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Original research article

Development and validation of UHPLC–MS/MS methods for determination of neonicotinoid insecticides in royal jelly-based products

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ABSTRACT

In this study, new methods have been proposed to determine seven neonicotinoid insecticides (dinotefuran, nitenpyram, thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid) in royal jelly-based products, by means of ultra-high-performance liquid chromatography coupled to a quadrupole-time-of-flight mass detector. Efficient sample treatments (with average analyte recoveries between 83% and 109%) involving solid-phase extraction (polymeric sorbent, Strata® X) and a dispersive liquid–liquid microextraction, were proposed to determine these neonicotinoids in liquid dietary supplements containing freeze-dried royal jelly and fresh royal jelly, respectively. Chromatographic analysis (8 min) was performed on a core-shell technology-based column (Kinetex® EVO C_{18}). Both methods were fully validated and the data demonstrated that they are consistent, reliable and have a wide linear range of applicability. Low limits of quantification, ranging from 2.5 to 9.5 μg/kg, were obtained in all cases, and it was necessary to employ matrix-matched standards for correct quantification of three of the compounds in each of the royal jelly-based products. Finally, the proposed methods were applied to neonicotinoid analysis of royal jelly-based products from different Spanish regions.

1. Introduction

Nowadays, the consumption of apicultural products (honey, royal jelly, propolis, beeswax or bee pollen) is gaining prominence, due to their bioactive compounds associated with beneficial properties to health (Ares et al., 2018; Martínez-Domínguez et al., 2016). Particularly noticeable is the significant increase in modern diets of the consumption of royal jelly, a thick and milky substance that is secreted from the mandibular glands of nurse bees (Jin et al., 2017; Wu et al., 2015), probably due to its wide range of biological functions, such as those of an antioxidant, anti-inflammatory, antiviral or antibacterial nature (Akamatsu and Mitsuhashi, 2013; Hryniewicka et al., 2016; Liming et al., 2009; Martínez-Domínguez et al., 2014, 2016; Wytrychowski et al., 2013; Wu et al., 2015). However, food alerts, caused by the detection of contaminants (pesticides or antibiotics) in beehive products, such as royal jelly, have recently affected their health image, as this could represent a potential risk for consumers (Ares et al., 2017; Jin et al., 2017; Tette et al., 2016). In this regard, concerns regarding the side-effects on health and the environment of neonicotinoids, which are the most widely-used insecticides in the world of insecticides, continue to increase, since they can be transferred to the latter and the food chain, with potentially adverse consequences for biodiversity and, for example, non-targeted organisms such as honeybees (Dankyi et al., 2015). As a consequence of the negative effects associated with neonicotinoid insecticides, international legislation, such as that of the European Union, has established stringent maximum residue levels (MRLs) for these substances in honey and other apicultural products, including royal jelly (10–200 μg/kg; European Union Pesticide Database, 2018).

To our knowledge, only one recent study exists of the analysis of neonicotinoid insecticides in royal jelly. This involves a multi-class methodology to determine more than 260 compounds, including six of the main neonicotinoid insecticides (imidacloprid-IMI; acetamiprid-ACET; clothianidin-CLO; thiacloprid-THIA; thiamethoxam-TMX; nitenpyram-NT), in green tea and a royal jelly liquid preparation (Martínez-Domínguez et al., 2016). In that study, neonicotinoids were determined by means of an ultra-high performance liquid chromatography (UHPLC) system equipped with a C_{18} based column coupled to a

Abbreviations: ACET, acetamiprid; AF, samples spiked after sample treatment; BF, samples spiked before sample treatment; CLO, clothianidin; DLLME, dispersive liquid–liquid microextraction; DN, dinotefuran; EIC, extracted ion chromatogram; FRJ, fresh royal jelly; IMI, imidacloprid; IS, internal standard; LDS, liquid dietary supplement; MRLs, maximum residue levels; *m/z*, mass-to-charge; NT, nitenpyram; QC, quality control; QTOF, quadrupole-time-of-flight; QuEChERS, quick, easy, cheap, effective, rugged and safe; RSD, relative standard deviation; SPE, solid phase extraction; THIA, thiacloprid; TMX, thiamethoxam; UHPLC, ultra-high performance liquid chromatography

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high resolution mass spectrometer after performing a solvent extraction (acetonitrile with formic acid) and a clean-up.

In view of the absence of specific procedures to determine neonicotinoids in royal jelly, it was decided that SPE and the sample preparation known as quick, easy, cheap, effective, rugged and safe (QuEChERS) would be initially tested as sample treatments. An SPE procedure usually provides good results in terms of sensitivity, recovery and matrix effect, although it also implies a significant cost in terms of reagents and equipment, especially on account of the SPE sorbents. In addition, it has been previously employed to determine other pesticides in royal jelly (Karazafiris et al., 2008; Martínez-Domínguez et al., 2014; Xia et al., 2014), However, the current trend in sample preparation techniques is focused on the simplification of these procedures to reduce costs, the number of reagents and the time spent on this step; these are some of the principles of the green analytical chemistry (Gałuszka et al., 2013) and the characteristics of the QuEChERS procedure. Moreover, in this study two different types of royal jelly (fresh (FRJ) and a liquid dietary supplement (LDS)) would be analyzed, and there was also the possibility that alternative sample treatments would be tested to obtain satisfactory results. Finally, it was decided that separation would be performed by UHPLC equipped with a C_{18} based stationary phase, as this usually provides better resolution and sensitivity in shorter running times than conventional HPLC (Valverde et al., 2016); meanwhile, a quadrupole-time-of-flight (QTOF) MS/MS detector was to be used in view of the good results obtained in recent studies involving neonicotinoids (Valverde et al., 2016).

The aim, therefore, of this study was to propose a specific analytical methodology to quantify seven neonicotinoid insecticides (dinotefuran-DN, NT, TMX, CLO, IMI, ACET and THIA), in two different royal jelly-based products (fresh royal jelly-FRJ; liquid dietary supplement-LDS) by means of UHPLC–MS/MS. We have optimized specific and efficient extraction/determination procedures with the aim of providing good recoveries, minimizing the potential matrix effect, and respecting as far as possible the principles of green analytical chemistry. To the best of our knowledge, this is the first study in which specific extraction, separation and detection procedures for neonicotinoids have been developed and optimized in different types of royal jelly. Further aims of the study involved validating the proposed method for the different royal jelly-based products, in accordance with current European legislation (European Commission Directorate-General for Health and Food Safety, 2015), and analyzing two different types of royal jelly-based products (FRJ and LDS) from different Spanish regions.

2. Materials and methods

2.1. Reagents and materials

Fluka-Pestanal analytical standards of ACET (Det. Purity 99.9%), CLO (Det. Purity 99.9%), DN (Det. Purity 98.8%), IMI (Det. Purity 99.9%), NT (Det. Purity 99.8%), THIA (Det. Purity 99.9%), TMX (Det. Purity 99.6%), and TMX-d3 (Det. Purity ≥ 98%) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). An isotope-labeled standard (TMX-d3) was chosen as internal standard (IS), since it has the same physical and chemical properties as the unlabeled analyte. Ethyl acetate, acetone, methanol, ethanol, dichloromethane, and acetonitrile (LC grade) were supplied by Lab Scan Ltd. (Dublin, Ireland). Chloroform (LC grade) was supplied by Scharlab S. L. (Barcelona, Spain); while, formic acid (98–100% pure), ammonium formate and magnesium sulfate anhydrous were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium chloride, sodium acetate, trisodium citrate dihydrate, and disodium hydrogen citrate sesquihydrate were supplied by Panreac (Barcelona, Spain); while, primary secondary amine (PSA) and C_{18} were purchased from Supelco (Bellefonte, PA). Strata® X (6 mL with 200 mg of sorbent) and Strata® C18-E (3 mL with 500 mg of sorbent) cartridges (Phenomenex, Torrance, CA), Isolute® HM-N diatomaceous earth packed (5 mL sample) cartridges (Biotage, Uppsala, Sweden), and a 10-port Visiprep vacuum manifold (Supelco, Bellefonte, PA), were used for the extractions. An ultrasonic bath both from J.P. Selecta S.A. (Barcelona, Spain), a vortex mechanical mixer from Heidolph (Schwabach, Germany), a 5810 R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany), a R-210/215 rotary evaporator from Buchi (Flawil, Switzerland) were employed for the extractions. Nylon syringe filters (17 mm, 0.45 µm) were from Nalgene (Rochester, NY), and ultrapure water was obtained using Millipore Milli-RO plus and Milli-Q systems (Bedford, MA).

2.2. Standards

Stock standard solutions of each neonicotinoid insecticide, at a concentration of 1000 mg/L, were prepared in methanol. These solutions were further diluted with a water and methanol (80:20, *v/v*) mixture in order to prepare the working solutions. Royal jelly samples were spiked before (BF samples) or after (AF samples) sample treatment with different amounts of the neonicotinoid insecticides and with 250 µg/kg and 83 µg/kg of the IS to prepare the FRJ and LDS matrix-matched standards, respectively; this is described in sub-section 2.3. Those samples were employed for validation (quality control (QC) samples and calibration curves), matrix effect, and treatment studies. Each QC sample was prepared with a royal jelly-based product (3.0 g LDS; 100 mg FRJ) spiked with three different concentrations of neonicotinoids within the linear range. These were as follows: QC1–LOQ µg/kg; QC2–83 µg/kg; high QC3–333 µg/kg for LDS samples; meanwhile, QC1–LOQ µg/kg; QC2–50 µg/kg; QC3–250 µg/kg for FRJ. The stock solutions were stored in glass containers in darkness at −20 °C; working and matrix-matched solutions were stored in glass containers and kept in the dark at 4 °C. All solutions remained stable for over two weeks.

2.3. Sample procurement and treatment

2.3.1. Samples

Two different types of royal jelly-based products (FRJ and LDS) would be investigated in the present study. FRJ samples (n = 7) were obtained from local beekeepers or markets (Valladolid, Spain); meanwhile, LDS (n = 5), which contained freeze-dried royal jelly, fructose and water as main constituents, were also purchased in local markets. In this study, all royal jelly-based products were examined in triplicate, and also underwent a preliminary analysis by UHPLC–MS/MS, in order to check for the presence of neonicotinoids. Once absence of neonicotinoids pesticides was confirmed in several samples, subsamples of the corresponding samples were used as blank samples to prepare matrix-matched samples for validation and sample treatment studies. All samples were stored at 4 °C before analysis.

2.3.2. Sample treatment

2.3.2.1. Liquid dietary supplement. Briefly, 3.0 g of sample was diluted in 10 mL of ammonium formate (10 mM) in water and the resulting solution was loaded onto a Strata® X cartridge previously conditioned with 5 mL of methanol and 5 mL of water at about 1 mL/min by means of a suction system. After 5 min of drying time, the analytes were eluted with 2 mL of a methanol and ethyl acetate (70:30, *v/v*) mixture. The resulting solution was evaporated to dryness at 60 °C in a rotary evaporator; the dry residue was reconstituted with 1 mL of a methanol and water (80:20, *v/v*) mixture, filtered through a nylon 0.45-mm filter, and injected (5 µL) into the UHPLC–MS/MS system. Fig. 1 outlines the steps of the SPE procedure used during the present study.

2.3.2.2. Fresh royal jelly. Briefly, 100 mg of sample were weighed in a 10-mL round-bottom tube, after which 1 mL of acetonitrile (dispersive solvent) and 250 µL of chloroform (extraction solvent) were added. The