POLYPHENOLS AND MINOR CONSTITUENTS IN HONEY
FROM NORTH MACEDONIA AND BULGARIA

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Abstract

Honey is a highly concentrated sugar solution that is a rich source of many bioactive compounds including numerous minor constituents. Among them, polyphenols such as flavonoids and phenolic acids are the most important classes of pharmacologically active compounds. Together with other minor constituents they are also considered potential chemical markers for the botanical and geographical origin of honey as well as for its authenticity.

A solid-phase extraction procedure and HPLC/DAD/MSⁿ method has been applied for identification and quantification of minor constituents in honey from the region of Bulgaria, North Macedonia, and Greece. Solid-phase extraction using Amberlite XAD-2 as an adsorbent was used for sample preparation enabling purification and concentration of the analysed compounds, mainly polyphenols. In total, 16 compounds were identified in the 21 extracts, 3 of them hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic acid), 5 flavonoids (naringenin, chrysin, galangin, pinocembrin and pinobanksin methyl ether) as well as gluconic acid, quinic acid, ascorbic acid, phenyllactic acid, kynurenic acid and abscisic acid in its two isomeric forms. Quantification of the identified polyphenols was made by an external calibration. The results showed that the developed method presented here is suitable for obtaining qualitative and quantitative data for polyphenols of honey.

Key words: honey, polyphenols, flavonoids, phenolic acids, solid-phase extraction, HPLC-DAD-MS

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Introduction. Honey is a primary product of honeybees, which is very well known for its nutritional, prophylactic and medicinal value. Chemically, it is a concentrated solution of sugars, mainly glucose and fructose containing also a wide range of minor constituents such as organic acids, proteins, amino acids, minerals, vitamins and enzymes [1]. Its composition largely depends on the plant species from which the nectar originates, on climate and geographical origin.

Polyphenols are plant secondary metabolites and one of the largest groups of natural products, which show a huge impact on human health [2]. Polyphenolic compounds possess antioxidant activity since they react as reducing substances, proton donors, and can form chelates with metal ions, and their analysis as trace compounds in the complex sugar matrix of honey is a challenging analytical problem that involves several steps starting with isolation of polyphenols from the complex honey matrix, followed by their separation, identification and quantification [3,4].

In recent years, numerous studies of honey polyphenols have been focused on the extraction of flavonoids and phenolic acids from honey using different adsorbents [3–9] with satisfactory analytical yields. PYRZINSKA and BIESAGA [4] and MICHALKIEWIC et al. [5] have compared the performance of different adsorbents (Oasis HLB, Strata X and C18) for extraction of flavonoids and phenolic acids and found C18 to be less efficient. Better performance was found for polymeric adsorbents with aromatic rings in their structure, which can exhibit π – π interactions with polyphenolic molecules. Separation of polyphenols is usually carried out with reversed-phase C18 columns, and gradient elution is performed by using acidified water and acetonitrile [6]. Identification is made by comparing the obtained retention times, UV-Vis and mass spectra with standards as well as with literature data.

The aim of the present study was to develop an analytical method for characterization of the polyphenolic profiles of eleven N. Macedonian, eight Bulgarian and two Greek honey samples of different botanical origin. The further idea was to evaluate the obtained results and check the possibility of using them for determining the botanical origin and authenticity of the honey samples.

Materials and methods. Reagents and standards. Reagents used were: methanol 25 HPLC grade (Fisher Scientific, UK), acetonitrile HPLC PLUS gradient grade (Carlo Erba, S. A. S, France); conc. hydrochloric acid, 37% (Merck, USA) and formic acid puriss. p. a. (Sigma Aldrich, USA). Ferulic acid, caffeic acid, *p*-coumaric acid were supplied from Genay (France); chlorogenic acid and naringenin from Carl Roth GmbH (Germany); gallic acid, galangin and rutin from Sigma Aldrich, quercetine trihydrate from TCI Europe (Belgium).

Samples and sample preparation. Honey samples were classified as multifloral, acacia, orange, chestnut, pine, forest honey or honeydew collected from North Macedonia (11), Bulgaria (8) and Greece (2). They were stored at room temperature without exposure to high temperatures and humidity. Solid-phase

extraction procedure using a column filled with 15 g of Amberlite XAD-2 was developed and optimized as follows: 10 g of honey sample was dissolved in 100 mL acidified water (previously adjusted to pH 2 with HCl) and stirred on a magnetic stirrer for 10 min. After applying the sample to the column, the column was washed with 25 mL of acidified water adjusted to pH 2 with HCl and then with 50 mL distilled water. Elution of the adsorbed compounds was carried out with 100 mL of methanol. Then the extract was concentrated under low pressure in a rotary evaporator at 40 °C, redissolved in 10 mL of 60% methanol, filtered through nylon 0.45 µm filter and applied to the HPLC system.

HPLC/DAD/MSⁿ. Agilent HPLC instrument, 1100 series was used with a UV-Vis diode array detector coupled to an ion trap mass spectrometer. The mobile phase consisted of 0.1% formic acid and acetonitrile with gradient elution as follows: 0–5 min 20% ACN, 5–15 min 30% ACN, 15–25 min 50% ACN, 25–35 min 90% ACN and 35–40 min back to 20% ACN. The flow rate was also gradient, starting with 1.0 mL/min from 1–15 min and increasing to 1.5 mL/min to 35 min, and then back to 1.0 mL/min. Sample volume was 100 µL. The column was Phenomenex Luna C18, 250 × 4.6 mm, 5 µm particle ID and it was kept at 40 °C. Spectral data were accumulated in the range 260–400 nm, and chromatograms were recorded at 266, 290, 310, 320, 350 and 370 nm. The mass spectrometer used was Agilent 6330 Ion trap with electrospray ionization in the negative ion mode. Nitrogen was used as spraying gas at a pressure of 50 psi and a flow rate of 12 L/min. The capillary temperature and potential were 325 °C and 4 kV, respectively. MS data were collected in the m/z range 100–1200 amu.

Compound identification. Identification of the extracted compounds was made by comparing the retention times and UV-Vis spectra and mass-spectral data with the spectra of standards and literature data.

Quantification of phenolics. Quantification of the identified polyphenols was made by HPLC using an external calibration.

Statistical analysis. The applied chemometric methods were accomplished using Excel and Simca15.

Results and discussion. Polyphenolic compounds in honey are present in traces in a very complex sugar matrix and the first step of the analysis aims to enable extraction and at the same time to eliminate the sugars and concentrate the polyphenolic compounds in the final extract.

Solid-phase extraction. A procedure for column solid-phase extraction (SPE) was developed. After several experiments (unpublished data), the following conditions were chosen as the most appropriate. The mass of adsorbent and column dimensions were set to 15 g and 16 × 2 cm. Because of the high sugar concentration, more than one washing step was introduced, so the final volumes of acidified water and distilled water for washing and methanol for elution were 25 mL, 50 mL and 100 mL, respectively. The extraction performed under these conditions was efficient leading to better and reproducible recovery, that was 80–

90% for hydroxycinnamic acids and rutin, 60–70% for naringenin, and around 40% for galangin and chrysine. The accuracy of the SPE method was confirmed by standard additions and also analysis of spiked honey samples.

Identification and quantification of phenolic compounds in honey samples. Identification of the extracted polyphenols was made by comparing the retention times and UV-Vis spectra with the spectra of standards and literature data. Structural characterization was made using masses of deprotonated molecular ions from the identified compounds and their characteristic fragments with literature data and standards. Eight polyphenolic compounds were found in the extracts, three of them hydroxycinnamic acids (caffeic, *p*-coumaric and ferulic acid), one flavone (chrysine), and one flavonol (galangin), two flavanones (naringenin and pinocembrin) and one flavanone (pinobanksin methyl ether). The phenolics were identified by comparing their MS spectra with literature data since they have been detected in honeys and other bee products such as propolis [6,9]. In addition, eight non-phenolic compounds were detected in most of the extracts: gluconic acid, quinic acid, ascorbic acid, kynurenic acid, 3-phenyllactic acid, and abscisic acid. Their identification was achieved by comparing their retention behaviour, UV-Vis and MS spectra with literature data. Both quinic and gluconic acid have been found in different honey types [10] and we detected them in all analyzed samples. Kynurenic acid has been identified mostly in honeys from arboreal plants [11,12]. Phenyllactic acid has often been identified in honey samples by its MS spectra [13]. Abscisic acid is a plant hormone that has been found in honey in two isomeric forms *cis-trans* and *trans-trans*, the former one being more common [9,14]. The retention data together with the UV-Vis and mass spectral data used for identification of all detected compounds are given in Table 1.

Multifloral honey samples were found to possess similar polyphenolic profiles, with the three hydroxycinnamic acids as well as naringenin, chrysin, pinocembrin and galangin. In the two chestnut honey samples, only flavonoids were found, whereas phenolic acids were not detected. Besides flavonoids, in these samples kynurenic acid was identified, it has been proposed as a potential chemical marker for the presence of chestnut flower nectar [11,12]. Pinocembrin, naringenin as well as phenyllactic and abscisic acid were detected in pine honey. Abscisic acid, known as a component of acacia honey [15] was present in the studied acacia honey sample in its two isomeric forms. In North Macedonian honeydew honeys, only flavonoids were identified, unlike honeydew honeys from Bulgaria in which hydroxycinnamic acids, phenyllactic, and abscisic acid were detected. Monofloral orange honey had similar profile as multifloral honeys with *p*-coumaric, caffeic acid, naringenin, pinocembrin, pinobanksin-5 methyl ether and abscisic acid.

The results of the quantitative analysis of the polyphenolic compounds in eleven N. Macedonian, eight Bulgarian and two Greek honey samples classified as multifloral, acacia, orange, chestnut, pine, forest honey or honeydew honey, are presented in Table 2. There were evident differences in the amounts of the

T a b l e 1

Retention time, UV-Vis absorption and MS/MS data for the detected and identified compounds

	Identified compounds	tr/min	$\lambda_{\max}$ /nm	$[M-H]^-$ (m/z)	MS^2 (m/z)
1	Gluconic acid	3.2	/	195	129
2	Quinic acid	4.2	/	191	111, 173
3	Ascorbic acid	6.3	/	175	157
4	Kynurenic acid	7.2	230, 246, 310	188	144
5	Caffeic acid	8.2	320	179	135
6	3-Phenyllactic acid	13.2	/	165	147
7	<i>p</i> -Coumaric acid	13.6	310	163	119
8	Ferullic acid	15.3	320	/	/
9	<i>trans,trans</i> -Absciscic acid	20.6	266	263	219, 153
10	Unidentified compound	21.5	/	199	155
11	<i>cis,trans</i> -Absciscic acid	22.2	266	263	219, 153
12	Unidentified compound	22.9	/	201	183, 139
13	Pinobanksin-5 methyl ether	24.2	290	285	267, 239
14	Naringenin	26.6	290	270	253
15	Chrysine	28.4	266	253	208
16	Pinocembrin	28.7	290	255	212
17	Galangin	29.1	266	269	254

polyphenolic compounds in different types of honey. Caffeic acid was present in all of the analyzed monofloral and multifloral honey samples and *p*-coumaric acid was the second most abundant quantified phenolic acid in flower honeys. There were no specific chemical markers found in honeydew honeys. The content of caffeic acid in multifloral honeys (average 16.0 mg/kg) was higher compared to honeydew honeys (average 5.1 mg/kg), the difference being statistically significant ($p < 0.05$). The total content of phenolic acids was greater in multifloral honey samples (mean value 20.44 mg/kg honey) than in honeydew (average 7.30 mg/kg, $p < 0.05$) and monofloral samples, where flavonoids were dominant. In order to check if the data obtained could be used to distinguish between honeydew and polyfloral honey, we used partial least squares discriminant analysis (PLS-DA). The 3D plot in Fig. 1 demonstrates that PLS-DA allows some separation of the samples according to their botanical origin.

Chestnut honey and pine honey were found to be rich in flavonoids with naringenin and pinocembrin present in all of these samples.

Naringenin and pinocembrin were present in all analyzed honey samples and chrysin was quantified in all multifloral honey samples.

Direct comparison of the obtained results with literature data is difficult due to the various extraction and detection techniques used in different studies.

T a b l e 2

Quantitative data for polyphenolic compounds in honey samples expressed as mg/kg honey^a

Honey sample	Phenolic acids			Flavonoids			
	Caffeic	<i>p</i> -Coumaric	Ferulic	Naringenin	Chrysine	Pinocembrin ^b	Galangin
Multifloral*	ND	2.28 ± 0.72	ND	4.45 ± 1.50	4.41 ± 162	1.97 ± 0.55	2.26 ± 0.69
Multifloral*	4.90 ± 0.29	37.55 ± 0.14	2.72 ± 0.12	6.65 ± 1.05	3.74 ± 0.07	2.08 ± 0.95	1.36 ± 0.02
Multifloral*	23.35 ± 1.38	8.19 ± 0.45	10.98 ± 0.26	30.14 ± 5.18	2.69 ± 0.19	10.03 ± 1.62	1.39 ± 0.04
Multifloral*	17.81 ± 0.09	5.52 ± 0.38	2.58 ± 0.21	13.17 ± 0.21	3.27 ± 1.18	5.41 ± 0.80	1.48 ± 0.18
Multifloral*	7.22 ± 0.19	4.75 ± 0.35	1.32 ± 0.04	16.15 ± 0.99	2.62 ± 0.72	8.29 ± 0.90	1.45 ± 0.09
Multifloral*	4.23 ± 0.24	3.11 ± 0.09	ND	12.11 ± 1.71	2.05 ± 0.43	4.18 ± 2.12	1.28 ± 0.00
Multifloral [•]	3.06 ± 0.08	2.09 ± 0.05	ND	1.99 ± 0.04	ND	0.85 ± 0.20	ND
Multifloral [#]	11.64 ± 1.46	5.27 ± 0.89	5.01 ± 1.58	13.84 ± 0.05	1.71 ± 0.03	2.03 ± 0.19	1.39 ± 0.09
Acacia*	4.88 ± 0.36	1.70 ± 0.08	2.68 ± 0.06	13.46 ± 1.38	1.75 ± 0.05	1.29 ± 1.25	1.29 ± 0.02
Orange	2.83 ± 0.58	1.93 ± 0.38	ND	2.04 ± 0.10	1.78 ± 0.01	1.09 ± 0.17	ND
Chestnut*	ND	ND	ND	4.24 ± 0.06	2.27 ± 0.62	1.77 ± 0.00	1.41 ± 0.21
Chestnut [#]	ND	ND	ND	5.73 ± 0.28	3.62 ± 0.05	1.68 ± 0.10	ND
Pine*	ND	ND	ND	1.26 ± 0.07	ND	1.59 ± 0.012	ND
Forest [#]	3.44 ± 0.60	8.87 ± 1.38	ND	4.99 ± 0.06	ND	2.52 ± 0.09	ND
Honeydew*	ND	ND	ND	1.96 ± 0.22	ND	0.84 ± 0.06	ND
Honeydew*	ND	ND	ND	1.56 ± 0.11	1.66 ± 0.05	0.86 ± 0.09	ND
Honeydew [#]	7.04 ± 0.63	8.63 ± 0.71	3.79 ± 0.17	18.99 ± 1.16	3.92 ± 1.66	10.15 ± 1.40	1.54 ± 0.46
Honeydew [#]	2.79 ± 0.56	4.90 ± 0.19	ND	5.47 ± 0.10	1.85 ± 0.00	2.66 ± 0.09	ND
Honeydew [#]	4.61 ± 0.11	6.58 ± 0.12	ND	8.29 ± 0.08	ND	2.36 ± 0.17	ND
Honeydew [#]	5.89 ± 0.50	4.64 ± 0.57	2.84 ± 0.11	11.51 ± 0.76	ND	3.49 ± 0.27	ND
Honeydew [#]	ND	ND	ND	20.42 ± 2.39	10.14 ± 0.01	6.89 ± 0.31	3.38 ± 0.31

^aMean value of 3 replicates ± SD, ^bExpressed as naringenin, *N. Macedonian, [#]Bulgarian, [•]Greek honey samples

Fig. 1. PLS- DA 3D score plot of polyfloral and honeydew honey samples

Naringenin and caffeic acid were the most commonly quantified compounds in multifloral honey samples. A similar content of these compounds was reported by KIVRAK et al. [17] with naringenin level of 3.42 mg/kg and caffeic acid of 40.85 mg/kg. GAŠIĆ et al. [18] analyzed Serbian multifloral honey and found pinocembrin, chrysin and galangin in all samples which corresponds with our data. Acacia honey was found to contain highest content of caffeic acid and naringenin. These results differ from the ones found by of DIMITROVA et al. [19], BERTONCELJ et al. [14], KEČKEŠ et al. [9], TOMÁS-BARBERÁN et al. [16], and TRUCHADO et al. [20], who found pinocembrin, pinobanksin and *p*-coumaric acid as most abundant. In our work, pine and chestnut honey were found to be very rich in flavonoids, whereas Bertoncelj et al. [14], Tomás-Barberán et al. [16] reported high percentages of phenolic acids for chestnut honey, as well as for pine honey, in addition to flavonoids.

Conclusion. The proposed solid-phase extraction in column with Amberlite XAD-2 as adsorbent of choice has proved to be an adequate technique for isolation of polyphenols from the complex matrix like honey. HPLC/DAD/MSⁿ was demonstrated as a powerful technique for identification and quantification of polyphenolic compounds with satisfactory level of accuracy and precision. Sixteen compounds have been identified, of which 8 polyphenolic compounds (*p*-coumaric acid, ferulic acid, caffeic acid, naringenin, pinocembrin, galangin, chrysin and pinobanksin-5-methyl ether), quinic acid, gluconic acid, kynurenic acid, phenyllactic acid, *cis-trans* abscisic, *trans-trans* abscisic and ascorbic acid. The results obtained confirm the established chemical marker for chestnut honey-kynurenic acid. In order to detect polyphenolic compounds as possible chemical markers,

it is necessary to study much more samples, and especially of unifloral honeys. The developed method presented here is suitable for obtaining qualitative and quantitative data for establishing polyphenolic profiles of honeys that can be further used for classification of honeys according to their botanical and/or geographical origin.

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