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Fast analysis of polyphenols in royal jelly products using automated TurboFlow™-liquid chromatography–Orbitrap high resolution mass spectrometry

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ABSTRACT

This study describes the development of a novel, simple and fast analytical method for the detection and quantification of polyphenols in royal jelly products, using an in-house database containing more than 50 compounds. The extraction method consisted of sample dilution, followed by a fast on-line system composed of turbulent flow chromatography (TurboFlow™) coupled to liquid chromatography (LC)–Exactive-Orbitrap analyzer. The total run time was 18 min, including automated extraction, analytical chromatography and re-equilibration. The method was validated obtaining limits of quantification (LOQ) ranging from 10 to 150 μg/kg. The linearity range was up to 2000 μg/L and determination coefficients (R^2) were higher than 0.994. Adequate recoveries were obtained at three concentration levels (500, 1000 and 2000 μg/kg). This method was applied to the analysis of nine samples and the concentration of polyphenols ranged from 14 (apigenin) to 18,936 μg/kg (ferulic acid).

1. Introduction

Royal jelly is a secretion produced by the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*) and it is an essential food for young larvae and for the queen herself. As a consequence of its complex composition of water, proteins, lipids, carbohydrates and proteins with a high content of essential amino acids and vitamins, as well as minerals, royal jelly shows several pharmacological activities such as neurotrophic [1,2], hipocholesterolemiant and hepatoprotective [3], hypotensive and blood pressure regulatory [4], antitumor [5], antibiotic [6], anti-inflammatory [7], immunomodulatory [8] and anti-allergic [6,9]. Moreover, its antioxidant effects and free radical scavenging capacity have incremented the interests of many consumers as well as researches for this natural product [6,10].

Therefore, many studies have been focused on the determination of antioxidant capacity and phenolic content of products derived from bees, such as propolis [11–13], pollen [14] and honey [15–19]. To carry out this task, some papers used gas chromatography (GC) for the analysis of phenolic acids in pollen [14], although liquid chromatography (LC) coupled to traditional detection techniques such as diode array detector [11,13,19,20] has been the most common technique for phenols analysis in propolis [11,13], pollen [20] and honey [19]. Nowadays, LC coupled to mass spectrometry (MS) is being the analytical technique selected to carry out the determination of phenolic acids and flavonoids in propolis [13] and honey [15,17].

Regarding the extraction of phenolic compounds, one of the main problems is the complexity of these products derived from bees (pollen, propolis, honey or royal jelly) because they contain very richly concentrated amount of nitrogenous materials, as proteins, minerals and amino acids, as well as other non-isolated and different trace elements. Several extraction procedures as liquid–liquid extraction (LLE) employing acidified water [15], organic solvents such as ethyl acetate [20] or mixtures of ethanol–water [12,13] have been used for the extraction of phenolic compounds from honey, pollen and propolis respectively. Moreover, LLE combined with other extraction techniques as microwave-assisted extraction (MAE) [13,17], ultrasonic extraction (UE) [14,17], pressurized liquid extraction (PLE) [11] and solid phase extraction (SPE) after a simply dilution with water [18] or after an acid or basic hydrolysis [16,19] have been used for the analysis of propolis [11,13], honey [16–19] and pollen [14]. These

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procedures provide an extract free from interfering matrix components such as sugars and other polar compounds. In all these cases, important steps of the extraction procedure are not automatic and involved a greater sample handling and therefore, sample throughput decreased.

However, automated or semi-automated extraction techniques, such as the so-called TurboFlow™ (turbulent flow chromatography, TFC), can be used in order to simplify extraction step [21]. TFC is an automatic extraction technique used for sample preparation that has a great potential for on-line sample pre-treatment, in terms of high sample throughput. It permits on-line sample extraction and clean-up, allowing the reduction of the overall analysis time [21]. This methodology has been employed in bioanalysis [22–24], and in less extent in food analysis. For instance, it has been used for the analysis of veterinary drugs in honey [25] or milk [26], or for the determination of pesticides in grapes, baby food and wheat flour matrices [27]. However, it has not been used for the extraction of polyphenols in food samples so far. Generally, TFC has been coupled to LC–tandem mass spectrometry (MS/MS), allowing the selectivity and sensitivity required to enable the identification and quantification of analytes in food and biological samples. However, this could be improved with the use of benchtop Exactive-Orbitrap mass spectrometry (Orbitrap-MS), which provides higher selectivity and similar sensitivity than triple quadrupole mass analyzer (QqQ) [28]. Taking into account the high mass resolving power provided by Orbitrap MS instruments (up to 100,000 FWHM, m/z 200) and mass accuracy (<5 ppm), this analyzer can allow the selective detection of phenolic compounds in complex samples, such as royal jelly.

Bearing in mind that there are very few reports analyzing the antioxidant effects of royal jelly [29], and up to our knowledge, there are no papers dealing with the quantitative analysis of polyphenols content in this matrix, the aim of this study is the development of the first quantitative analysis of phenolic compounds employing an online extraction procedure in royal jelly using TFC–UHPLC–Orbitrap-MS. The combination of TFC extraction and UHPLC–Orbitrap-MS provides a fast and simple method, increasing sample throughput.

2. Material and methods

2.1. Chemicals and reagents

All standards were purchased from Extrasynthese (Genay, France), Sigma–Aldrich (Madrid, Spain), ChromaDEX (Irvine, CA, USA) and Fluka (Steinheim, Germany). All standards were of high purity grade (≥90%).

The concentration of the individual stock standard solutions ranged from 120 to 500 mg/L. Stock solutions were stored at −18 °C in the darkness, and they were renewed every 6 months. Three working standard solutions were prepared by appropriate dilution of all individual stock solutions in methanol (MeOH) at the following concentrations: 5000 μg/L, 500 μg/L and 50 μg/L. These solutions were kept in amber bottles, and stored at −18 °C in the darkness.

Acetone HPLC (purity ≥99.8%), MeOH (LC–MS grade) and 2-propanol (LC–MS grade) were purchased from Sigma–Aldrich. Acetonitrile (ACN) LC–MS grade was supplied from Fisher Scientific (Fair Lawn, NJ, USA). Water (LC–MS grade) and formic acid (LC–MS grade) were purchased from Scharlau (Barcelona, Spain). Ammonium acetate (purity 97%) was obtained by Panreac (Barcelona, Spain).

For accurate mass calibration, a mixture of acetic acid, caffeine, Met–Arg–Phe–Ala–acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrate and Ultramark 1621 (fluorinated phosphazines) (ProteoMass LTQ/FT-Hybrid ESI negative) from Supelco (Bellefonte, PA, USA) were used in the Orbitrap analyzer for calibration.

Millipore Millex-LG filters (0.20 μm, Millipore, Carrigtwohill, Ireland) were used for samples filtration.

2.2. Chromatography: TFC clean up and LC separation

The TFC system consisted of an Aria TLX-1 system (Thermo Fisher Scientific, Franklin, MA, USA) equipped with a CTC HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) maintained at 10 °C, and a low-pressure mixing quaternary pump for online extraction (loading pump), a high-pressure mixing quaternary pump for analytical separation (eluting pump), and a three-valve switching device unit with a six-port valve and 100 μL loop.

The system was controlled by software Aria 1.6 (Thermo Fisher Scientific). The automated online sample extraction was performed on a Cyclone P (50 mm × 0.5 mm, 60 μm particle size, 60 Å pore size) column from Thermo Fisher Scientific (Franklin, MA, USA) and a Waters (Milford, MA, USA) Acquity C18 column (2.1 mm × 100 mm, 1.7 μm particle size) column was employed as analytical column using an LC system, Transcend 600 (Thermo Fisher Scientific, San Jose, CA, USA).

Chromatographic conditions are based on a previous work developed in our laboratory [30]. Thus, the analyses were performed with a mobile phase prepared from an aqueous solution of ammonium acetate 30 mM, pH 5 (eluent E) and MeOH (eluent F) at a flow rate of 0.2 mL/min. The gradient elution and separation conditions are described in Table 1, obtaining a total running time, including the extraction and the elution phase, of 18 min. The analytical column temperature was set at 30 °C.

2.3. Mass spectrometry: Orbitrap-MS analysis

MS detection was performed using a single-stage Orbitrap mass spectrometer (Exactive™, Thermo Fisher Scientific, Bremen, Germany) with heated electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in positive (ESI+) and negative ionization (ESI−) mode. The HESI-II source parameters included spray voltage, 4 kV (−4 kV in ESI−); sheath gas (N_2, >95%), 35 (adimensional); auxiliary gas (N_2, >95%), 10 (adimensional); skimmer voltage, 18 V (−18 V in ESI−); capillary voltage, 35 V (−35 V in ESI−); tube lens voltage, 95 V (−95 V in ESI−); heater temperature, 305 °C; capillary temperature, 300 °C. The automatic gain control (AGC) was set at a target value of 1×10^6. The full scan spectra were acquired employing four acquisition functions as follows: (1) full MS, ESI+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (2) all-ion fragmentation (AIF), ESI+, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s; (3) full MS, ESI− without fragmentation (HCD collision cell was switched off), mass resolving power = 25,000 FWHM; scan time = 0.25 s; (4) AIF, ESI−, with fragmentation (HCD on, collision energy = 30 eV), mass resolving power = 10,000 FWHM; scan time = 0.10 s.

Considering the scan time of the four acquisition functions, and the polarity switching (approx. 0.27 s), an overall scan rate of 0.56 Hz was achieved. Mass range in the full scan experiments was set from m/z 100 to 1000, while for MS/MS, it was set from m/z 70 to 700. All the analyses were performed without lock mass. The Orbitrap was calibrated every two weeks with mass accuracy standards (see Section 2.1). Data acquisition and processing were carried out using Thermo Scientific Xcalibur™ (Thermo Fisher Scientific, Les Ulis, France) version 2.2.1 software and Qual and Quan Browser.