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Trace analysis of sulforaphane in bee pollen and royal jelly by liquid chromatography–tandem mass spectrometry

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ABSTRACT

In this study, we investigate for the first time the presence of sulforaphane (SFN) residues in two of the most currently consumed food/dietary supplements, royal jelly and bee pollen. Chromatography-tandem mass spectrometry (LC–MS/MS) was the method employed, the mass spectrometer consisting of an ion-trap mass analyzer used with electrospray ionization (ESI) in positive ion mode. An efficient sample treatment involving a solvent extraction with methanol, centrifugation, and concentration in a rotary evaporator was proposed. In all cases average analyte recoveries were between 92 and 106%. Chromatographic analysis (16 min) was performed on a core–shell technology based column (Kinetex C_{18}, 150 × 4.6 mm, 2.6 μm, 100 Å). The mobile phase consisted of 0.02 M ammonium formate in water and acetonitrile, with a flow rate of 0.5 mL/min in gradient elution mode. The fully validated method was selective, linear from 8 to 1000 μg/kg (bee pollen), or from 10 to 1250 μg/kg (royal jelly), precise and accurate; relative standard deviation (% RSD) and relative error (% RE) values were below 10%. Low limits of detection (LOD) and quantification (LOQ) were obtained, namely, 3 μg/kg (LOD) and 8 (bee pollen) and 10 (royal jelly) μg/kg (LOQ). The method was applied for SFN analysis in several royal jelly and bee pollen samples. SFN was detected at trace levels in some bee pollen samples (<23 μg/kg) examined, whilst SFN went undetected in the royal jelly samples analyzed.

1. Introduction

Food supplements, also called dietary or nutritional supplements, whose consumption has increased considerably in recent years, are marketed as foods containing concentrated sources of nutrients and are claimed to supplement the intake of these nutrients in a normal diet. Consumers, then, may choose to supplement their intake of certain nutrients through food/dietary supplements [1]. Yet it should be mentioned that nutritional supplements may contain bioactive compounds shown to have consequences for human health [2]. Currently, many studies concerning bioactive compounds with anticancer properties focus on a single compound, namely, sulforaphane (SFN) [3]; this isothiocyanate is present in cruciferous plants [4]. SFN is of interest due to its potential role not only in preventing cancer, but also chronic and degenerative diseases such as diabetes, atherosclerosis and cardiovascular disorders [5,6]. Despite being found in some food supplements, mostly broccoli extracts, with an amount usually ranging from 400 to 2000 μg per capsule (0.1–0.5% in weight), SFN has yet to be reported in naturally occurring dietary supplements not originating from cruciferous plants. The present study investigates the possibility of SFN existing in two of the most widely consumed food supplements, royal jelly and bee pollen. These matrices were selected because SFN has been previously detected in honey [7,8], and because nowadays natural products from insects are receiving attention in the food industry [9]. Consequently, it may be surmised that SFN residues are also present in other bee products, such as beeswax or bee pollen. An analysis of SFN could be of significant interest for verifying its presence as since, to the best of our knowledge, no research has been published in which this compound has been analyzed in these matrices. In addition, it might help to determine the existence of SFN in products to be consumed by humans, who would be positively affected by their beneficial health properties.

Abbreviations: AF, samples spiked after sample treatment; BF, samples spiked before sample treatment; ESI, electrospray ionization; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; QC, quality control; RE, relative error; SFN, sulforaphane; S/N, signal to noise ratio; SPE, solid phase extraction.

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Although SFN has never been investigated in bee pollen and royal jelly, it has been widely determined in other matrices such as *Brassica* vegetables. Its extraction has generally been performed with organic solvents [10–18], and in several of those studies [10,11,14] solid phase extraction (SPE) with silica based cartridges was also undertaken in order to purify the plant extracts. However, other SPE sorbents have been employed to analyze SFN in biological matrices, such as polymeric [19], C_{18} [20], and C_2 [21]. Occasionally gas chromatography (GC) [13,15,16,21] has been employed to determine this substance, but LC has been the main choice [7,10–12,14–16,18–22] due to thermal degradation in the injection ports of GC equipment [11], and the normally longer analysis times. Although C_{18} based analytical columns and UV or diode array detectors have largely been used for LC studies, in our study tandem mass spectrometry (MS/MS) detection was employed to enhance selectivity and sensitivity.

Our aim was to develop a new, robust LC–MS/MS method to determine SFN in royal jelly and bee pollen. Pioneer specific and efficient extraction and determination procedures have been proposed. An analytical column based on core–shell technology was used for separation; this was particularly significant as there are relatively few reports and applications regarding the use of this type of column for performing the task. A second goal was to validate this method and apply it for analyzing samples from local markets (bee pollen and royal jelly) and organic apiaries (bee pollen); this would support the claim that SFN might be found in these matrices.

2. Materials and methods

2.1. Reagents and materials

SFN standard, ammonium formate, sodium hydroxide, formic and hydrochloric acids were obtained from Sigma Aldrich Chemie Gbmh (Steinheim, Germany). LC grade ethanol, hexane, diethyl ether, methanol, dichloromethane, and acetonitrile were supplied by Panreac Química S.A.U. (Barcelona, Spain). Syringe filters (17 mm, Nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA), ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA,USA), a vortex reax control was obtained from Heidolph (Schwabach, Germany), and a vibromatic mechanical shaker and a ultrasonic bath with heating were purchased from J.P. Selecta S.A. (Barcelona, Spain). A R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), a filtration system (Millipore), Strata C18-E (3 mL with 500 mg of sorbent) and Strata X (6 mL with 200 mg of polymeric sorbent) from Phenomenex (Torrance, CA, USA), a vortex mechanical mixer from Heidolph (Schwabach, Germany) and a 10-port Visiprep vacuum manifold (Supelco, St. Louis, MO, USA) were used for the extractions.

2.2. Standard solutions

Standard stock solution (500 mg/L) was prepared in acetonitrile. This was further diluted with methanol to prepare the intermediate and working solutions. Bee pollen (0.5 g) and royal jelly (1.6 g) were spiked before (BF samples) or after (AF samples) sample treatment with different amounts of SFN to prepare the matrix-matched standards; this is described in sub-section 2.3. The samples were employed for validation (quality control (QC) samples and calibration curves), matrix effect, and treatment studies. Each QC sample was prepared with 0.5 g of bee pollen and 1.6 g of royal jelly spiked with three different concentrations of SFN within the linear range. These were as follows: QC level 1–13 μg/kg (royal jelly) and 20 μg/kg (bee pollen); QC level 2–125 μg/kg (royal jelly) and 200 μg/kg (bee pollen); QC level 3–625 μg/kg (royal jelly) and 1000 μg/kg (bee pollen). The stock solution was stored in glass containers in darkness at −20 °C; intermediate, working and matrix-matched solutions were stored in glass containers and kept in the dark at +4 °C. All solutions were stable for over two weeks (data not shown).

2.3. Sample procurement and treatment

2.3.1. Samples

Commercial bee pollen ($n = 5$, **C1–C5**) and royal jelly samples ($n = 5$, **R1–R5**) were purchased in local markets (Valladolid, Spain); these were from different Spanish regions. Corbicular bee pollen samples ($n = 5$, **O1–O5**) from organic apiaries were supplied by the Centro Apícola Regional of Marchamalo (Castilla la Mancha, Spain). They were mixed and dried at +45 °C in an oven, ground and pooled for optimum sample homogeneity, and subsequently stored in darkness at +4 °C until analysis. The royal jelly samples required no special treatment and were stored in the dark at +4 °C prior to being analyzed. All the samples underwent preliminary assaying by LC–MS/MS to check for the presence of SFN. Once it was confirmed that there was no residual trace of the latter, subsamples of bee pollen and royal jelly were used as blanks to prepare matrix-matched standards.

2.3.2. Sample treatment

Briefly, 0.5 g of bee pollen sample and 12 mL of methanol were transferred to a centrifuge tube, and the mixture was shaken for 10 min at maximum speed (960 oscillations per minute) in a vibromatic; meanwhile, 1.6 g of homogenized royal jelly sample and 5 mL of methanol were also transferred to a centrifuge tube, the mixture being shaken for 1 min in a vortex mechanical shaker and then put in an ultrasonic bath for 5 min. The resulting mixtures were centrifuged for 10 min at +10 °C and 11,000 rpm. The supernatant was collected and evaporated to dryness at +45 °C in a rotary evaporator; the dry residue was reconstituted with 1 mL (bee pollen) or 2 mL (royal jelly) of methanol, filtered through a nylon 0.45 μm filter, and injected into the LC–MS/MS system. Fig. 1 outlines the analytical methods used during the present study.

2.4. LC–MS/MS system

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC–MSD Trap XCT instrument (ion-trap mass analyzer) was used in conjunction with electrospray ionization (ESI) in positive ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump, an autosampler and a thermostated column compartment. The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and MSD Trap Control version 4.2. Data were analyzed by Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies. A Kinetex fused-core type column (C_{18}, 150 × 4.6 mm, 2.6 μm, 100 Å) was employed for LC analysis, and this was protected by a C_{18} guard column; both were acquired from Phenomenex. After an optimization study, the mobile phase was selected; this was 0.02 M ammonium formate in water (solvent **A**) and acetonitrile (solvent **B**) applied at a flow rate of 0.5 mL/min in the following gradient mode: (**i**) 0–6 min (**A–B**, 68:32, v/v); (**ii**) 6–8 min (**A–B**, 5:95, v/v); (**iii**) 8–11 min (**A–B**, 5:95, v/v); (**iv**) 11–13 min (**A–B**, 68:32, v/v); (**v**) 13–16 min (A–B, 68:32, v/v). Injection volume and column temperature were set at 10 μL and +25 °C, respectively. All ESI–MS/MS analyses were performed in positive mode by means of multiple reaction monitoring (MRM) in ultra scan mass range mode; this enabled smart fragmentation and scanning from m/z 50 to 200. Optimal MS/MS conditions were set as follows: capillary voltage, 4300 V; drying gas (N_2) flow, 12 L/min; drying gas (N_2) temperature, 225 °C; nebulizer pressure, 30 psi; trap drive, 35;