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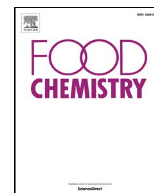

Nitrogen compounds in *Phacelia tanacetifolia* Benth. honey: First time report on occurrence of (−)-5-*epi*-lithospermoside, uridine, adenine and xanthine in honey

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ABSTRACT

Lacy phacelia (*Phacelia tanacetifolia* Borkh.) honey composition was screened by UHPLC-DAD-QqTOF-MS. The targeted analysis revealed 6 major nitrogen compounds including aromatic amino acids (tyrosine, phenylalanine), purine derivatives (adenine, xanthine), nucleoside (uridine) and rare non-cyanogenic cyanoglucoside, (−)-5-*epi*-lithospermoside ((2*Z*)-2-[(4*R*,5*R*,6*S*)-4,5-dihydroxy-6-(β-D-glucopyranosyl)oxycyclohex-2-en-1-ylidene]acetonitrile). Their identity was confirmed by different analytical tools: HRMS, co-chromatography with standard compound or comprehensive NMR experiments. All the compounds, except amino acids, were reported and determined in honey for the first time. The amount of the compounds was quantified in 16 unifloral phacelia samples: adenine (18.45 ± 4.63 mg/kg), xanthine (10.53 ± 2.98 mg/kg), uridine (42.84 ± 9.26 mg/kg), tyrosine (14.66 ± 10.22 mg/kg), (−)-5-*epi*-lithospermoside (70.61 ± 31.37 mg/kg) and phenylalanine (20.41 ± 11.99 mg/kg). The (−)-5-*epi*-lithospermoside content is significantly correlated with *P. tanacetifolia* pollen percentage ($R^2 = 0.5612$, $p < 0.001$) and it is proposed as a potential marker of botanical origin for phacelia honey.

1. Introduction

Phacelia honey is a less common type of honey, characterized by delicate flavor and light amber color. It is obtained from nectar of lacy phacelia (*Phacelia tanacetifolia* Benth.), an annual plant that belongs to the Boraginaceae family (but also classified in closely related Hydrophyllaceae) native to California and Texas (Farkas & Zajácz, 2007; von der Ohe & von der Ohe, 1999). The plant is used as cover crop, green manure, but also as excellent bee pasture providing high nectar yield in any warm season of the year (Farkas & Zajácz, 2007; Trzybiński, 2010; von der Ohe & von der Ohe, 1999). Recently, the plant gains more popularity as green fertilizer, used especially in organic agriculture or as animal feed, that can be cultivated as intercrop or post-crop suitable for non-plough cultivation (Farkas & Zajácz, 2007; Ponichtera, 2016; Trzybiński, 2010; von der Ohe & von der Ohe, 1999). Thus, the increasing availability of large areas of this excellent nectariferous plant, allows more often to obtain unifloral phacelia honey that is still a quite rare variety. Therefore, importance of investigating its composition in terms of potentially beneficial compounds as well as markers of botanical origin useful to assure its quality are becoming more up-to-date. Until now, very little is known about chemical composition of this honey type. Persano Oddo et al. (2004) as well as von der Ohe and von der Ohe (1999) reported various physico-chemical parameters such as electrical conductivity, total acidity and sugar composition of phacelia honey. Petrus, Schwartz, and Sontag (2011) reported the presence of several flavonoids in phacelia honey samples from Austria. Špánik et al. (2012) reported headspace volatile profile of this honey type, analyzed by solid-phase micro extraction coupled to two dimensional gas chromatography time of flight mass spectrometry (SPME–GC × GC–TOF–MS). Recently, color characteristics as well as more detailed volatile profile was reported for phacelia honey. It was dominated by *trans*-linalool oxide, hexan-1-ol and lavender lactone (headspace) and (*E*)-/(*Z*)-3-oxo-*retro*-α-ionol, vomifoliol (solvent extracts) (Kuś, Jerković, Marijanović, Kranjac, & Tuberoso, 2018). However, beside the research mentioned there is no other detailed data available reporting phytochemical profile of phacelia honey, particularly regarding non-volatile compounds. Therefore, in continuation of our research, focusing on characterization of phytochemical profiles as well as in search of chemical markers of botanical origin and bioactive compounds in honey, the scope of current research was to identify and quantify the main nonvolatile components in unifloral phacelia honey by targeted UHPLC-DAD analysis supported by UHPLC-QqTOF-MS,

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MS/MS and NMR for identification of the unknown compounds.

2. Materials and methods

2.1. Honey samples

Sixteen samples of unifloral lacy phacelia (*Phacelia tanacetifolia* Benth.) honey were obtained from professional beekeepers in different parts of Poland in 2016–2017. All the samples after acquisition from beekeepers were stored in hermetically closed glass jars in the dark at 4 °C. Melissopalynological analysis was performed according to the International Commission for Bee Botany (Louveaux, Maurizio, & Vorwohl, 1978). Microscopical examination was carried out on a Hund H 500 light microscope (Wetzlar, Germany) attached to a digital camera (Motic m 1000, Motic Deutschland GmbH, Wetzlar, Germany) and coupled to an image analysis system (Motic Images Plus software, Motic Deutschland GmbH, Wetzlar, Germany) for the morphometry of pollen grains. The honey samples were considered as unifloral when relative percentage of *Phacelia* pollen was higher than 60%, according to data reported by von der Ohe, Oddo, Piana, Morlot, & Martin (2004).

2.2. Reagents

Acetonitrile (gradient and LC-MS grade), formic acid, phosphoric acid, all of analytical grade were bought from Honeywell (Fluka), Seelze, Germany. Adenine and uridine standards, deuterium oxide were bought from Sigma-Aldrich, Steinheim, Germany. Tyrosine and Phenylalanine were from Merck, Darmstadt, Germany and xanthine from Reanal, Budapest, Hungary. Chloroform, methanol, magnesium sulphate, all of analytical grade, were bought from Chempur, Piekary Śląskie, Poland. Ultrapure H_2O (< 0.06 µS/cm) was obtained using Hydrolab HLP20UV (Hydrolab, Straszyn, Poland) water purification system.

2.3. UHPLC-DAD and UHPLC-QqTOF-MS analysis

The analyses were performed using UHPLC-DAD Thermo Scientific™-Dionex™ system UltiMate™ 3000 fitted with a pump module LPG-3400SD, an autosampler module WPS-3000TSL, column thermostate TCC-3000SD and a diode array detector UV, DAD-3000 (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA) set at 210 and 254 nm. The separation was obtained with a Phenomenex Kinetex® EVO C18 110 Å column (150 mm × 2.10 mm, 2.6 µm, Phenomenex, Torrence, CA, USA) thermostated at 35 °C using 0.2 M phosphoric acid in water (solvent A) and acetonitrile (solvent B) as mobile phase at a constant flow rate of 0.4 mL/min. The gradient (v/v) was generated, starting with 100% of solvent A and decreasing to 95% in 4 min, to 5% within 0.5 min, and remaining at this concentration for 3 min, increased to 100% within 0.5 min and washed for another 5 min. The system was stabilized for 10 min with 100% A, before each injection. The injection volume was 5 µL. The obtained chromatograms and spectra were elaborated with a Chromeleon v7.2 SR5 software (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA). Standard solutions were prepared in methanol or water (xanthine), and the working standard solutions in ultrapure water. The calibrations curves were prepared in concentration range concentrations of 0.2–30 mg/L and the correlation values were 0.9999–1.0000. Before analysis, the honey samples were diluted with ultrapure water (1:5, w/v), filtered through H-PTFE membrane (0.2 µm, Ø 25 mm, Macherey Nagel™, Düren, Germany), and injected in UHPLC. The limits of detection (LOD) and quantification (LOQ) were determined in agreement with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidance note (EMEA, 1995). LOD and LOQ were calculated according the equations $LOD = 3.3\,\sigma/S$ and $LOQ = 10\,\sigma/S$, where σ is the standard deviation of the blank and S is the slope of the appropriate calibration plot.

UHPLC-QqTOF-MS analyses for cleaned fractions obtained from honey were performed in similar setting and conditions of UHPLC-DAD analyses, except of solvent A, which was substituted by 0.1% formic acid in water. ESI-HRMS analysis was performed with Compact™ QqTOF mass spectrometer (Bruker Daltonic, Bremen, Germany). The following settings of MS detector were applied: positive mode, the ion source temperature was set at 100 °C, nebulizer gas pressure was set at 2.0 bar, dry gas flow 8.0 L/min and temperature 210 °C. The capillary voltage was set at 4.50 kV. The collision energy was set on 8.0 eV and for MS/MS, the collision energy was 35 eV. The internal calibration was obtained using sodium formate clusters at concentration 10 mM prior to each analysis.

2.4. Isolation of (−)-5-epi-lithospermoside

An aliquot of 100 g of honey was dissolved in ultrapure water, centrifuged and filtered through H-PTFE membrane (0.2 µm, Ø 25 mm, Macherey Nagel™, Düren, Germany). The solution was extracted by SPE, using Strata® SDBL 100 µm styrene-divinylbenzene columns (Phenomenex, Torrence, CA, USA) and the fraction washed with 10% MeOH was collected. The obtained extract was evaporated under vacuum using rotary evaporator (Büchi, Flawil, Switzerland) and freeze-dried using 2–4 LD Alpha lyophilizer (Martin Christ, Osterode am Harz, Germany) and subsequently purified by LC packed with silica gel Si-60 (0.045–0.075 mm 325–200 mesh ATSM; Macherey-Nagel™, Düren, Germany) using different ratios of $CHCl_3$-MeOH. The fraction containing compound, later identified as (-)-5-*epi*-lithospermoside (**5** – the number corresponds to that used in Table 2, Figs. 3 and 4) was washed using $CHCl_3$-MeOH (6:4). After drying using rotary-evaporator at vacuum, it was re-dissolved in water and purified using semi-preparative HPLC. The separation was obtained using LiChroCART® Lichrospher® RP-18 100 Å column (250 mm × 10 mm, 10 µm, Merck, Darmstadt, Germany) thermostated at 35 °C using flow 5 mL/min and 10% acetonitrile in ultrapure water as isocratic eluent. The joined collected fractions were filtered through H-PTFE membrane (0.2 µm, Ø 25 mm, Macherey-Nagel™, Düren, Germany), concentrated under vacuum using rotary-evaporator and dried in vacuum desiccator over silica gel. An amount of 9.9 mg of (−)-5-*epi*-lithospermoside was obtained and its purity was established by HPLC and ^{1}H NMR as described below.

2.5. NMR experiments and optical rotation measurement

NMR spectra were obtained on a 300 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) using HOD signal as a standard for ^{1}H and TMS for ^{13}C-experiments. ^{1}H, ^{13}C, DEPT-90 and -135 COSY, HSQC, HMBC, NOESY, NMR spectra were measured in D_2O. Optical rotation was measured with P-2000 polarimeter (Jasco, Easton, PA, USA) in water solution, concentration denoted in g/100 mL.

2.6. Statistical analysis

Statistical analyses were performed using the Statistica 64 v13.1 software (StatSoft Inc., Tulsa, OK, USA). The Pearson's product-moment correlation was used to estimate the relationships between the investigated parameters and their significance was assessed in two-tailed test at the level of significance $p < 0.05$ and $p < 0.001$.

3. Results and discussion

3.1. Characterization of (−)-5-epi-lithospermoside

Compound (**5**) visible as high peak eluting about 2.9 min was characterized by NMR, MS/MS, UV and HRMS. Its molecular mass was found to be [M] = 329.1109 Da and corresponded to molecular formula $C_{14}H_{19}NO_8$ (calculated 329.1111 Da). The main ions were (HRMS): *m/z* 352.1002 $[M + Na]^+$, 330.1182 $[M + H]^+$, 168.0656 $[M - 162 + H]^+$

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