The determination of phenolic profiles of Serbian unifloral honeys using ultra-high-performance liquid chromatography/high resolution accurate mass spectrometry

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A B S T R A C T

Polyphenolic profiles of 44 unifloral Serbian honeys were analyzed using ultra-high-performance liquid chromatography (UHPLC) coupled with hybrid mass spectrometer which combines the Linear Trap Quadrupole (LTQ) and OrbiTrap mass analyzer. Rapid UHPLC method was developed in combination with a high sensitivity accurate mass scan and a simultaneous data dependent scan. The honey samples were of different botanical origin: acacia (*Robinia pseudoacacia*), sunflower (*Helianthus annuus*), linden (*Tilia cordata*), basil (*Ocimum basilicum*), buckwheat (*Fagopyrum esculentum*), oilseed rape (*Brassica napus*), and goldenrod (*Solidago virgaurea*). The presence of 43 compounds, mainly flavonoids, was proven in all honey samples by their characteristic mass spectra and fragmentation pattern. Relatively high amounts of chrysin, pinocembrin and galangin were identified in all honey extracts. *p*-Coumaric acid was not detected in basil, buckwheat and goldenrod honey extracts. A larger amount of gallic acid (max value 1.45 mg/kg) was found in the sunflower honey, while a larger amount of apigenin (0.97 mg/kg) was determined in the buckwheat honey in comparison with other honeys. The samples were classified according to the botanical origin using pattern recognition technique, Principal Component Analysis (PCA). The LTQ OrbiTrap technique was proven to be reliable for the unambiguous detection of phenolic acids, their derivatives, and flavonoid aglycones based on their molecular masses and fragmentation pattern.

1. Introduction

Honey is a natural sweet product produced by honeybees. It is essentially an aqueous solution of saccharides, primarily glucose and fructose, and other substances, such as organic acids, amino acids, proteins, polyphenolic compounds, minerals and other chemicals. Polyphenolic compounds, mainly phenolic acids and flavonoids, were recognized as the major constituents responsible for health-promoting properties of honey. These phytochemicals were studied for their antimicrobial, anti-inflammatory, antimutagenic, antitumor, antioxidative activity, and many other effects on human health (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008). Their identification and quantification are of great interest as they make a significant contribution to the overall bioactivity of honey. Phenolic compounds were also used to classify honeys according to their botanical origin (Truchado, Ferreres, Bortolotti, Sabatini, & Tomás-Barberán, 2008; Truchado et al., 2010).

Analytical procedures used to determine polyphenols in a honey sample include their extraction from the matrix as well as

their separation and quantification (Michalkiewicz, Biesaga, & Pyrzyńska, 2008). Different profiling methods can be used for the determination of phenolic compounds (Harnly, Bhagwat, & Lin, 2007). Liquid chromatography (LC) is considered to be the most useful separation technique for the analysis of polyphenols in different samples (de Rijke et al., 2006). Coupled with various detection techniques, such as a diode array detector (DAD) and/or mass spectrometry (MS), it enables both identification and quantification of polyphenols (Cuyckens & Claeys, 2004; Stobiecki, 2000). Since phenolic components can vary greatly, the suitable technique is liquid chromatography coupled with various types of mass detection (Daher & Gülaçar, 2008; Pulcini, Allegrini, & Festuccia, 2006). LC–MS enables high selectivity, sensitivity and universality when analyzing various polyphenolic components in their complex matrices. The current standard is LC–MS/MS (liquid chromatography with a triple quadrupole ion analyzer) which is used for the identification of target analytes based on characteristic ion transition (Stephens et al., 2010). However, such an approach cannot make much of a contribution to the study of complex samples such as honey. One of the shortcomings of this technique is that it cannot be used for the identification of predefined compounds, which limits the number of polyphenolic components which can be

identified. Liquid chromatography coupled with mass analyzers based on ion trap technology which enable MS^n analysis enables the identification of a larger number of polyphenolic compounds (Pulcini et al., 2006). The modern analytical trend is UHPLC coupled with high resolution and accurate mass spectrometry, as well as the use of hybrid mass analyzers (Makarov & Scigelova, 2010).

In this study, qualitative and quantitative content of polyphenols was determined using liquid chromatography coupled with a hybrid mass spectrometer which consists of a linear ion trap and an Orbitrap mass analyzer. The LTQ OrbiTrap MS has several advantages compared to triple quadrupole MS and quadrupole/time-of-flight MS (Hogenboom, van Leerdam, & de Voogt, 2009; Makarov & Scigelova, 2010). A mass detector with a triple quadrupole mass analyzer is not a suitable detector for full scan measurements, and it is not possible to analyze components for which we have not predefined their ion transition using this detector. The application of this hybrid technique enables a simultaneous determination of qualitative content, quantification, MS^n analysis based on high resolution accurate mass measurement and data dependent experiment. HESI in negative mode is a more suitable ionisation technique due to better ionisation, i.e. greater sensitivity to polyphenolic components.

As each melliferous plant tends to have a distinctive phytochemical profile, the content of polyphenolic compounds depends on both botanical and geographical origin of honey, as well as on the climatic conditions of a region. A review of the analytical methods for determination of the botanical origin of honey samples points to the significance of such constituents and their profiles for proving the authenticity of honey samples (Bogdanov & Martin, 2002; Kaškonienė & Venskutonis, 2010; Oelschlaegel et al., 2012; Pyrzyńska & Biesaga, 2009; Tomás-Barberán, Martos, Ferreres, Radovic, & Anklam, 2001). In paper published by Oelschlaegel et al. (2012) numerous manuka honeys were analyzed by UPLC-PDA-MS/MS after solid-phase extraction and compared to other kinds of honey to define characteristic marker substances. According to the obtained profiles the individual honey samples were assigned to different groups. The determination of a polyphenolic profile of honey is a complex task, so it is essential to develop separation and detection techniques which would enable an unambiguous determination of as many components as possible. Tandem mass spectrometry is the detection method of choice when a comprehensive analysis of non-target analytes is needed.

The aim of the present work was to determine a characteristic polyphenolic profile of several varieties of Serbian unifloral honey for the first time. A systematic characterisation of Serbian honey had been started and classification of the most important unifloral honeys based on physicochemical parameters (water content, electrical conductivity, free acidity, optical rotation and pH) and multivariate chemometric methods were published (Lazarević, Andrić, Trifković, Tešić, & Milojković-Opsenica, 2012). Sensitive and specific UHPLC–HESI–MS^n was selected for the study of the botanical origin of honey regarding its polyphenolic content. Samples of different botanical origin were collected for that purpose. PCA, a useful multivariate statistical technique for data evaluation, was utilised in order to select and establish floral markers of the botanical origin of Serbian honeys.

2. Experimental

2.1. Chemicals and materials

All reagents and solvents used were of analytical grade. Acetonitrile and methanol (both of them HPLC grade), NaCl, ethyl acetate and formic acid were purchased from Merck (Darmstadt, Germany). Ultra pure water (Thermofisher TKA MicroPure water

purification system, 0.055 μS/cm) was used to prepare standard solutions and blanks. Phenolic standards, quercetin, apigenin, kaempferol, luteolin, chrysin, pinocembrin, galangin and quercetin-3-O-rutinoside (rutin) were purchased from Fluka AG (Buch, Switzerland), while catechin, myricetin, cis, trans-abscisic, p-coumaric, caffeic, 3-O-caffeoylquinic acid, protocatechuic, and gallic acid were supplied by Sigma Aldrich (Steinheim, Germany). Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA).

2.2. Preparation of polyphenolic standards

A 1000 mg/L stock solution of a mixture of all phenolic standards was prepared in methanol. Dilution of the stock solution with methanol yielded the working solution at concentrations of 0.10, 0.25, 0.50, 0.75, and 1.00 mg/L. All stock and working solutions were stored in the dark at 4 °C and were stable for at least 3 months. Calibration curves were obtained by plotting the peak areas of the compounds identified relative to the peak area against the concentration of the standard solution. Calibration curves revealed good linearity, with R^2 values exceeding 0.99 (peak areas vs. concentration).

2.3. Honey samples

Forty-four honey samples were obtained in 2010 directly from the beekeepers from different regions of Serbia. The samples were provided by The Association of the Beekeeping Organizations of Serbia (the SPOS) (www.spos.info). The botanical origins of the samples were specified by the SPOS based on the information provided by beekeepers and sensory characteristics, and confirmed by physicochemical analyses and chemometrics (Lazarević et al., 2012). The honey samples were: acacia (Robinia pseudoacacia), sunflower (Helianthus annuus), linden (Tilia cordata), basil (Ocimum basilicum), buckwheat (Fagopyrum esculentum), oilseed rape (Brassica napus), and goldenrod (Solidago virgaurea).

2.4. Extraction of polyphenolics

The honey samples were stored in the dark at 4 °C prior to the analysis. The method used for extraction and isolation of polyphenolics from the honey was a modification of the method described in (Trautvetter, Koelling-Speer, & Speer, 2009). Honey samples (50 g of each) were diluted with 50 mL of 2% sodium chloride solution and extracted with 50 mL of ethyl acetate five times. All fractions were collected, and after drying over the molecular sieves for 45 min, ethyl acetate was evaporated on the rotary evaporator (40 °C). After concentrating the sample to about 1 mL, the residue was dissolved in 5 mL methanol/water (3:2, v/v) and filtered through a 0.45 μm membrane filter to be analyzed by UHPLC–HESI–MS/MS.

2.5. LTQ OrbiTrap mass spectrometer

All experiments were performed using a Thermo Scientific liquid chromatography system which consisted of a quaternary Accela 600 pump and Accela Autosampler, connected to a linear ion trap–Orbitrap hybrid mass spectrometer (LTQ OrbiTrap XL, Thermo Fisher Scientific, Bremen, Germany) with heated electrospray ionisation (HESI).

Separations were performed on a Hypersil gold C18 (50 × 2.1 mm, 1.9 μm) from Thermo Fisher Scientific. The mobile phase consisted of (A) water + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. A linear gradient program at flow rate of 0.400 mL/min was used 0–5 min from 5% to 95% (B), 5–6 min 95% (B) then 5% (B) for 3 min. The injection volume was 5 μL.