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 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.

Keywords: DLLME; Food analysis; Food composition; Food safety; Insecticides; Neonicotinoids; Royal jelly; SPE; UHPLC-MS/MS.

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, which is 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, 2017). 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 this 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 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 an 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 Gbmh (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 to Supelco (Bellefonte, PA, USA). Strata® X (6 mL with 200 mg of sorbent) and Strata® C18-E (3 mL with 500 mg of sorbent) cartridges (Phenomenex, Torrance, CA, USA), Isolute® HM-N diatomaceous earth packed (5 mL sample) cartridges (Biotage, Uppsala, Sweden), and a 10-port Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA), 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, USA), and ultrapure water was obtained using Milipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA).

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 -20ºC; working and matrix-matched solutions were stored in glass containers and kept in the dark at 4ºC. 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 4ºC before analysis.

2.3.2. Sample treatment

2.3.2.1. Liquid dietary supplement

Briefly, 3.0 g of sample was diluted with 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 60ºC 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. Figure 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 was 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 tube was then shaken for 30 s in a vortex device, following by 10 min of sonication at 40ºC in an ultrasound device, and 3 min of centrifugation at 5ºC and 2500 rpm. The lower layer (chloroform extract) was removed by using a syringe, and then the extraction procedure was repeated. After the second extraction, both chloroform extracts were combined and evaporated to dryness under a nitrogen stream. The dry extract was reconstituted with 100 µL of a methanol and water (80:20, v/v) mixture, and the resulting solution was passed through a nylon filter (0.45 µm). After which, a 5 µL aliquot was injected into the UHPLC-MS/MS system. Figure 1 outlines the dispersive liquid-liquid microextraction (DLLME) procedure used during the present study.

2.4. UHPLC-MS/MS system

An Acquity™ UHPLC system (ACQUITY, Waters, Milford, MA, USA) and a QTOF mass spectrometer (maXis impact, Bruker Daltonik, Bremen, Germany) were coupled through an electrospray (ESI) interface, which was operated in the positive mode ionization mode. The UHPLC instrument was equipped with a vacuum degasser, a binary solvent pump, an autosampler, and a thermostated column compartment. Data were acquired and processed with software Data Analysis 4.1 and Qualitative Analysis from Bruker Daltonik.

2.4.1. UHPLC conditions

A Kinetex® EVO fused-core type column (C_{18}, 50 × 2.1 mm, 1.7 µm, 100 Å) was employed for UHPLC analysis, and this was protected by a Kinetex® EVO C_{18} guard column. Both were acquired from Phenomenex. After optimization studies, the mobile phase composition and the flow rate, the injection volume and the column temperature were selected; mobile phase was composed of 0.1% (v/v) formic acid in acetonitrile (solvent A) and 0.1% (v/v) formic acid in water (solvent B) applied at a flow rate of 0.3 mL/min in the following gradient mode: (**i**) 0.0-1.5 min (**A–B**, 10:90, v/v); (**ii**) 1.5-2.5 min (**A–B**, 80:20, v/v); (**iii**) 2.5-3.5 min (**A–B**, 80:20, v/v); (i**v**) 3.5–4.0 min (A–B, 90:10, v/v); (**vi**) 4.0–4.5 min (A–B, 90:10, v/v); (**vii**) 4.5–5.0 min (A–B, 20:80, v/v); (**viii**) 5.0–6.0 min (A–B, 10:90, v/v); (**ix**) 6.0–8.0 min (A–B, 10:90, v/v). Injection volume and column temperature were set at 5 µL and 30ºC, respectively.

2.4.2. QTOF conditions

The optimal conditions were set as follows after several experiments (flow injection analysis in infusion mode, 80 µL/min) were conducted: capillary voltage, 4000 V; drying gas (nitrogen) flow, 12 L/min; drying gas (nitrogen) temperature, 250ºC; nebulizer pressure, 2 bars. Spectra were acquired in a mass range of mass/charge (*m/z*) 50–350. The *m/z* scale of the mass spectra was calibrated daily by infusing a sodium formate and

sodium acetate mixture. Compounds showed an intense $[M+H]^+$ (precursor ions) on their full-scan spectra, which was selected as a precursor to obtain product ions for MS/MS analyses, which were carried out by using an isolation width of 10 *m/z* and variable collision energies (10–30 eV; see Table 1). A window of ±0.01 *m/z* for the extracted ion chromatograms (EIC) was used in order to extract the exact mass.

2.5. Method Validation

In order to compare the proposed method with other procedures (Martínez-Domínguez et al., 2016), validation was in line with current European legislation (European Commission Directorate-General for Health and Food Safety, 2015) as well as with recent studies (Valverde et al., 2016). Moreover, several of the main elements of uncertainty (Konieczka and Namieśnik, 2010) were taken into account when optimizing and validating this method, such as the amount of sample used, the recovery value of the analytical procedure and precision (relative standard deviation (RSD) repeatability). Validation was performed with standard and matrix-matched solutions, which were treated according to the chosen procedures for each type of royal jelly (LDS-SPE; FRJ-DLLME). Finally, it should be noted that basic but efficient chemometric statistical tools from Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA, USA), Data Analysis 4.1 and Qualitative Analysis both from Bruker Daltonik, were employed to acquire, process and analyze the data in order to validate the method.

2.5.1. Selectivity

To determine the selectivity of the proposed method, a set of extracts from non-spiked samples (n=6) was injected onto the chromatographic system, and the results were compared with those obtained for spiked samples.

2.5.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) were experimentally determined by the injection of a number of blank samples (n=6), in which it was previously confirmed that there were no insecticide residues, and the magnitude of background analytical response at elution time in each sample for the two different types of royal jelly was measured. The LODs and LOQs were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively.

2.5.3. Matrix effect

In order to ascertain how the matrix influenced ESI ionization, a comparison was made of the results (analyte peak area/IS area) with standard working solutions and blank royal jelly samples spiked at three different concentrations (QC levels) following sample treatment (AF samples).

2.5.4. Linearity studies

Matrix-matched standard calibration curves were used to quantify DN, ACET and CLO, or DN, CLO and THIA neonicotinoid insecticides in LDS and FRJ, respectively. In contrast, the other neonicotinoid insecticides could be quantified with standard calibration curves in all other cases. Blank royal jelly was treated accordingly with the proposed procedure and spiked with variable amounts of the seven neonicotinoids over an analytical range between LOQ and 333 µg/kg (calibration levels of LOQ, 10, 25, 50, 83, 167, 333 µg/kg) and LOQ and 250 µg/kg (calibration levels of LOQ, 10, 25, 50, 100, 150, 250 µg/kg) for matrix-matched calibration curves for LDS and FRJ, respectively. The analytical ranges prepared for the standard calibration curves were between LOQ and 1000 µg/L (calibration levels of LOQ, 30, 75, 150, 300, 600, 1000 µg/L) for LDS and LOQ and 250 µg/L (calibration levels of LOQ, 10, 25, 50, 100, 150, 250 µg/L) for FRJ. Neonicotinoid concentrations were the same in the standard (µg/L) and matrix-matched (µg/kg) solutions, in line with the proposed sample treatment and

unit conversion. Calibration curves (n = 6) were constructed by plotting the signal on the *y*-axis (analyte peak area/IS area) against the analyte concentration on the *x*-axis.

2.5.5. Precision

Intra-day precision experiments were performed concurrently by repeated sample analysis using blank royal jelly samples spiked before sample treatment (BF samples) at three different concentrations (low, medium and high QC levels), either on the same day of (n=6) (intra-day precision experiments), or over three consecutive days (n=6) (inter-day precision).

2.5.6. Trueness

This was evaluated by the mean recoveries (as a measure of trueness), calculated by comparing the results (analyte peak area/IS area) obtained from blank royal jelly samples spiked at three different concentrations (low, medium and high QC levels), either prior to (BF samples) or following (AF samples) sample treatment.

3. Results and discussion

3.1. Optimization of the sample treatment

3.1.1. Liquid dietary supplements

Firstly, QuEChERS methodology was examined, since we have recently obtained good results in terms of extraction efficiency and influence of the matrix effect when analyzing neonicotinoids in a different bee matrix (bee pollen) (Valverde et al. 2016). After performing some preliminary experiments and selecting the optimal QuEChERS conditions (data not shown), recoveries ranged in most cases between 70% and 93%, although for NT and DN they were slightly lower (~ 60%). However, it was also observed that an interface was formed following centrifugation, which made it difficult to collect the supernatant and subsequently affected the reproducibility of the results. Several attempts

were made to remove this interface by varying the centrifugation conditions and adding aluminium salts, yet despite this it remained. Consequently, we opted for an SPE procedure, as this has provided satisfactory results in previous studies with other pesticides in royal jelly (see Introduction) and, from our experience, it has successfully been used to analyze these compounds in other bee matrices (honey and beeswax; Sánchez-Hernández, et al., 2016; Yáñez et al., 2013). Firstly, the type of cartridge that would be used for SPE was determined. As a result of the physicochemical properties of neonicotinoids and our research experience (Sánchez-Hernández et al., 2016; Yáñez et al., 2013), we examined the suitability of polymeric (Strata® X) and diatomaceous material (Isolute® HMN). Following several experiments, it was observed that the polymeric sorbent (Strata® X) provided highest recoveries in most cases (see Supplementary Material, Figure 1S), as well as slightly cleaner chromatograms in all cases. In addition, although diatomaceous based cartridges do not require as many steps as Strata® X, overall organic solvent consumption is much higher. We therefore chose the polymeric sorbent to optimize extraction. Next, the sample amount (1-5 g), solvent (water; ammonium formate (10 mM) in water; ammonium hydroxide 1% (v/v) in water; formic acid 1% (v/v) in water), and solvent volume (5-15 mL) were selected. These were chosen on the basis of preliminary experiments. After several tests, 3.0 g of LDS and 10 mL of ammonium formate (10 mM) in water were deemed the optimal values, as in this way the highest signal to noise (S/N) ratio for securing maximum sensitivity was obtained (data not shown). Prior to the diluted sample being loaded onto the SPE cartridges, certain parameters were evaluated for optimal extraction procedure. First of all, different amounts of methanol and water were tested in order to precondition the cartridge; 5 mL of both applied sequentially was the most suitable amount. However, as royal jelly is a complex matrix that contains several substances, direct elution of the cartridges usually resulted in matrix interference and

unclean chromatograms, and consequently a washing stage to avoid such problems was usually required. Several water and methanol mixtures (100:0, 90:10, 80:20, 70:30, 50:50, v/v) and amounts (5-15 mL) were tested for this purpose, as these have provided good results in previous studies (Sánchez-Hernández et al., 2016). It was found that in all cases two of the neonicotinoids (DN and NT) were lost during washing, whilst no significant improvement was observed in the matrix effect or in the removal of the interferences (data not shown). Thus, it was decided that the washing step could be eliminated from the SPE procedure. Optimal drying times for the cartridges were also determined and, as no differences were observed between times of 5-20 min, a 5-minute drying period was chosen to avoid delays in the extraction procedure. Different mixtures of acetonitrile or methanol with water or ethyl acetate (100:0, 80:20, 70:30, 50:50; v/v) were assayed to elute neonicotinoids from the cartridges. The best overall results in terms of extraction efficiency were obtained when a methanol and ethyl acetate (70:30, v/v) mixture was employed (see Supplementary Material, Figure 2S). Following testing of the elution amounts (ranging from 1.0-5.0 mL), it was also found that 2 mL of the selected mixture was appropriate for obtaining satisfactory recoveries (> 85%). The solution was transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 60ºC. No loss of neonicotinoids was observed during the evaporation step. Different amounts (0.5-2.0 mL) of a methanol and water (80:20, v/v) mixture, which were selected on the basis of the good results obtained in previous research (Valverde et al., 2016), were assayed in order to obtain the best results. Since it was observed that amounts of solvent in excess of 1 mL did not improve the recovery percentages, 1 mL of the mixture was deemed appropriate to reconstitute the dry residue.

3.1.2. Fresh royal jelly

As a result of the good results obtained with polymeric SPE cartridges for neonicotinoid extraction in LDS, we decided to test them with FRJ. However, once the sample was mixed with ammonium formate (10 mM) in water, a viscous solution was obtained which caused the obstruction of the cartridges when the sample was loaded onto them. Larger amounts of the ammonium formate solution were tested (20 and 30 mL), but the problem remained. Therefore, some tests were conducted with lower sample weights (0.25-2.50 g), yet once again it was not possible to solve the problem. Consequently, the SPE procedure was discarded and the suitability of the QuEChERS approach was examined as the sample treatment for this matrix. Some preliminary experiments were carried out, and after the centrifugation stage an interface was also formed, which in this case was even more dense than in the LDS. Several tests (solvent amounts, sample weights, extraction and centrifugation times) were performed with the aim of removing this interface, but the results were unsuccessful in all cases; as a result, the use of QuEChERS was discarded. Therefore, it was necessary to find an alternative procedure to the one most commonly adopted as sample treatment for determining neonicotinoids in FRJ. A new literature survey was, then, undertaken, and a recent study was found in which neonicotinoid insecticides were successfully determined in a similar matrix (honey liqueur) by means of dispersive liquid-liquid microextraction (DLLME) (Jovanov et al., 2014). Thus, we decided to check the performance of DLLME as the sample treatment. For optimal conditions a series of parameters that affected DLLME efficiency (e.g. sample amount, type and amount of extraction and dispersive solvents, time of vortex mixing, centrifugation and soaking in the ultrasonic bath; Jovanov et al., 2013) were established. Firstly, the amount of FRJ was selected. After varying the sample amount from 50 to 500 mg, 100 mg was chosen for further investigations because there were no significant differences between the final recoveries and the S/N ratios for higher amounts of the

sample, whilst it was easier to handle smaller amounts throughout sample treatment. Next, an appropriate extracting solvent, the major parameter for the DLLME process, was chosen. Three different extraction solvents were investigated: acetone, chloroform and dichloromethane, chloroform displayed better overall extraction recovery values (> 70%) than those obtained with the other solvents (see Supplementary Material, Figure 3S). Following this, the dispersive solvent was selected. Three different solvents (acetonitrile, methanol and ethanol) were examined, and the best results in terms of extraction efficiency were obtained with acetonitrile (see Supplementary Material, Figure 4S). Once the extraction and dispersive solvents had been selected, the influence of their corresponding amounts on the recovery percentages was studied. The amounts varied from 0.05 to 1.0 mL (0.05, 0.1, 0.5, 1.0 mL; extraction solvent) and from 1.0 to 2.5 mL (1.0, 1.5, 2.0, 2.5 mL; dispersive solvent) for optimal sample recovery. Extraction recovery increased for all the neonicotinoids with the increase of the extraction and dispersive solvents by up to 0.5 mL and 2.0 mL, respectively; a further increase, meanwhile, did not significantly affect the extraction recoveries (data not shown). Subsequently, the influence of certain extraction parameters, such as agitation source (vortex and/or ultrasound) and extraction time (5-20 min) were sequentially tested in order to obtain the optimal conditions. Following extraction, the mixture was centrifuged at 2500 rpm and 5ºC for 5 minutes. The results showed that the best recovery percentages (> 75%) were obtained with 30 seconds of vortex agitation, followed by 10 minutes in an ultrasound bath at 40ºC (data not shown). Finally, we considered whether the number of extractions (one or two) would have an influence on extraction efficiency. The results obtained with the above-mentioned procedure were compared with those following an initial extraction with 0.25 mL of chloroform and 1.0 mL of acetonitrile; subsequent to this, the lower layer (chloroform extract) was removed with a syringe and the extraction procedure was repeated with new

volumes of extraction (0.25 mL) and dispersive (1.0 mL) solvents. It was found that extraction recoveries were significantly better with two extractions rather than one (see Supplementary Material, Figure 5S). The chloroform extracts obtained in both extractions were mixed and evaporated to dryness under a nitrogen stream. Different amounts (50-200 μL) of a methanol and water (80:20, v/v) mixture were tested to dissolve the dry extract, and 100 μL was seen to be sufficient to achieve satisfactory results. Finally, the suitability of the DLLME procedure for LDS analysis was examined, but this was not appropriate since an emulsion was formed when the extraction (chloroform) and dispersive (acetonitrile) solvents were added, making it impossible to collect the chloroform extract in a proper way.

3.1.3. Comparison of the proposed sample treatments

In order to check the effectiveness of the proposed sample treatments, insecticide responses, which were the peak areas (analyte peak area/IS area) obtained from blank samples spiked at three different neonicotinoid concentrations (QC levels), either prior to (BF samples) or following (AF samples) sample treatment, were compared. Recovery values ranged from 83%-107% when the SPE approach for LDS was employed and 90%-109% in the case of DLLME for FRJ (see Table 2). The recovery values indicated that the sample treatment procedures selected were appropriate and efficient for all the concentrations and the two types of royal jelly products assayed. These results have demonstrated that the proposed procedures are an efficient, shorter, and greener alternative to the existing procedure for analyzing these pesticides in royal jelly, since the recovery values (83-109%), overall procedure time (< 30 minutes), and volume/amount of reagents, especially organic solvents (SPE-8 mL; DLLME-2.6 mL), are comparable with or better than the reported values (70%-152%; > 2 hours; 7 mL; Martínez-Domínguez et al., 2016). Finally, it can be concluded that different

methodologies should be employed as sample treatments according to the nature of the royal jelly-based products, as it has been demonstrated that the sample treatments proposed for each of the royal jelly products could not be employed with the other product (see subsection 3.1.2).

3.2. UHPLC-MS/MS optimization

3.2.1. UHPLC

We decided to optimize the separation of the insecticides in royal jelly with a Kinetex® EVO (C_{18}, 50 x 2.1 mm, 1.7 µm, 100 Å) column and 0.1% (v/v) formic acid in ACN and 0.1% (v/v) formic acid as mobile phase components due to the good performance showed in the analysis of neonicotinoid insecticides in bee pollen (Valverde et al, 2016). Several experiments were conducted in which standard and matrix matched solutions were injected with diverse mobile phases and flow rates so as to elute neonicotinoids rapidly whilst preventing co-elution (data not shown). The shortest analysis time was obtained with the chromatographic conditions described in subsection 2.4.1. With such conditions the overall run time was 8.0 min, which, to our knowledge, is the fastest proposal that has been published in relation to neonicotinoid analysis in royal jelly, eluting the last of the insecticides in less than 3.5 min (see Figures 2 and 3).

3.2.2. MS/MS

Regarding optimization of the QTOF conditions, ESI in positive mode was chosen to conduct the experiments as a result of our previous experience (Valverde et al., 2016). To establish the optimal MS/MS conditions, several experiments (flow injection analysis) were conducted in order to choose the optimum parameters and achieve the maximum sensitivity by the infusion mode of standard and matrix matched solutions (see subsection 2.4.2 and Table 1). Neonicotinoids showed an intense $[M+H]^+$ (precursor ions) on their

full-scan spectra, which were were selected as precursor ions to obtain product ions for MS/MS analyses (see Table 1), and also as confirmation ions. The product ions with the highest signals were used for quantification; meanwhile, the second products ions with the higher signals were used for confirmation (see Table 1).

3.3. Method validation

3.3.1. Selectivity

No chromatographic interference was observed at analyte retention times in any of the blank samples analysed of the two types of royal jelly-based products (see Figures 2 and 3). For identification of neonicotinoid peaks in spiked samples, their mass spectra in standard solutions and spiked samples were compared; the concentrations were similar and the same conditions were employed for measurement. There was a considerable similarity between both types of mass spectra. However, slight differences in the intensity of several ions were observed and certain low intensity ions appeared in some cases, especially in FRJ, as can be observed in the mass spectra obtained for TMX, CLO and IMI (see Supplementary Material, Figure 6S).

3.3.2. Limits of detection and quantification

As can be seen in Table 3, low LODs and LOQs were obtained in all cases, ranging LODs from 0.8 to 2.4 µg/kg (LDS) and 1.1 to 3.0µg/kg (FRJ); meanwhile, the LOQs varied between 2.5 and 8.0µg/kg (LDS) or 3.7 to 9.4 µg/kg (FRJ). It should be highlighted that these LOQ values are much lower than the MRLs established by the European Commission for some of these pesticides in honey and other apiculture products (50-200 µg/kg; European Union Pesticide Database, 2016), and also significantly lower than LOQs previously reported (5-20 µg/kg; Martínez-Domínguez et al., 2016).

3.3.3. Matrix effect

Certain differences were observed in neonicotinoid responses at the three concentrations (QC levels) assayed for each type of royal jelly product (see Table 2). It was seen that DN, CLO and ACET displayed responses lower than 80% in some LDS analyses, while DN, CLO and THIA represented the neonicotinoids with lower responses (< 80%) in FRJ samples. In addition, significant differences (+15%) in the responses were observed for certain compounds (NT, CLO, ACET and THIA) in accordance with the type of sample. To confirm these findings the slopes of the standard and matrix-matched calibration curves were compared (see Table 3), and overlapping was seen to occur at the confidence intervals for NT, TMX, IMI and THIA in LDS samples, and for NT, TMX, IMI and ACET in FRJ samples. However, this was not case for DN and CLO in both types of samples, and ACET or THIA in LDS and FRJ samples, respectively. As can be seen, these findings matched perfectly with the responses summarized in Table 2. It was, therefore, concluded that the matrix did not significantly affect the MS/MS signal of NT, TMX, IMI, ACET (FRJ) or THIA (LDS), results which complied with the criteria of the European Commission for pesticide residue analysis (± 20% of the response from standard solutions; SANTE, 2015). Meanwhile, a significant matrix effect (signal suppression) was observed for some of the compounds in both kinds of royal jelly samples (DN, CLO, ACET-LDS; THIA-FRJ). This is an important result because a significant matrix effect has been reported in the only previous publication, in which these compounds were determined in royal jelly (Martínez-Domínguez et al., 2016). In addition, it has also been corroborated the need to evaluate the matrix effect prior to quantification of different royal jelly-based products, which is frequently ignored, in order to correctly quantify the samples.

3.3.4. Linearity studies

As mentioned in subsection 2.5.5, different calibration curves were used to quantify neonicotinoids in accordance with the royal jelly-based product. Matrix-matched standard calibration curves should be used to quantify DN, ACET and CLO, or DN, CLO and THIA neonicotinoid insecticides in LDS and FRJ, respectively, as matrix interference affected analyte ionization, causing a suppression of the MS/MS signal (> 20%; see Table 2), while the slopes of the standard and matrix-matched calibration curves did not overlap at the confidence intervals (see Table 3). Meanwhile, standard calibration curves could be employed in all other cases, as no significant matrix effect was observed (see subsection 3.3.3). (see Table 2). Moreover, the graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied; the coefficient of the determination values (R^2) was above 0.99 in all cases (see Table 3). Finally, the lack of bias was confirmed by a *t* test and a study of the distribution of residuals (data not shown).

3.3.5. Precision

Precision, which was expressed as %RSD, was at all times (intra- and inter-day experiments) lower than 10% (see Supplementary Material, Table 1S), which is below the limits established by existing regulations (%RSD ≤ 20; SANTE, 2015) and the values reported in the only study in which neonicotinoids were determined in royal jelly (< 25%) (Martínez-Domínguez et al., 2016).

3.3.6. Trueness

Mean recoveries (as a measure of trueness), which were calculated as described in subsection 3.1.3, ranged from 83% to 109% with %RSD values lower than 10% in all cases (see Table 2). These values fulfilled the requirements established by the European Commission (SANTE, 2015) for pesticide residue analysis (recovery percentages

between 70% and 120% and %RSD ≤ 20), and are similar to or better than the recoveries obtained in previous works (Martínez-Domínguez et al., 2016).

3.4. Application of the method

The validated method was applied to determine potential residues of neonicotinoids in FRJ and LDS samples (see subsection 2.3.1). All of these were analyzed in triplicate, and the IS was added at the same concentration as in the matrix-matched samples. No residues of the insecticides under study were detected in any of the samples. This does not, however, mean that the applicability of the method would be limited, since international organizations such as the European Commission have already established MRLs for these compounds in honey and related matrices. As a result, it is reasonable to suppose that neonicotinoid residues could appear in royal jelly samples from different regions/countries, and, therefore, it is of great importance the development of sensitive and selective methods to detect such pesticides in this matrix on account of the low concentrations expected.

4. Conclusions

Different analytical methods to simultaneously identify and quantify seven neonicotinoids in royal jelly-based products (FRS and a LDS) have been developed, optimized and validated with a view to proposing the most appropriate methodology. The extraction methods suggested, based on SPE (LDS) and DLLME (FRJ) procedures, have proven to be rapid (< 30 min), efficient (recoveries between 83% and 109%) and to require a low consumption of organic solvents, especially DLLME (< 3 mL), as this is recommended according to the principles of green analytical chemistry. It should also be mentioned that both procedures were much faster, and with a similar (SPE) or

significantly lower (DLLME) consumption of organic solvents in comparison with the only previous proposal. UHPLC separation of the insecticides was achieved with a core-shell technology-based column (Kinetex® EVO) in 8 minutes, which is the fastest proposal that has been published in relation to neonicotinoid analysis in royal jelly. Both methods were fully validated and the data demonstrated that they are consistent, reliable and have a wide linear range of applicability. It was necessary to employ matrix-matched standards for correct ESI-MS/MS quantification of three of the compounds in each of the royal jelly-based products (DN, CLO, ACET (LDS) and THIA (FRJ)), due to the significant signal suppression observed. It should be mentioned that the LODs and LOQs obtained with the proposed method are much lower than the MRLs established by the European Commission for honey and other apiculture products, and than the values proposed in the only previous study in which these compounds were determined in royal jelly. Moreover, several FRJ and LDS samples were analyzed with the proposed sample treatments. No residues of the insecticides under study were detected in any of those samples. In conclusion, the proposed method offers an innovative means for determining neonicotinoid residues at trace level in a complex matrix such as royal jelly. Moreover, we have also demonstrated that those methods should be specifically developed and optimized for the various royal jelly-based products, as the sample treatments proposed for each of the royal jelly products could not be employed with the other product due their different composition.

Non-standard 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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Declaration of interest: none

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Figure captions

Figure 1.- Analytical procedures work-up flow charts: (**A**) dispersive liquid-liquid microextraction (DLLME); (**B**) solid phase extraction (SPE).

Figure 2.- Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) chromatograms (extracted ion chromatogram (EIC) in positive mode using the quantification ions; see Table 1) obtained from: (**A**) non-spiked fresh royal jelly (FRJ) samples; (**B**) spiked (50 µg/kg) FRJ samples. The UHPLC-MS/MS conditions are summarized in subsection 2.4 and Table 1.

Figure 3.- Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) chromatograms (extracted ion chromatogram (EIC) in positive mode using the quantification ions; see Table 1) obtained from: (**A**) non-spiked liquid dietary supplement (LDS) samples; (**B**) spiked (83 µg/kg) LDS samples. The UHPLC-MS/MS conditions are summarized in subsection 2.4 and Table 1.

Figure 1

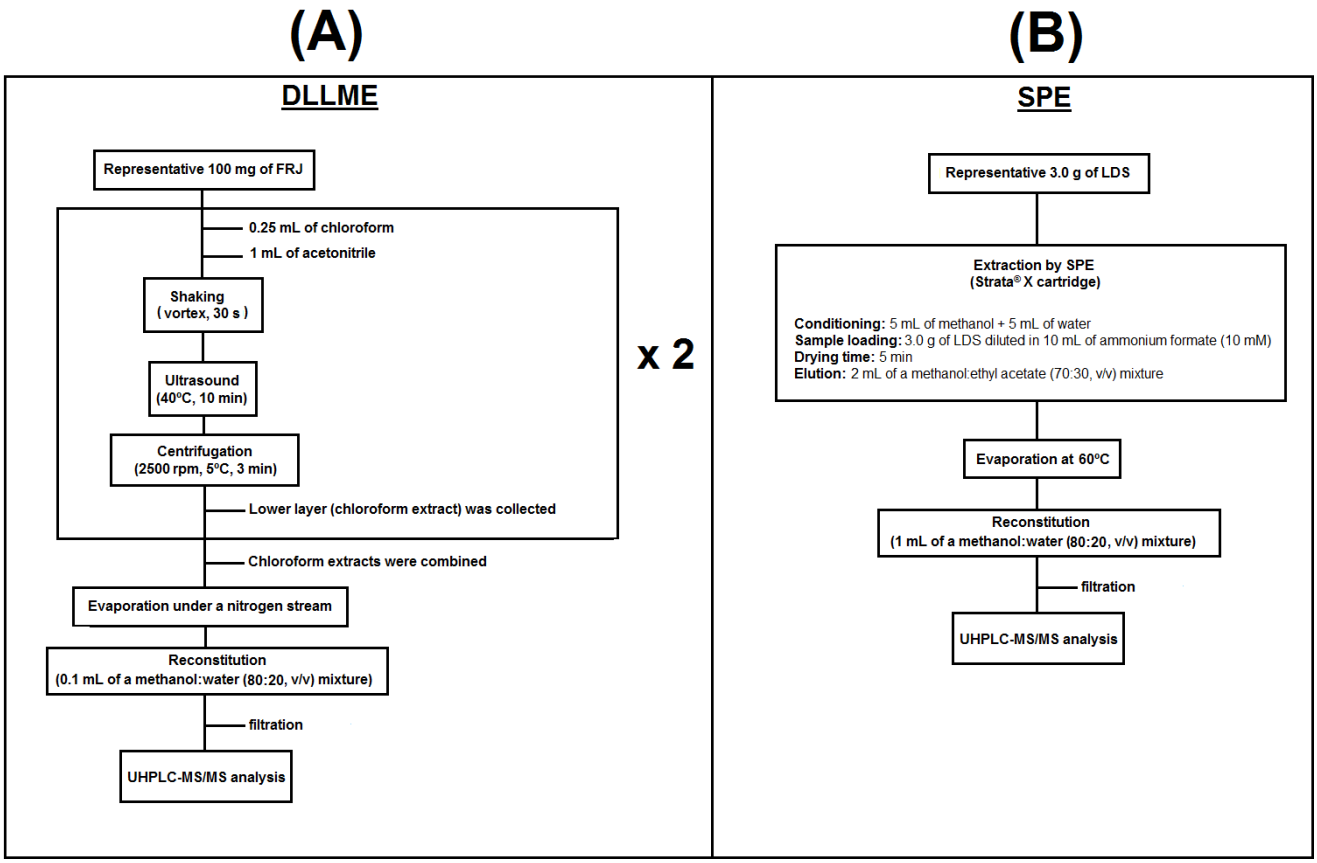
(A)
(B)
DLLME
SPE
Representative 100 mg of FRJ
0.25 mL of chloroform
1 mL of acetonitrile
Shaking
(vortex, 30 s)
Ultrasound
(40ºC, 10 min)
Centrifugation
(2500 rpm, 5ºC, 3 min)
Lower layer (chloroform extract) was collected
x 2
Chloroform extracts were combined
Evaporation under a nitrogen stream
Reconstitution
(0.1 mL of a methanol:water (80:20, v/v) mixture)
filtration
UHPLC-MS/MS analysis
Representative 3.0 g of LDS
Extraction by SPE
(Strata® X cartridge)
Conditioning: 5 mL of methanol + 5 mL of water
Sample loading: 3.0 g of LDS diluted in 10 mL of ammonium formate (10 mM)
Drying time: 5 min
Elution: 2 mL of a methanol:ethyl acetate (70:30, v/v) mixture
Evaporation at 60ºC
Reconstitution
(1 mL of a methanol:water (80:20, v/v) mixture)
filtration
UHPLC-MS/MS analysis

Figure 2

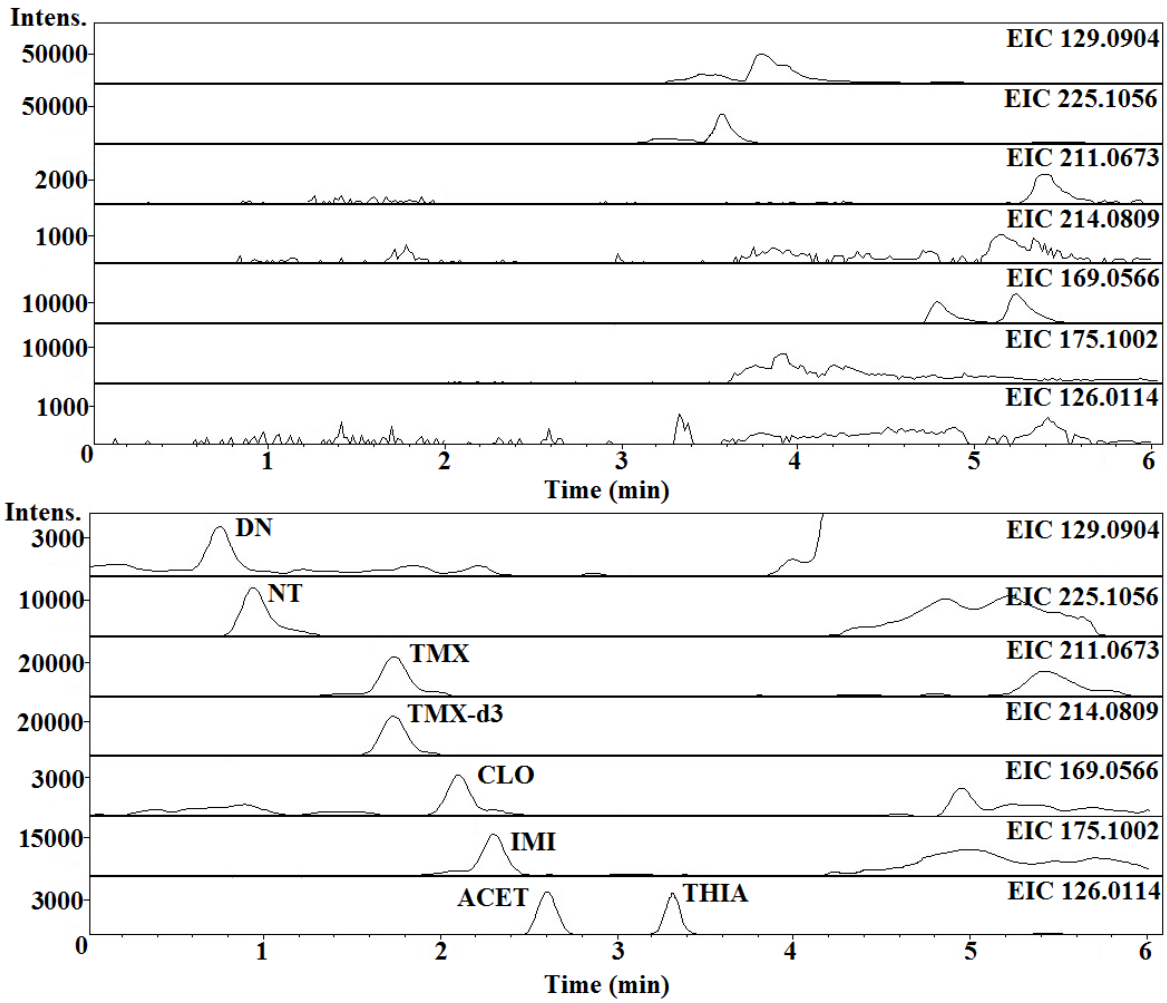
Intens.
50000
EIC 129.0904
50000
EIC 225.1056
2000
EIC 211.0673
1000
EIC 214.0809
10000
EIC 169.0566
10000
EIC 175.1002
1000
EIC 126.0114
0
1
2
3
4
5
6
Time (min)
Intens.
3000
DN
EIC 129.0904
10000
NT
EIC 225.1056
20000
TMX
EIC 211.0673
20000
TMX-d3
EIC 214.0809
3000
CLO
EIC 169.0566
15000
IMI
EIC 175.1002
3000
ACET
THIA
EIC 126.0114
0
1
2
3
4
5
6
Time (min)

Figure 3

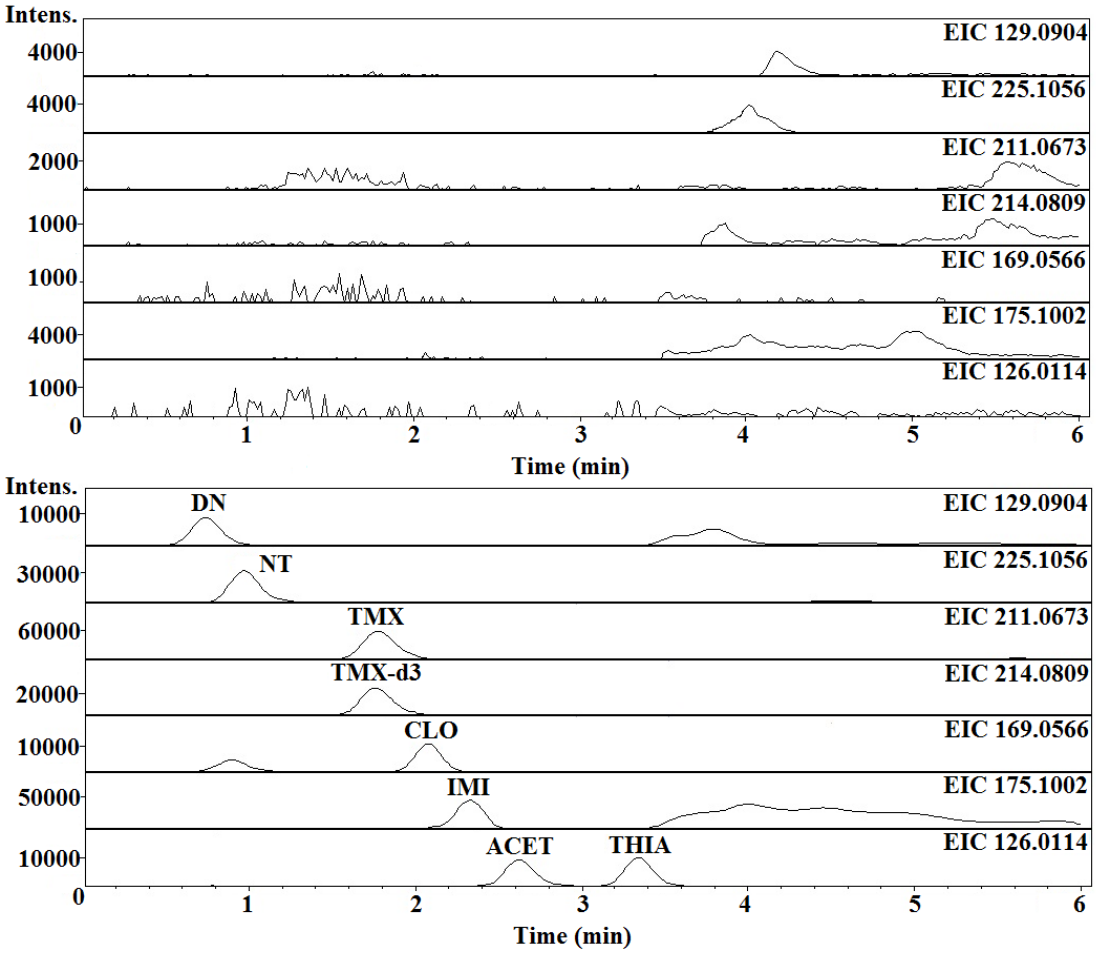
Intens.
4000
4000
2000
1000
1000
4000
1000
0
EIC 129.0904
EIC 225.1056
EIC 211.0673
EIC 214.0809
EIC 169.0566
EIC 175.1002
EIC 126.0114
1
2
3
4
5
6
Time (min)
Intens.
10000
30000
60000
20000
10000
50000
10000
0
DN
NT
TMX
TMX-d3
CLO
IMI
ACET
THIA
EIC 129.0904
EIC 225.1056
EIC 211.0673
EIC 214.0809
EIC 169.0566
EIC 175.1002
EIC 126.0114
1
2
3
4
5
6
Time (min)

Table 1.-Specific QTOF parameters employed for each of the neonicotinoid insecticides.

Compound	Precursor ions (*m/z*)	Product ions (*m/z*)	CE (*eV*)
DN	203.1163[A]	113.1039[A]	15
		129.0908[B]	15
NT	271.0988[A]	99.0920[A]	15
		225.1059[B]	15
TMX	292.0215[A]	131.9621[A]	15
		211.0604[B]	15
TMX-d3 (IS)	295.0396[A]	131.9675[A]	15
		214.0687[B]	15
CLO	250.0166[A]	131.9622[A]	15
		169.0495[B]	15
IMI	256.0537[A]	175.0932[B]	25
		209.0542[A]	25
ACET	223.0780[A]	56.1002[A]	30
		126.0117[B]	25
THIA	253.0342[A]	126.0118[B]	20
		186.0154 [A]	20

[A]Confirmation ions; [B]Quantification ions; **CE**, collision energy

Table 2.- Evaluation of the efficiency (recoveries) of the sample treatment and the matrix effect (comparison of responses). Data obtained as described in subsections 2.5, 3.1 and 3.3 (n = 6).

	Liquid dietary supplement (LDS)						**Fresh royal jelly (FRJ)**					
	Evaluation of the sample treatment			**Evaluation of the matrix effect**			**Evaluation of the sample treatment**			**Evaluation of the matrix effect**		
	Mean (%) ± RSD (%)			**Mean (%) ± RSD (%)**			**Mean (%) ± RSD (%)**			**Mean (%) ± RSD (%)**		
	QC1	**QC2**	**QC3**	**QC1**	**QC2**	**QC3**	**QC1**	**QC2**	**QC3**	**QC1**	**QC2**	**QC3**
DN	103 ± 2	97 ± 4	94 ± 2	68 ± 3	75 ± 4	71 ± 2	107 ± 5	100 ± 3	104 ± 2	54 ± 4	62 ± 5	57 ± 3
NT	109 ± 5	99 ± 2	104 ± 4	80 ± 4	84 ± 5	82 ± 5	91 ± 4	90 ± 4	86 ± 5	94 ± 4	101 ± 2	98 ± 4
TMX	107 ± 2	102 ± 3	98 ± 2	97 ± 2	104 ± 3	99 ± 2	83 ± 5	91 ± 2	88 ± 5	97 ± 5	98 ± 3	104 ± 3
CLO	101 ± 3	92 ± 4	97 ± 6	62 ± 5	68 ± 3	70 ± 6	100 ± 3	106 ± 7	103 ± 5	53 ± 4	48 ± 6	45 ± 7
IMI	101 ± 3	97 ± 4	95 ± 2	101 ± 4	96 ± 3	97 ± 5	92 ± 2	85 ± 5	90 ± 4	92 ± 4	95 ± 4	102 ± 5
ACET	108 ± 3	105 ± 2	106 ± 2	74 ± 4	78 ± 2	71 ± 2	103 ± 3	105 ± 3	100 ± 4	96 ± 5	89 ± 3	90 ± 6
THIA	94 ± 2	92 ± 5	90 ± 3	90 ± 4	87 ± 5	92 ± 3	106 ± 2	103 ± 2	99 ± 5	70 ± 4	71 ± 5	74 ± 3

LDS: QC1-LOQ (see Table 3); **QC2**- 83 µg/kg; **QC3**-333 µg/kg.

FRJ: QC1-LOQ (see Table 3); **QC2**-50 µg/kg; **QC3**-250 µg/kg.

Table 3.- Calibration curve data (n = 6), LOD and LOQ values obtained for neonicotinoid insecticides in royal jelly-based products.

		Liquid dietary supplement					Fresh royal jelly				
Compound	**Calibration curve**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**	**Analytical range[A]**	**Slope confidence intervals**	**R^2**	**LOD[B]**	**LOQ[B]**
DN	Standard	7.5-333	$1.5\times10^{-3} \pm 1.0\times10^{-4}$	0.996	2.4	7.5	9.4-250	$1.3\times10^{-3} \pm 1.0\times10^{-4}$	0.992	3.0	9.4
	Matrix-matched		$1.1\times10^{-3} \pm 6.8\times10^{-4}$	0.995				$7.8\times10^{-4} \pm 3.7\times10^{-5}$	0.991		
NT	Standard	3.7-333	$5.7\times10^{-3} \pm 9.6\times10^{-4}$	0.999	1.1	3.7	5.4-250	$5.8\times10^{-3} \pm 9.6\times10^{-5}$	0.999	1.6	5.4
	Matrix-matched		$4.7\times10^{-3} \pm 3.1\times10^{-4}$	0.999				$5.6\times10^{-3} \pm 4.4\times10^{-4}$	0.994		
TMX	Standard	4.0-333	$4.9\times10^{-3} \pm 1.1\times10^{-4}$	0.999	1.2	4.0	1.8-250	$5.1\times10^{-3} \pm 1.1\times10^{-4}$	0.997	1.8	6.0
	Matrix-matched		$4.8\times10^{-3} \pm 6.1\times10^{-4}$	0.999				$5.2\times10^{-3} \pm 1.2\times10^{-4}$	0.998		
CLO	Standard	4.8-333	$2.4\times10^{-3} \pm 3.3\times10^{-5}$	0.999	1.4	4.8	7.0-250	$1.4\times10^{-3} \pm 4.6\times10^{-5}$	0.996	2.2	7.0
	Matrix-matched		$1.6\times10^{-3} \pm 4.0\text{x}10^{-5}$	0.996				$6.6\times10^{-4} \pm 6.9\times10^{-5}$	0.991		
IMI	Standard	4.4-333	$2.9\times10^{-3} \pm 5.2\times10^{-4}$	0.999	1.3	4.4	4.5-250	$3.5\times10^{-3} \pm 0.3\times10^{1}$	0.999	1.4	4.5
	Matrix-matched		$2.8\times10^{-3} \pm 3.6\times10^{-4}$	0.997				$3.1\times10^{-3} \pm 2.9\times10^{-5}$	0.991		
ACET	Standard	2.5-333	$3.3\times10^{-3} \pm 8.6\times10^{-5}$	0.998	0.8	2.5	3.7-250	$3.5\times10^{-3} \pm 3.1\times10^{-5}$	0.999	1.1	3.7
	Matrix-matched		$2.5\times10^{-3} \pm 1.3\times10^{-6}$	0.991				$3.2\times10^{-3} \pm 1.8\times10^{-4}$	0.995		
THIA	Standard	4.3-333	$6.4\times10^{-3} \pm 2.4\times10^{-4}$	0.999	1.3	4.3	7.3-250	$8.4\times10^{-3} \pm 4.1\times10^{-4}$	0.999	2.3	7.3
	Matrix-matched		$5.7\times10^{-3} \pm 6.6\times10^{-4}$	0.996				$6.1\times10^{-3} \pm 1.9\times10^{-3}$	0.991		

[A]Neonicotinoid concentrations (µg/kg) were same in the standard and matrix-matched samples according to the proposed sample treatment and the unit conversion.

[B]LOD and LOQ values were calculated in matrix (µg/kg).

Electronic Supplementary Material

DEVELOPMENT AND VALIDATION OF UHPLC-MS/MS METHODS FOR DETERMINATION OF NEONICOTINOID INSECTICIDES IN ROYAL JELLY-BASED PRODUCTS

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Table 1S.- Summary of precision studies for the neonicotinoid determination in spiked blank royal jelly samples (n = 6).

	Liquid dietary supplement (LDS)						Fresh royal jelly (FRJ)					
	Intraday precision (%RSD)			Interday precision (%RSD)			Intraday precision (%RSD)			Interday precision (%RSD)		
	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3
DN	6	7	5	7	9	8	5	6	4	9	9	8
NT	7	4	4	9	7	5	5	4	5	8	8	6
TMX	8	6	5	9	5	6	4	5	7	7	6	8
CLO	6	6	7	8	5	7	7	8	9	9	8	9
IMI	2	4	6	5	7	8	6	5	7	8	6	8
ACET	3	2	7	8	6	9	5	4	5	7	7	9
THIA	2	6	3	5	7	6	4	7	5	6	8	9

LDS: QC1-LOQ (see Table 3); **QC2**- 83 µg/kg; **QC3**-333 µg/kg.

FRJ: QC1-LOQ (see Table 3); **QC2**-50 µg/kg; **QC3**-250 µg/kg.

Figure 1S.- Evaluation of the extraction efficiency (recoveries) obtained for LDS spiked at QC2 (83 µg/kg) after testing different SPE sorbents (Strata® X and Isolute® HMN). Data represent the mean ± the relative standard deviation of the mean (narrow bars; n = 3).

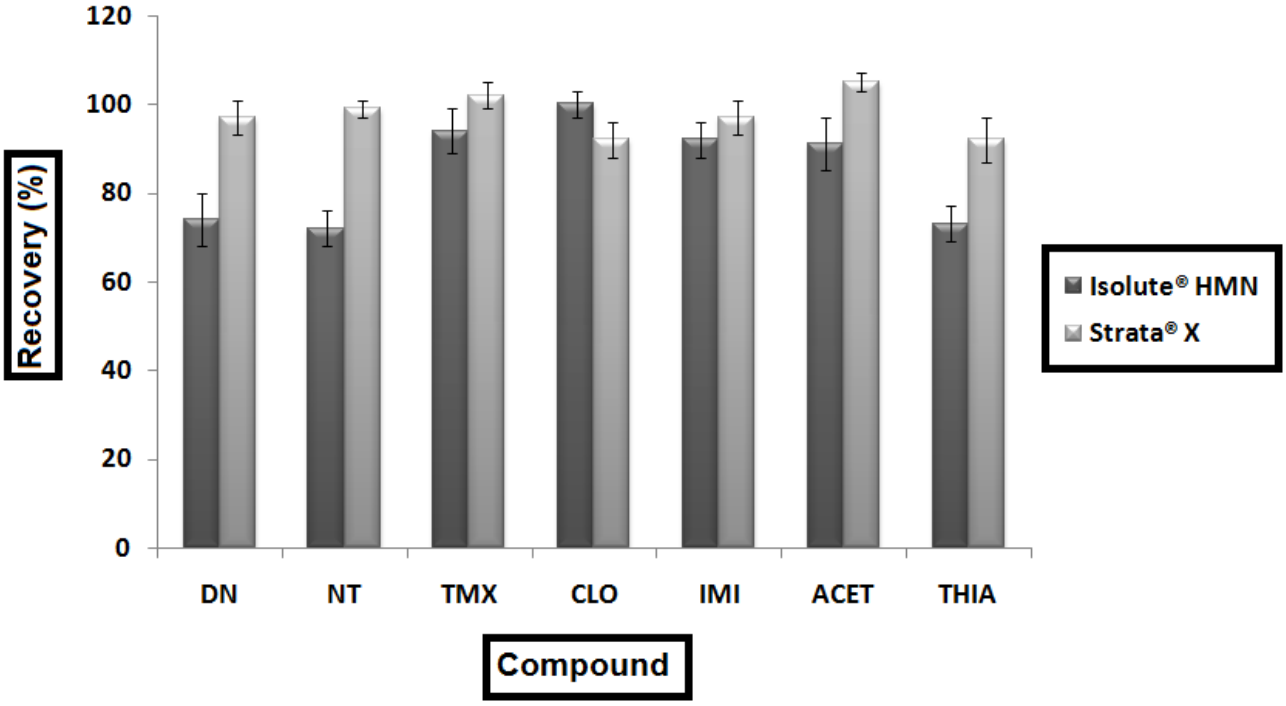


Figure 2S.- Evaluation of the extraction efficiency (recoveries) obtained for LDS spiked at QC2 (83 µg/kg) after testing with the elution solvents that provided the best results. Data represent the mean ± the relative standard deviation of the mean (narrow bars; n = 3) obtained with 2 mL of the (70:30, v/v) methanol and ethyl acetate mixture and 5 mL of the other solvents.

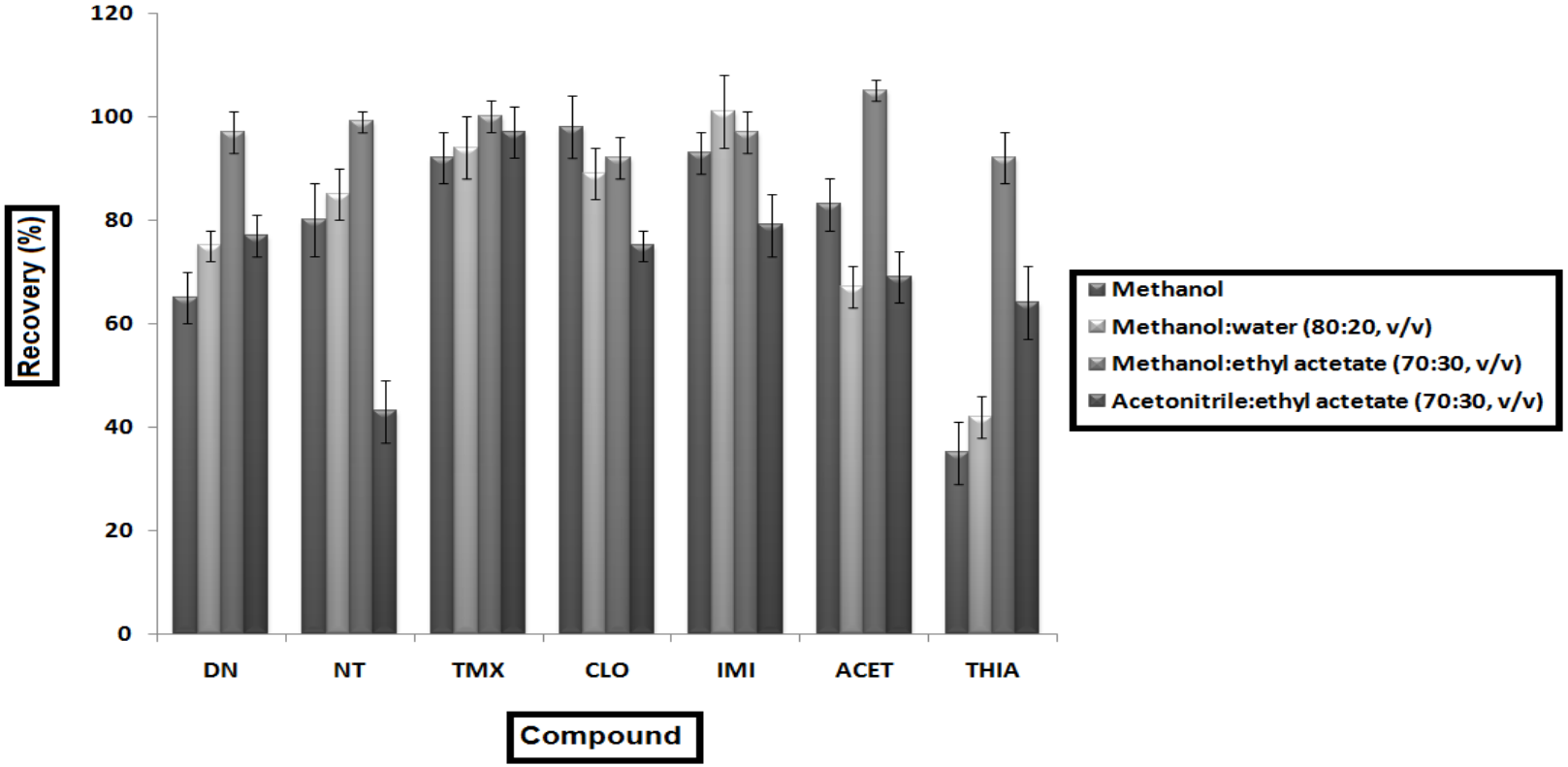


Figure 3S.- Evaluation of the extraction efficiency (recoveries) obtained for FRJ spiked at QC2 (50 µg/kg) after testing with 0.5 mL of different extraction solvents. Data represent the mean ± the relative standard deviation of the mean (narrow bars; n = 3).

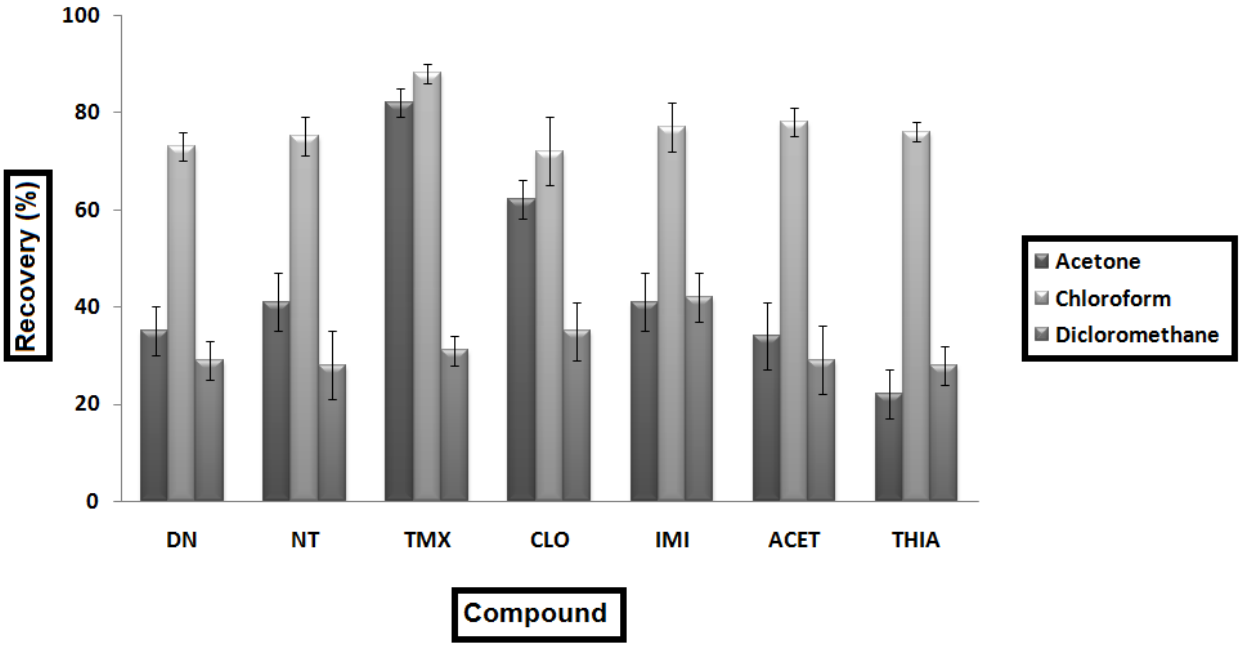


Figure 4S.- Evaluation of the extraction efficiency (recoveries) obtained for FRJ spiked at QC2 (50 µg/kg) after testing with 2.0 mL of different dispersive solvents. Data represent the mean ± the relative standard deviation of the mean (narrow bars; n = 3).

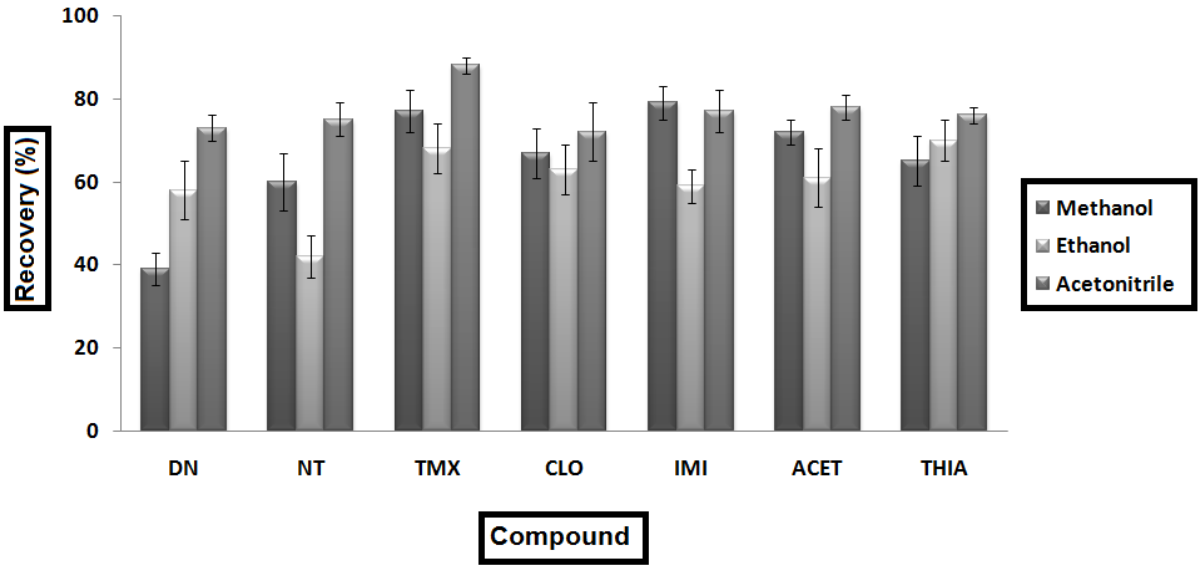


Figure 5S.- Evaluation of the extraction efficiency (recoveries) obtained FRJ spiked at QC2 (50 µg/kg) after performing one or two extractions. It must be specified that the overall dispersive and extraction solvent volumes were 0.5 mL and 2.0 mL in both cases, respectively. Data represent the mean ± the relative standard deviation of the mean (narrow bars; n = 3).

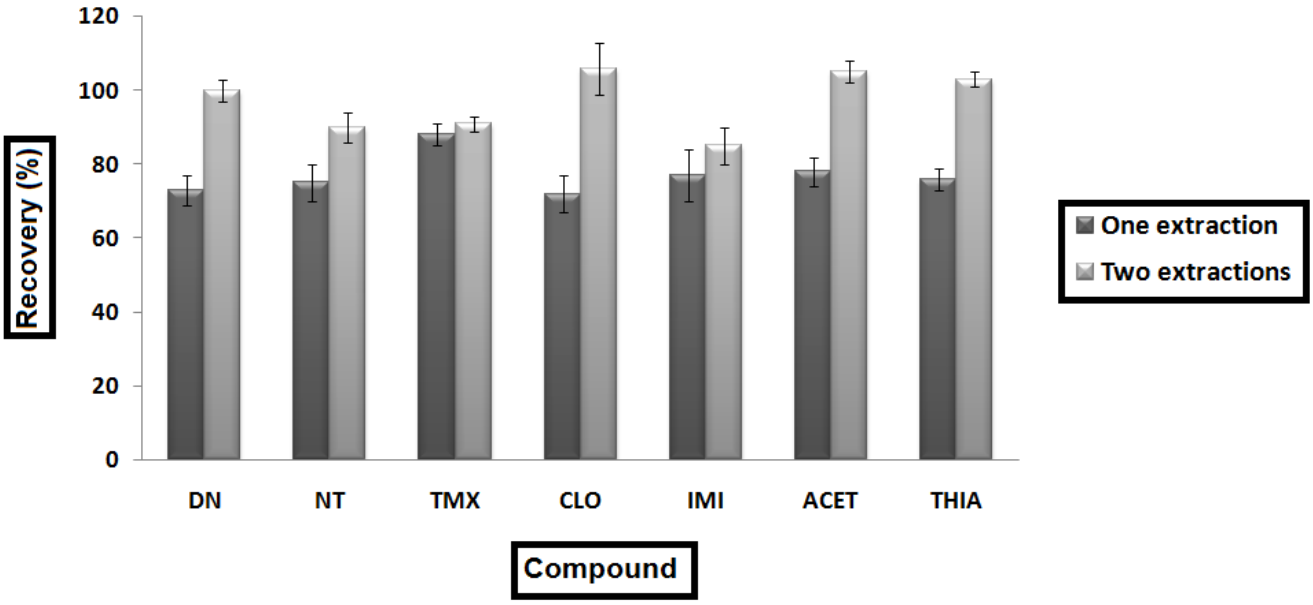
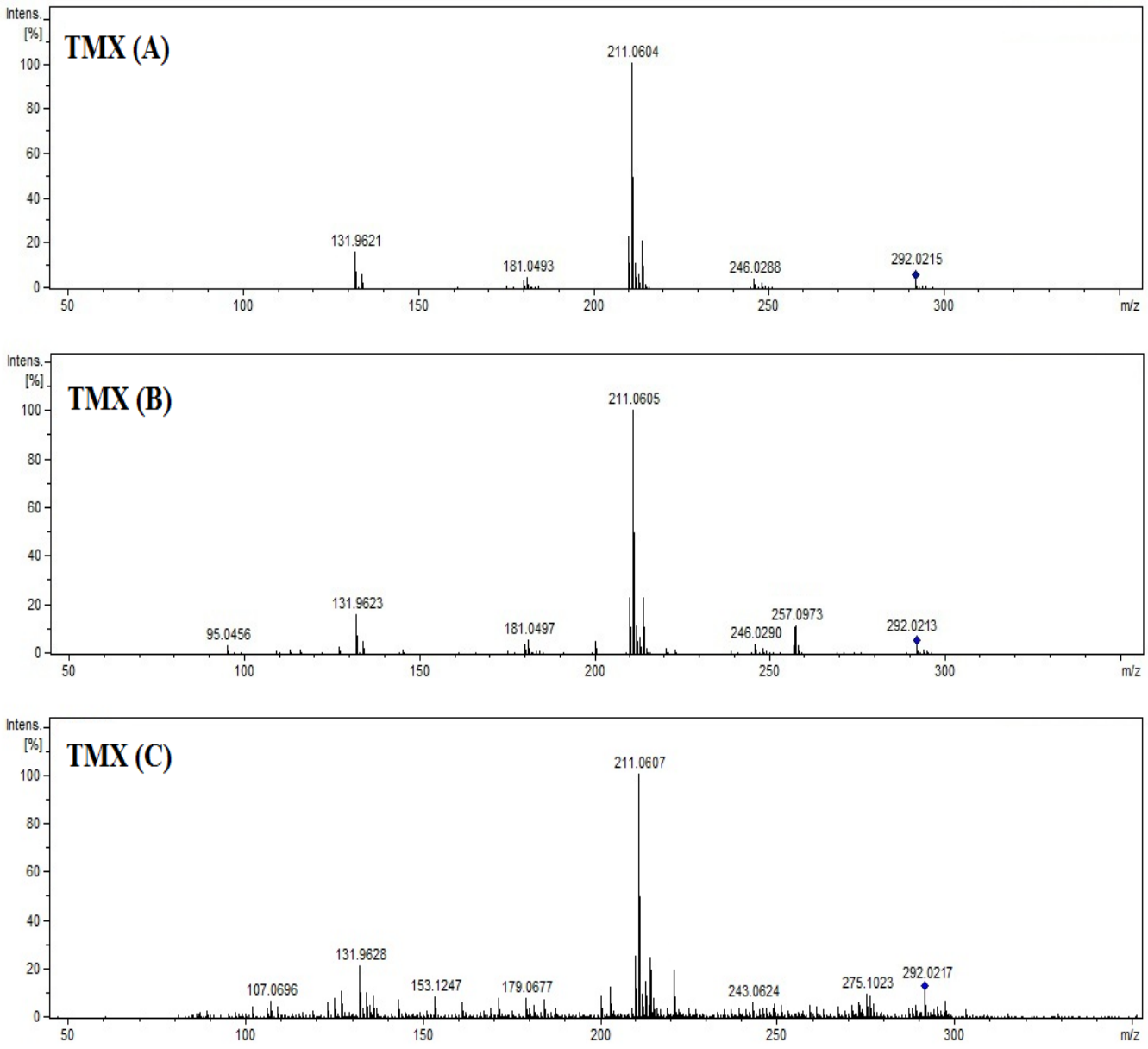
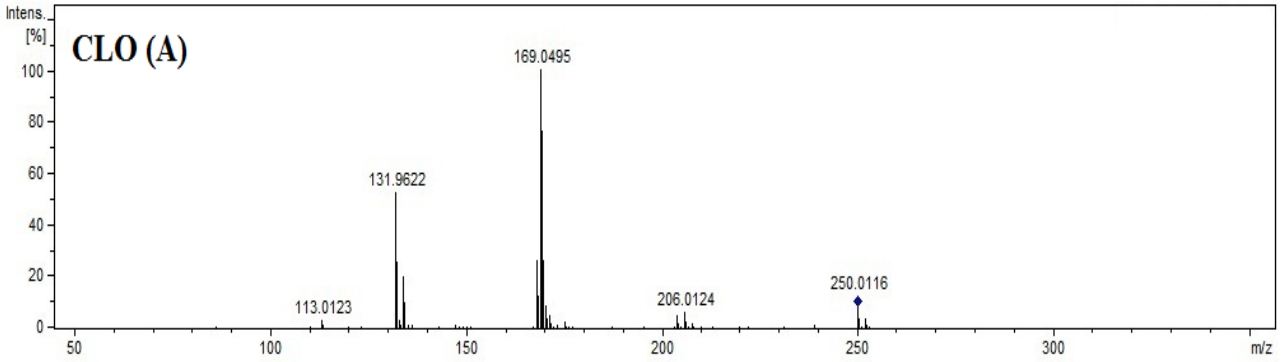
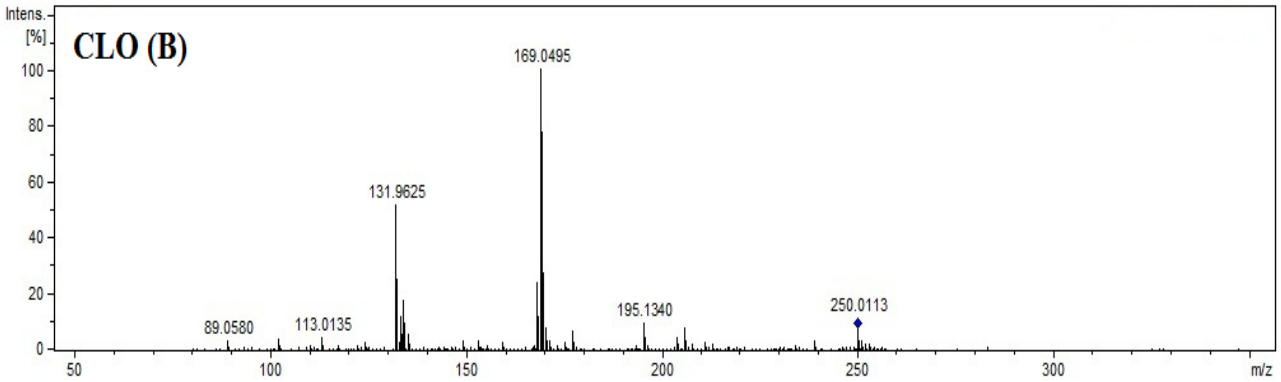
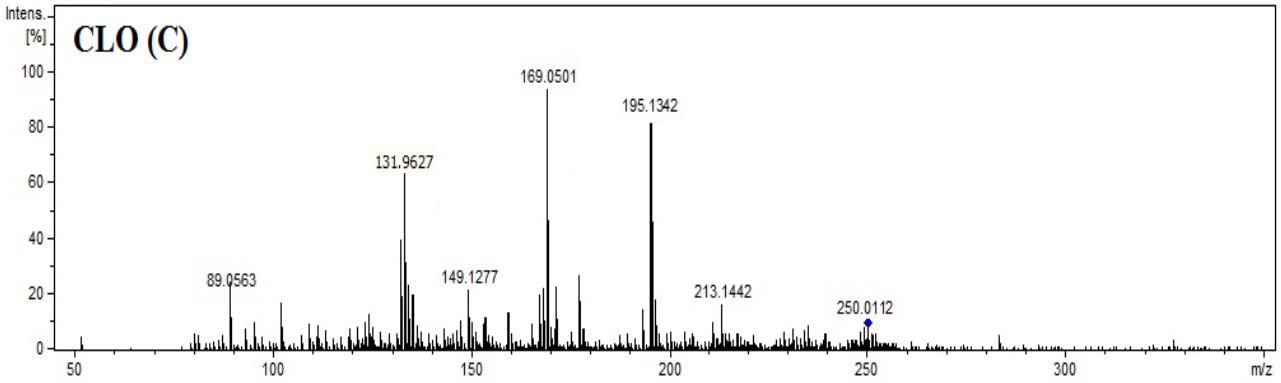


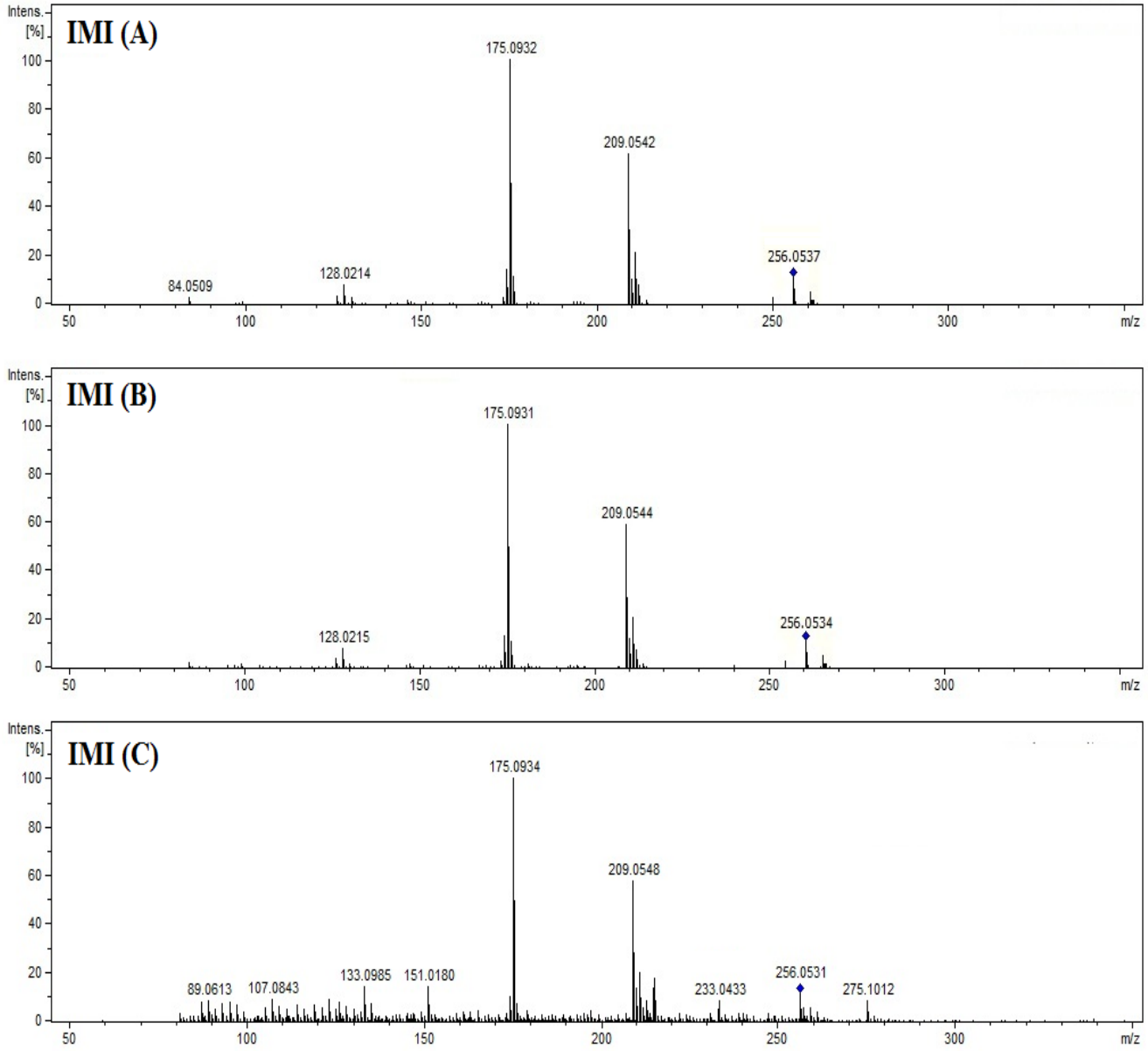
Figure 6S.- Full scan ESI-MS/MS spectra of TMX, CLO and IMI in: (**A**) standard solution (150 µg/L); (**B**) spiked (50 µg/kg) LDS sample; (**C**) spiked (150 µg/kg) FRJ sample. It must be remarked that the neonicotinoids concentrations were the same in the standard and spiked samples according to the proposed sample treatment and the unit conversion ESI-MS/MS conditions are summarized in subsection 2.4 and Table 1.




CLO (A)
Intens. [%]
169.0495
131.9622
113.0123
206.0124
250.0116
m/z


CLO (B)
Intens. [%]
169.0495
131.9625
89.0580
113.0135
195.1340
250.0113
m/z


CLO (C)
Intens. [%]
169.0501
195.1342
131.9627
89.0563
149.1277
213.1442
250.0112
m/z


IMI (A)
Intens.
[%]
175.0932
209.0542
256.0537
84.0509
128.0214
m/z
IMI (B)
175.0931
209.0544
256.0534
128.0215
IMI (C)
175.0934
209.0548
89.0613
107.0843
133.0985
151.0180
233.0433
256.0531
275.1012