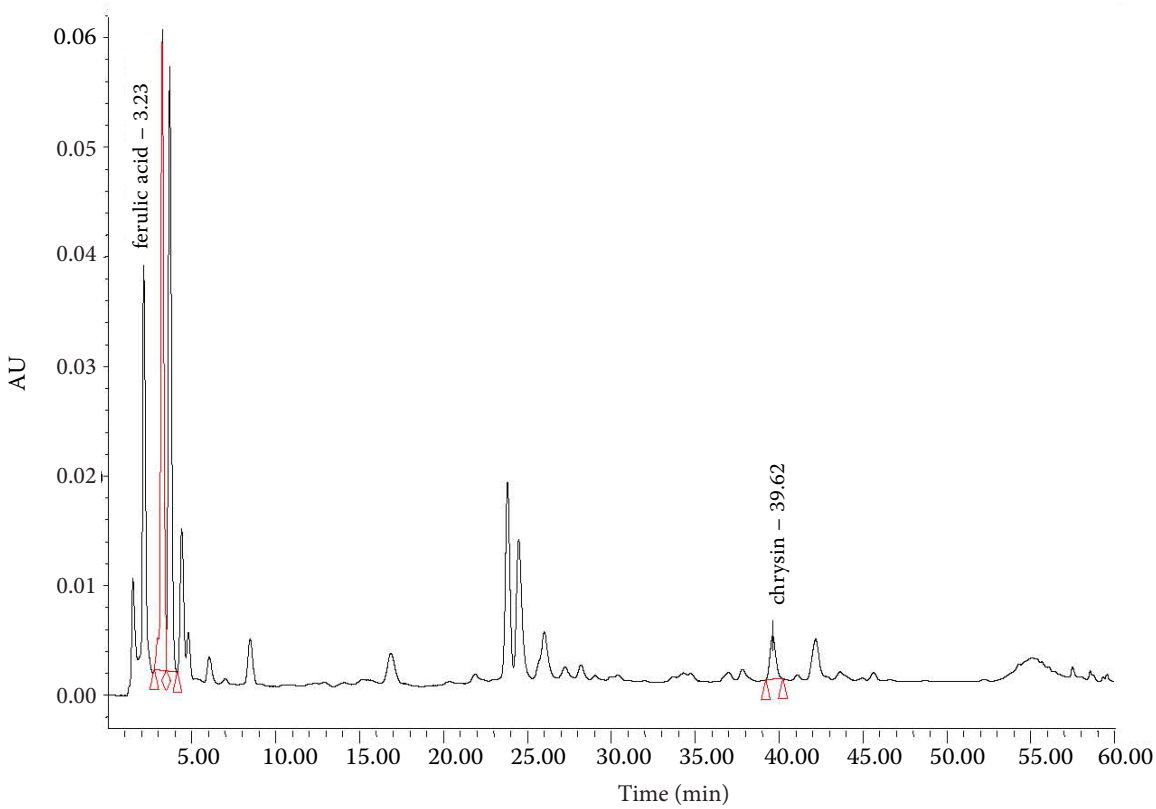

Figure 3. HPLC-DAD chromatogram of honey sample No. 30 (λ = 340 nm)

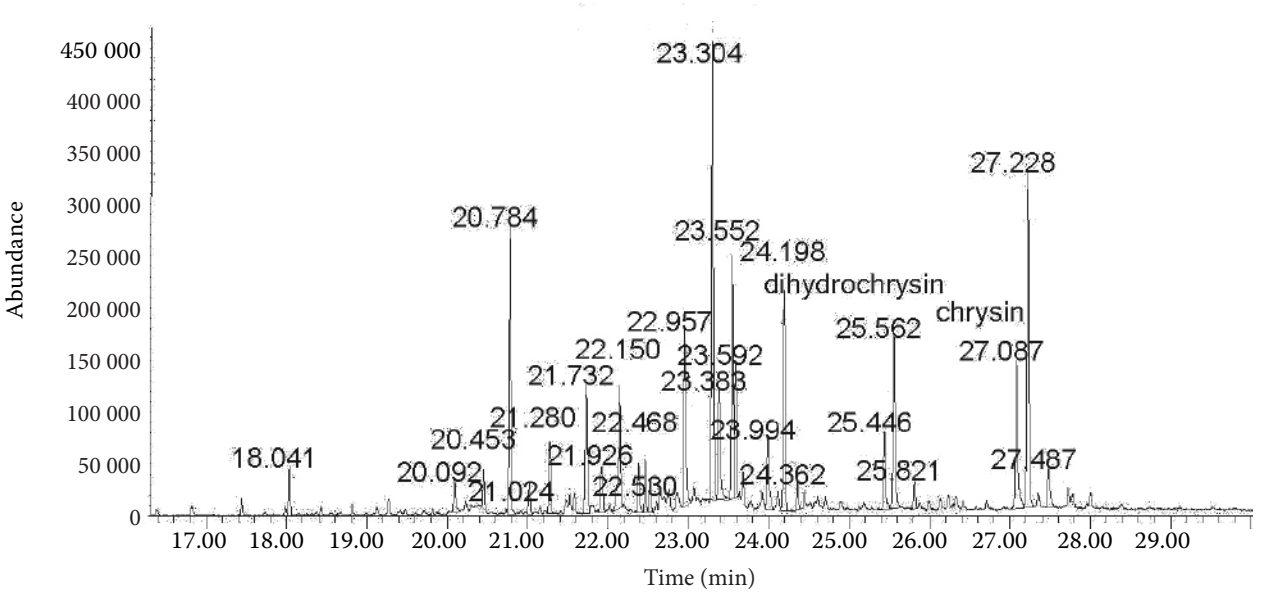

Figure 4. GC-MS chromatogram of the fraction 4 obtained from honey mixed samples by preparative HPLC-UV-VIS

rotary evaporator and analysed by GC-MS. The compounds were identified by NIST library search and by comparison with the authentic samples (Table 6). In the first phenolic fraction, 16 compounds were detected and among them ferulic acid, 3,4-dimethoxycinnamic acid, 4-hydroxybenzaldehyde, vanillin, syringaldehyde, salicylic acid, syringic acid, 2-(4'-methoxyphenyl)ethan-1-ol and 4-hydroxyacetophenone. In the second fraction, benzoic and dihydrocinnamic acids were found. The flavones, namely chrysin and dihydrochrysin, were typical of the fourth fraction (Figure 4). In the fifth fraction, fatty acids such as decanoic acid–methyl ester, dodecanoic acid–methyl ester, tetradecanoic acid, palmitic acid–methyl ester, oleic acid–methylester, and stearic acid–methylester were found.

CONCLUSION

Honeydew honey had a darker colour as compared to floral honey, whereas the unifloral honey samples analysed were significantly coloured in comparison to multifloral honey. In the dark coloured kinds of honey, higher values of TP and TF were determined; however, the relation between DHF and the honey colour was not demonstrated. TP ranged between 7.37–16.71 mg GAE/100 g (average 11.02 mg GAE/100 g), TF between 0.53–1.23 mg QE/100 g (average 0.66 mg GAE/100 g), and DHF between 1.87–6.61 μg QE/100 g (average 4.32 μg QE/100 g). The honey kind (origin), location, and date of honey harvest showed evident effects on TP, TF, and DHF, and the individual phenolics in the analysed honey samples. Cluster analysis revealed that, concerning TP and TF, floral and mixture honey kinds were statistically similar, whereas on the basis of DHF were more similar mixture and honeydew honey samples. Out of the individual phenolics, the most abundant were phenolic acids – isomers of ferulic acid (0.13 mg/100 g), and of flavonoids chrysin (0.06 mg/100 g). Based on GC-MS of the honey samples and standards, other phenolics were also found, such as 3,4-dimethoxycinnamic acid, 4-hydroxybenzaldehyde, vanillin, syringaldehyde, salicylic acid, syringic acid, dihydrochrysin, tectochrysin, and galangin.

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