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Analytical Methods

Flavonoid pattern of sage (*Salvia officinalis* L.) unifloral honey

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Abstract

The aim of the present paper was to determine the flavonoids in monofloral sage (*Salvia officinalis* L.) honey which is characteristic and specific for the area of Croatian coast and islands. For that purpose 38 sage honey samples from two production seasons were analysed. After specific pollen content determination, and analyses of selected physicochemical parameters which confirmed that samples are in compliance with national and international regulations and can be regarded as unifloral sage honeys, flavonoid fraction was isolated and analysed using RP-HPLC/DAD method. The HPLC analysis showed that all examined sage honey samples contain quercetin (3,3′,4′,5,7-pentahydroxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), apigenin (4′,5,7-trihydroxyflavone), chrysin (5,7-dihydroxyflavone) and galangin (3,5,7-trihydroxyflavone), as well as *p*-coumaric (*trans*-4-hydroxycinnamic acid) and caffeic acid (3,4-dihydroxycinnamic acid). Total amount of identified flavonoids varied from 109.4 μg/100 g of honey to 589.9 μg/100 g of honey, with the average of 288.5 μg/100 g of honey. All analysed honey samples showed common and specific flavonoid profile which could be the basis for differentiating sage from other monofloral honeys.

Keywords: Honey; Sage (*Salvia officinalis*); HPLC; Flavonoids

1. Introduction

Honey is a natural product, one of rare natural foodstuffs that are available all over the world, and the only natural sweetener that can be used in human nutrition without any preparation. As such, it represents a type of food more and more preferred by the consumers, and it can easily comply to the name of "organic food". Its value on the market varies on the basis of floral origin, and while in some Northern European countries honeydew is favorite type and highly prized than blossom honey (Bogdanov, Ruoff, & Persano Oddo, 2004; Prodolliet & Hischenhuber, 1998), consumers in Croatia prefer blossom monofloral honeys what causes rising of their price. That price variability due to the consumers' preference is probably one of the biggest temptations and so honey is still constant aim of food adulteration, especially in respect of the botanical origin. Anyhow, since identification of honey sources is a difficult task there is still no adequate analytical method available for the unequivocal determination of botanical origin of honey (Anklam & Radovic, 2001). The only official method for the botanical origin determination in Croatia, as well as in European countries, is a melissopalynological analysis, which is liable to subject influence (Ministry of Agriculture Forestry, 2000; The Council of the European Union, 2002; Von der Ohe, Persano Oddo, Piana, Morlot, & Martin, 2004). The Commission of the EU encourages researchers in this area of research to develop new methods for honey authentication (Anklam, 1998). The ultimate aim is to find alternative method for undoubtful determination of honey botanical origin which should be easy and possible to set in any routine laboratory. With this aim different honey characteristics (sugar content (Cotte, Casabianca, Chardon, Lheritier, & Grenier-Loustalot, 2003; Gómez Bárez et al., 2000), amino acids content (Hermosín, Chicón, & Cabezudo, 2003; Iglesias, De Lorenzo, Del Carmen Polo, Martín-Álvarez, & Pueyo, 2004), volatile compounds (Ampuero, Bogdanov, & Bosset, 2004; Benedetti, Mannino, Sabatini, & Marcazzan, 2004), regular parameters

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(Devillers, Morlot, Pham-Delègue, & Doré, 2004)) have been investigated. With the same purpose this research is pointed on the phenolic profile of sage (*Salvia officinalis* L.) honey. Namely, flavonoids and other phenolics are compounds widely spread in the plants. Considering the fact how honey is produced it is obvious that different types of honey have different flavonoid profile, depending on the plant which was used as a main source of nectar for its production. Though some of the flavonoid compounds are present in more or less all honey types, there are flavonoids which are specific for some plant type and as such could be used as markers for that type of monofloral honey. Different authors have earlier reported phenolic content and possible markers for some widely spread types of honey (Gil, Ferreres, Ortiz, Subra, & Tomás-Barberán, 1995; Martos, Ferreres, & Tomás-Barberán, 2000; Soler, Gil, García-Viguera, & Tomás-Barberán, 1995; Tomás-Barberán, Martos, Ferreres, Radovic, & Anklam, 2001; Yao, Datta, Tomás-Berberán, Ferreres, Martos, & Singanusong, 2003; Yao et al., 2004), but since common sage honey is autochthonous type of honey, and in Europe is produced only in the parts of Croatian coast and islands, it has never been widely investigated. Therefore, the aim of the present work was to determine flavonoid content of collected samples and to assess the applicability of sage honey flavonoid profile in botanical origin determination.

2. Materials and methods

2.1. Honey samples

Thirty-eight samples of sage honey from two production seasons were provided by the beekeepers from Mediterranean parts of Republic Croatia (Croatian coast and islands). Therewith, samples were subjected to analysis of physicochemical parameters and pollen analysis, and than stored till the flavonoid analysis. Since flavonoids are relatively stable compounds, resistant to heat, oxygen and moderate degrees of acidity honey samples were prior to analysis stored in dark place at room temperature (Peterson & Dwyer, 1998).

2.2. Pollen analysis

Though beekeepers themselves according to their best knowledge and the location of hives declared honey as monofloral sage honey, all the samples were subjected to pollen analysis according the method of Louveaux, Maurizio, and Vorwohl (1978). The aim of that analysis was to confirm that analysed samples could be declared as sage monofloral honey. Namely, according to the Croatian regulations minimum of 20% sage pollen in indissoluble matter is necessary if honey is about to be declared as sage honey (Ministry of Agriculture and Forestry, 2000). For this purpose, only orienting analysis with identification of characteristic pollen grains was performed. 200–300 pollen grains were counted and number of *S. officinalis* L. grains expressed as a share of the pollen grains counted. Identification of pollen grains was made by reference to the literature data (Von der Ohe & Von der Ohe, 2003) and/or personal comparative preparations.

2.3. Physicochemical analysis

Physicochemical parameters were determined according the methods prescribed by the Croatian Regulations (Ministry of Agriculture and Forestry, 2000) and according to the Harmonised methods of the European Honey Commission (Bogdanov, Martin, & Lüllmann, 1997). Moisture content was determined using refractometric method, free acidity by titration of honey sample solution with 0.1 M sodium hydroxide to pH 8.30, and electrical conductivity was measured at 20.0 °C in a 20% (w/v) solution of honey (dry matter basis) in a water with conductivity <1 μS/cm.

2.4. Flavonoids extraction

Flavonoids were determined according to the method developed by Ferreres, Tomás-Barberán, et al. (1994). Honey sample (*ca.* 50 g) was diluted with five parts of acidified water (pH adjusted on 2–3 with HCl). Solution was than passed through a glass column (25 × 2 cm) filled with Amberlite XAD-2 resins (pore size 9 nm, particle size 0.3–1.2 mm, Supelco, Bellefonte). During this passing the various phenolic compounds remained in the column, while sugars as well as other polar compounds were eluted with the aqueous solvent. Further, the column was washed with 100 ml of acidified water, and 300 ml of distilled water. The whole phenolic fraction was eluted with *ca.* 300 ml of methanol and taken to dryness under the reduced pressure. The dry residue was redissolved in 5 ml of distilled water and partitioned with ethyl ether (3 × 5 ml). The ether extracts were combined and ether removed under the reduced pressure. At the end of the extraction procedure, dry residue containing flavonoid fraction was redissolved in 0.5 ml of methanol and analyzed by HPLC.

2.5. HPLC analysis of honey flavonoids

For this purpose Varian HPLC system consisting of ProStar 230 Solvent Delivery Module, ProStar 500 Column Valve Module, Pro Star 310 UV/Vis Detector and ProStar 330 Photodiode Array Detector coupled to a computer with the ProStar 5.5 Star Chromatography Workstation and PolyView2000 Ver. 6.0 Software was used. For the separation of sample flavonoid components LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany, 12.5 × 0.4 cm I.D., 5 μm particle size) was used. The mobile phase consisted of a mixture of water and formic acid (95:5) (solvent A) and methanol (solvent B) at a flow rate of 1 ml/min. To achieve better separation gradient elution was used starting with 30% of methanol which remained isocratic for the first 15 min, and than followed by gradient to obtain 40% of methanol at 20 min, 45% of methanol at 30 min,

60% of methanol at 50 min, 80% of methanol at 52 min, and which than again become isocratic until the end of analysis in the 60 min. Chromatograms were recorded at two wavelengths (340 and 290 nm). The injection volume was 10 μl.

The identification of separated honey flavonoids was based on a comparison of chromatographic data (retention times and UV spectra) with authentic markers, while quantification was performed through external calibration data with the same compounds.

Authentic markers available at the market were used for chromatographic comparison of data. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), luteolin (3′,4′,5,7-tetrahydroxyflavone) and myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) were supplied by Sigma, while chrysin (5,7-dihydroxyflavone), apigenin (4′,5,7-trihydroxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), galangin (3,5,7-trihydroxyflavone) and isorhamnetin (3′-methoxy-3,4′,5,7-tetrahydroxyflavone) were by Fluka (Buchs/Schweiz, Switzerland). *p*-Coumaric acid (*trans*-4-hydroxycinnamic acid), ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenic acid dilactone) and caffeic acid (3,4-dihydroxycinnamic acid) were also supplied by Fluka.

Formic acid (Fluka) and methanol (Merck) were HPLC grade, while other chemicals were analytical grade.

2.6. Data analysis

Mean values and standard deviations (SD) were calculated using computer programs Statistica 7.0 (Statsoft Inc.) and Microsoft Excel 2000 (Microsoft Corp.).

3. Results and discussion

Results of pollen analysis conducted prior to flavonoid determination showed relatively high content of sage pollen grains (20–65%, average 34%) (Table 1). These values are in agreement with those published earlier for the sage honey produced during the period from 1999 till 2005 in the same area (Kenjerić et al., 2006). Also, it is known fact that sage is under-represented species, i.e. honey produced from sage flowers contains smaller percentage of sage pollen grains in the sediment than the percentage of the corresponding nectar in honey. As such, according to the Croatian legislation it needs 20% of sage pollen grains to be declared as monofloral sage honey (Ministry of Agriculture and Forestry, 2000), while according to the Louveaux et al. (1978) even 10% of *Salvia* (European) pollen grains are enough. From the respect of frequency classes defined by the same author *S. officinalis* L. pollen grains are in most of the samples (79%) present as secondary pollen (16–45% of the total pollen), while its presence in the frequency of predominant pollen (> 45% of the total pollen) was found only in 8 (21%) samples mostly produced in the season 2002.

Analysis of physicochemical parameters confirmed that samples are of good quality and therefore it was concluded that chosen samples will give a representative flavonoid profile for this honey type.

Table 1
Specific pollen content and flavonoids content (μg/100 g of honey) in sage honey samples produced in Mediterranean part of Croatia

Sample code	*	Flavonoids (μg/100 g of honey)								
		1	2	3	4	5	6	7	8	Total
M-04-02	23	–	126.1	9.6	0.0	30.2	–	189.1	206.1	561.1
M-07-02	47	–	65.6	8.3	28.3	15.2	–	105.9	43.5	266.8
M-21-02	48	–	42.6	14.9	76.3	15.1	–	58.0	68.6	275.5
M-22-02	55	–	16.8	7.5	35.0	13.7	–	30.5	30.2	133.7
M-23-02	49	–	56.3	22.1	13.0	19.0	–	84.3	86.8	281.5
M-24-02	50	–	31.7	12.7	41.9	14.9	–	54.5	52.3	208.0
M-46-02	35	–	23.1	23.6	24.0	20.1	–	28.7	32.0	151.5
M-56-02	32	–	48.1	18.9	12.6	14.0	–	88.7	89.1	271.4
M-67-02	65	–	102.2	10.0	26.7	21.1	–	63.9	67.8	291.7
M-82-02	32	–	60.8	29.7	19.8	18.6	–	87.9	86.0	302.8
M-01-03	24	–	42.8	15.7	45.7	18.6	–	106.5	31.0	260.3
M-07-03	32	–	41.4	16.3	37.9	25.5	–	252.3	73.7	447.1
M-24-03	21	–	70.3	54.1	67.4	51.6	–	56.5	21.6	321.5
M-27-03	31	–	46.6	10.1	25.8	48.1	–	81.8	28.9	241.3
M-37-03	33	–	55.3	21.8	18.1	28.8	–	119.4	44.7	288.1
M-38-03	21	–	33.8	14.3	29.7	22.7	–	173.1	60.5	334.1
M-44-03	20	–	50.9	16.1	38.9	30.8	–	86.0	27.5	250.2
M-58-03	22	–	107.2	14.7	29.1	29.0	–	181.3	51.4	412.7
M-60-03	21	–	53.1	18.7	43.8	32.7	–	95.5	22.5	266.3
M-87-03	20	–	131.5	18.7	43.3	45.8	–	174.0	68.8	482.1
M-101-03	30	–	92.7	10.0	26.0	20.0	–	134.0	59.3	342.0
M-102-03	21	–	41.0	47.7	26.1	29.1	–	60.6	23.2	227.7
MB-01	29	–	147.6	23.9	27.8	37.9	–	250.1	102.8	589.9
MB-02	47	–	16.7	2.4	14.5	15.0	–	48.9	12.0	109.4
MB-03	37	–	36.2	19.2	25.7	31.3	–	146.1	60.7	319.1
MB-04	35	–	36.2	19.8	22.9	31.2	–	179.2	81.3	370.4
MB-05	35	–	28.6	113	26.7	77.0	–	71.0	22.6	237.1
MB-06	42	–	34.8	19.2	26.0	23.3	–	125.3	56.7	285.2
MB-07	41	–	34.1	20.3	28.5	16.6	–	62.4	30.1	191.9
MB-10	28	–	33.8	20.8	29.1	16.6	–	78.4	32.9	211.5
MB-11	24	–	37.2	7.6	30.2	11.1	–	57.0	18.3	161.2
MB-14	39	–	58.3	32.1	47.3	59.2	–	162.8	91.3	450.8
MB-15	57	–	20.1	4.1	16.4	17.8	–	81.7	18.2	158.1
MB-16	20	–	34.5	6.4	33.4	18.2	–	58.0	19.6	170.0
MB-17	21	–	60.6	8.5	34.8	17.2	–	40.6	14.1	175.7
MB-18	22	–	41.9	11.2	30.8	23.2	–	39.9	14.8	161.6
MB-19	45	–	138.4	19.1	48.8	41.1	–	96.1	36.4	379.7
MB-20	31	–	81.6	15.6	44.1	108.4	–	92.5	30.2	372.4
Mean	34	–	57.4	17.3	31.5	29.2	–	102.7	50.5	288.5
SD	12	–	34.6	10.4	14.4	19.3	–	57.3	36.5	114.3

* – % of sage pollen grains, 1 – myricetin, 2 – quercetin, 3 – luteolin, 4 – kaempferol, 5 – apigenin, 6 – isorhamnetin, 7 – chrysin, 8 – galangin.

The HPLC analysis of phenolic fraction showed that all analysed samples have a common flavonoid profile as presented in Fig. 1. The RP-HPLC/DAD chromatograms recorded at 340 nm confirmed the presence of quercetin, luteolin, kaempferol, apigenin, chrysin and galangin in all analyzed samples, while myricetin and isorhamnetin were not present in any of the analysed samples. The content of the individual as well as the total identified flavonoids in the analysed sage honey samples is shown in Table 1. Total identified flavonoids content ranged from 109.4 μg/100 g of honey (MB-02) to 589.9 μg/100 g of honey (MB-01) giving the average value of 288.5 μg/100 g of honey.

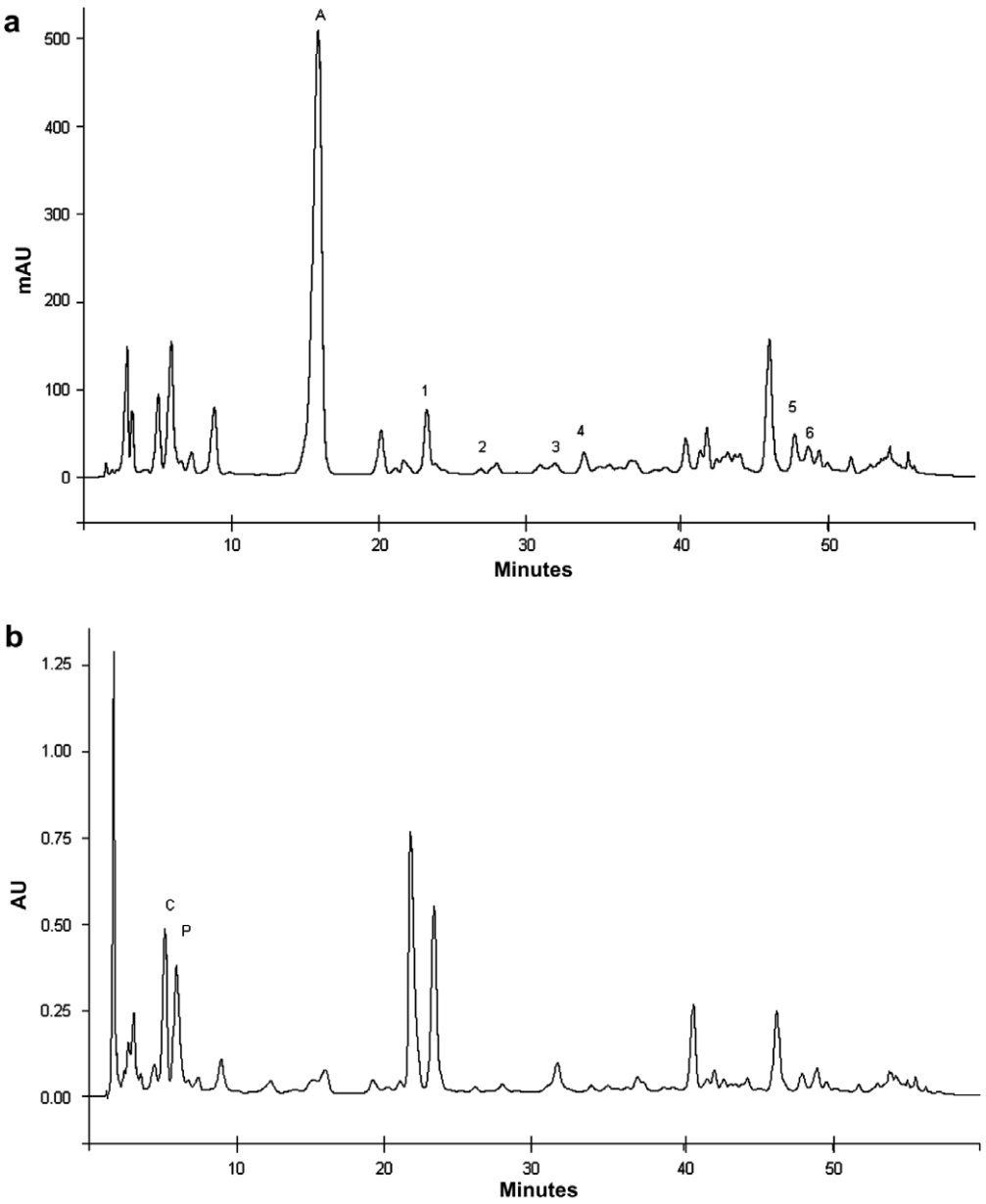

Fig. 1. Typical HPLC phenolic profile of Croatian sage honey recorded at: (a) 340 and (b) 290 nm. Peaks: quercetin (1), luteolin (2), kaempferol (3), apigenin (4), chrysin (5), galangin (6), abscisic acid (A), caffeic acid (C) and *p*-coumaric acid (P).

Since sage honey is produced only in Mediterranean part of Republic of Croatia any literature data from other authors in this field of research were not found for comparison. Therefore, the obtained values were compared with those of other Mediterranean honey types. Ferreres, Andrade, and Tomás-Barberán (1994) reported much higher values (500–2000 μg/100 g of honey) for rosemary (*Rosmarinus officinalis*) and lavender (*Lavandula* spp.) honey produced in Spain, but if pinobanksin (3,5,7-trihydroxyflavanone) and pinocembrin (5,7-dihydroxyflavanone) as compounds from propolis with their share of 100–550 μg/100 g of honey and 100–400 μg/100 g of honey respectively are excluded form this amount the values are much lower. Namely, pinocembrin and pinobanksin were left out of our research due to the fact that their source is propolis, and as such they are useful in geographical origin determination, but not in botanical origin determination (Tomás-Barberán, Ferreres, Blázquez, Garciá-Viguera, & Tomás-Lorente, 1993) which was the secondary aim of our research. Ferreres, Andrade, et al. (1994) reported amounts of the flavonoids identified in the present paper varied from 150 to 980 μg/100 g of honey with the average of 560 μg/100 g of honey. On the other hand, for other research conducted at the heather (*Erica* spp.) honey samples produced in Portugal the same authors reported 60–500 μg of flavonoids/100 g of honey (Ferreres, Andrade, et al., 1994).

Considering the amounts of identified particular compounds it is noticeable that flavone chrysin dominates with

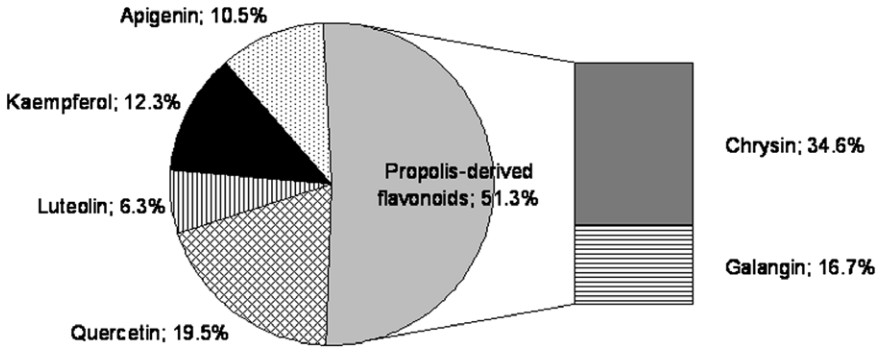

Fig. 2. Mean share (%) of individual flavonoid compounds in total identified flavonoids.

the average of 34.6% (102.7 μg/100 g of honey) of total flavonoids, though amounts of this particular flavone, as same as the amount of the total identified flavonoid compounds are very variable. Together with flavone galangin, chrysin makes propolis-derived fraction with average share of 51.3% of total identified flavonoids (Fig. 2). Amounts of pollen-nectar derived compounds are much lower, and their values are similar with those reported earlier by different authors for some Mediterranean honey types (Ferreres, Andrade, et al., 1994; Gil et al., 1995).

Comparison of the flavonoid profile of analyzed sage honey samples with profiles of other European unifloral honey types reported earlier by Tomás-Barberán et al. (2001) showed that sage honey can be differentiated from all of them. Namely, as in non-of earlier reported flavonoid profile, domination of only one peak was not noted. From the comparison of the retention time and characteristic UV spectra with literature data obtained under the same conditions of analysis (Tomás-Barberán et al., 2001) this peak was identified as abscisic acid (A), plant hormone which is known to be present in floral nectars as a protective constituent of plants against environmental stresses. Since one of its functions is a protection of plants during the droughts it is normal to expect its high concentrations in sage. Namely, sage is a plant widespread in the Mediterranean part of The Republic of Croatia (coast and islands) and during its flowering season the rain falls are rather rare. The land where sage grows contains very small amounts of soil and rather great part of rocks. Therefore, the soil is very dry, and it is to be expected that plant needs a protection to grow and flower under these conditions. To enable unbiased estimation of obtained results, samples for this research were collected during two climatically opposite seasons. As discussed in previously published paper (Kenjerić, Mandić, Primorac, Bubalo, & Perl, 2007), according to the report of Croatian Meteorological and Hydrological Service season 2002 was in Croatia extremely warm and 80% of area was very rainy, while season 2003 was the opposite according to the rainfalls and 80% of Republic area was extremely dry in combination with extremely high temperatures (Meteorological and Hydrological Service, Republic of Croatia, 2003, 2004). Namely, as same as phenolic compounds, flavonoids production in plants varies widely in dependence on the type of stress they suffer, and Bell (1980) proposed that flavonoids synthesis should be considered as a plant defence mechanism against stress. Balakumar, Vincent, and Paliwal (1993) reported that multiple stress conditions (for example, high levels of UV light irradiation are usually accompanied by drought and high temperatures) could have synergistic and/or antagonistic responses. Still, though concentrations of analysed particular flavonoids have changed, their share in total concentration has stayed unchanged, resulting in a same typical profile (Kenjerić et al., 2007) with the domination of one peak over the chromatogram.

Presence of this compound was earlier reported for a few types of honey (eucalyptus (Yao, Jiang, Singanusong, Datta, & Raymont, 2003), heather (Ferreres, Andrade, & Tomás-Barberán, 1996), rapeseed and lime-tree (Tomás-Barberán et al., 2001)), but never in such amounts that it dominates over the chromatogram.

Chromatogram recorded at 290 nm confirmed the presence of caffeic (C) and *p*-coumaric acid (P), while elagic acid was not present in any of the analyzed samples, confirming the results of Tomás-Barberán et al. (2001) that ellagic acid is a botanical marker of heather honey.

4. Conclusion

Taking into account results of this research, as well as those presented earlier by different authors, it was concluded that sage honey does not contain any specific marker compound. Nevertheless, unifloral sage honey has specific and unique phenolic profile characterised by the domination of only one peak over the chromatogram, which corresponds to abscisic acid. This profile enables differentiation of sage honey from all other honey types.

Content of total flavonoids varied in a wide range (109.4–589.9 μg/100 g of honey), and propolis derived flavonoids made in average a half of that amount.

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