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# Development and validation of ultra high performance-liquid chromatography–tandem mass spectrometry based methods for the determination of neonicotinoid insecticides in honey

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ABSTRACT

In this study, the feasibility of two sample treatments has been evaluated for the determination of seven neonicotinoid insecticides in honey from different botanical origins using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). A solid phase extraction with a polymeric sorbent (Strata® X) is proposed for analyzing dark honeys, while a QuEChERS (quick, easy, cheap, effective, rugged and safe) approach is recommended for light honeys. Chromatographic analysis (6 min) was performed on a core-shell column (Kinetex® EVO $C_{18}$). The proposed methods were fully validated using two different MS/MS systems: quadrupole-time-of-flight and triple quadrupole. The results showed that the best overall analytical performance was achieved using triple quadrupole, mainly due to its better sensitivity and the reduced influence of the matrix onto the analyte signals. The methods developed were applied to the analysis of commercial honey samples from different regions of Spain, as well as from experimental apiaries.

## 1. Introduction

Honey, one of the most used products of the hive, is a natural, unprocessed and easily digested food that has been part of the human diet since ancient times (Ares et al., 2017), and it is mainly composed of glucose, fructose and sucrose (Dong, Xiao, Xian, & Wu, 2018). It is a highly valuable natural food product due to its characteristic flavor, nutritional value and therapeutic applications; this has led to a significant increase in its consumption in the last years (Juan-Borrás, Domenech, & Escriche, 2016). However, food alerts caused by the detection of contaminants, e.g. insecticides such as the family of neonicotinoids, have recently affected its healthy image, as they could represent a potential risk for consumers (Ares et al., 2017; Tette et al., 2016). Neonicotinoids are among the most widely used insecticides in the world due to their broad spectrum of efficacy, their systemic and translaminar action, and their pronounced residual activity and unique mode of action (Valverde, Bernal, Martín, Nozal & Bernal, 2016). However, concerns regarding the side effects on health and the environment of this family of insecticides continue increasing, since they can be transferred to the environment and the food chain, with potential adverse consequences for biodiversity, and for example non-target organisms, such as honeybees. As a consequence of those negative effects associated with the use of neonicotinoid insecticides, International institutions, such as the European Union, have established stringent maximum residue levels (MRLs) for these substances in honey (50–200 μg/kg; European Union Pesticide Database, 2017). Therefore, efficient, selective and sensitive methods are needed for the simultaneous determination of these pesticides in honey.

In order to achieve accurate and reliable analytical data, an efficient pre-concentration/separation step is usually required prior to the determination of neonicotinoid residues in honey (see Supplementary Material, Table S1), even using sensitive detection systems, such as tandem mass spectrometry (MS/MS). After dilution with an aqueous solution honey can be extracted using protocols similar to those applied to water samples, as solid phase extraction (SPE) (Calatayud-Vernich, Calatayud, Simó, & Picó, 2016; Campillo, Viñas, Férez-Melgarejo, & Hernández-Córdoba, 2013; Gblylik-Sikorska, Sniegocki, & Posyniak, 2015; Sánchez-Hernández et al., 2016; Tanner & Czerwenka, 2011). Current trends in sample preparation techniques are focused on the simplification of this step in order to reduce costs, the amount of reagents and time spent, which are some of the principles of green analytical chemistry (Calatayud-Vernich et al., 2016; Gałuszka, Migaszewski, & Namieśnik, 2015). In recent years, (QuEChERS; quick, easy, cheap, effective, rugged and safe) based procedures have been

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predominately used for the extraction of pesticides in food matrices, and in particular of neonicotinoids from honey (Codling, Al Naggar, Giesy, & Robertson, 2016; Galeano et al., 2013; Jovanov et al., 2015; Laaniste et al., 2017; Shendy, Al-Ghobashy, Mohammed, Alla, & Lofty, 2016; Tanner & Czerwenka, 2011; Tette et al., 2016; Tomasini et al., 2012). The simple steps involved and the relatively low cost of reagents and equipment allow its application in most laboratories. Another possibility is the employ of liquid-liquid microextraction (LLME), which overcomes some of the problems of conventional liquid-liquid extraction (large volumes of organic solvents, time and steps) (Campillo et al., 2013 Jovanov et al., 2013; Rezaee, Yamini, & Faraji, 2010; Vichapong, Burakham, Santaladchaiyakit, & Srijanarai, 2016; Vichapong, Burakham, & Srijaranai, 2015).

Due to their thermolability, low volatility and high polarity, neonicotinoid residues in honey have usually been determined by high-performance liquid chromatography (HPLC) in reverse phase mode with $C_{18}$ columns. HPLC coupled with tandem mass spectrometry (MS/MS) (see Supplementary Material, Table S1) has been predominately used due to its excellent performance in terms of sensitivity, selectivity and robustness, as well as the reliable identification and quantification of the analytes. In the last years, ultra-high performance liquid chromatography (UHPLC) has been also employed in this field because of the better resolution and sensitivity attained and shorter running times (Galeano et al., 2013; Tette et al., 2016; Sánchez-Hernández et al., 2016).

The aim of this study was to propose a specific analytical methodology to quantify seven of the most commonly employed neonicotinoid insecticides (dinotefuran-DN, nitenpyram-NT, thiamethoxam-TMX, clothianidin-CLO, imidacloprid-IMI, acetamiprid-ACET, and thiacloprid-THIA), with special emphasis to IMI and TMX, in honeys from three different botanical origins (multifloral, rosemary and heather) using UHPLC–MS/MS. In order to propose the most suitable sample treatment, relevant parameters (extraction efficiency, organic solvent consumption, overall time, cost and number of steps) of two of the most employed approaches (SPE and QuEChERS) were evaluated. Honey samples from different botanical origins were tested and the methodology optimized in order to evaluate matrix effects as their different chemical composition may strongly affect the insecticide determination. The final objective was the selection of the most appropriate sample treatment according to the honey botanical origin. The analytical performance of two different MS/MS systems (quadrupole-time-of-flight-QTOF; triple quadrupole-QqQ), was also evaluated. To the best of our knowledge, this is the first study in which a simultaneous comparison is made for different combinations of sample treatments and MS analyzers, considering honeys from three different botanical origins (multifloral, rosemary and heather). The proposed methods for the different honeys were validated and eventually applied to samples from different regions of Spain as well as from experimental apiaries located close to cultivars in which a TMX treatment had been applied.

## 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 and acetonitrile (HPLC grade) were supplied by Lab Scan Ltd. (Dublin, Ireland). Formic acid (98–100% pure), ammonium acetate, ammonium hydroxide, 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 provided by Supelco (Bellefonte, PA, USA). Meanwhile, Strata® X (3 mL with 600 mg of sorbent) SPE cartridges (Phenomenex, Torrance, CA, USA), and a 10-port Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA), were used in the SPE procedure. A vibromatic mechanical shaker, a thermostated ultrasound system, and a drying oven, both supplied by 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), and an R-210/215 rotary evaporator from Buchi (Flawil, Switzerland) were employed for all extractions. Nylon syringe filters (17 mm, 0.45 µm) were from Nalgene (Rochester, NY, USA), and ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA, USA).

### 2.2. Standards

Standard stock solutions (~1000 mg/L) were prepared by dissolving approximately 10 mg of each neonicotinoid insecticide, accurately weighed, in 10 mL of methanol. These solutions were further diluted with a water and methanol mixture (80:20, v/v) in order to prepare the working solutions. Honey samples (5.0 g) were spiked before (BF samples) or after (AF samples) sample treatment with different amounts of the neonicotinoid insecticides and with 50 µg/kg of the IS to prepare the matrix-matched standards, as described in 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 5.0 g of honey spiked with the neonicotinoids at three concentration levels within the corresponding linear range for each MS/MS (QTOF and QqQ). These were as follows: low QC-LOQ; medium QC-10 µg/kg for QqQ and 50 µg/kg for QTOF; high QC-50 µg/kg for QqQ and 300 µg/kg for QTOF. The stock solution was 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 were stable for over two weeks.

### 2.3. Sample procurement and treatment

Several honey types were selected according to their different color, composition and botanical origin. Samples from different regions of Spain, in which a neonicotinoid treatment had been employed in some crops, were kindly donated by the “Centro Apícola Regional-CAR” at Marchamalo (Guadalajara, Spain). Their botanical origin was confirmed by melissopalynological analysis, and corresponded to: rosemary, *Rosmarinus officinalis* (n = 6); multifloral (n = 6); and heather, *Erica* spp (n = 6). In addition, multifloral honey samples (n = 10) collected from controlled apiaries were also supplied by CAR. Apiaries were located close to experimental crops, previously treated with TMX dressed rapeseeds (1 L per 100 kg of Cruiser 350 FS (Syngenta, Madrid, Spain) containing TMX-35%, w/v. In this study, all honey samples were examined in triplicate, and also underwent a preliminary analysis by HPLC–MS/MS in order to check for the presence of neonicotinoids. Once absence was confirmed in the samples, different subsamples were generated and used to prepare matrix-matched standards for validation and sample treatment studies. The blank honey samples were stored in a fresh (4 °C) and dark place before analysis. Two different sample treatments (SPE and QuEChERS) were developed and compared. Fig. 1 outlines the steps of the selected procedures used during the present study.

### 2.4. UHPLC–MS/MS system

#### 2.4.1. UHPLC conditions

The chromatographic system consisted of an Acquity™ UHPLC system (Waters, Milford, MA, USA) equipped with an online vacuum

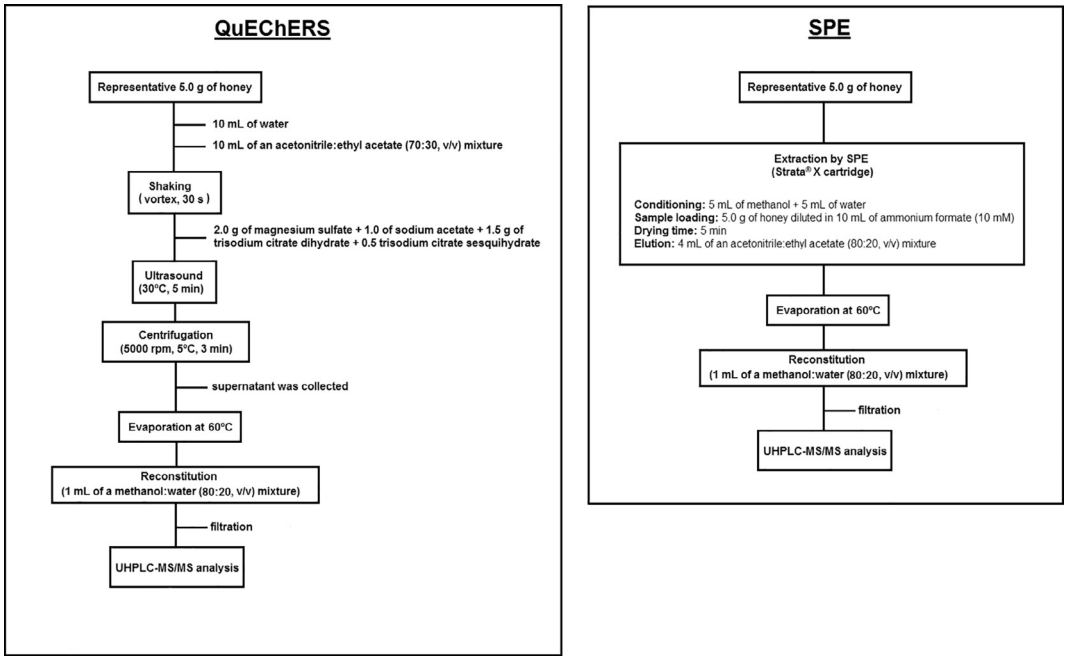


**Fig. 1.** Analytical procedures (QuEChERS and SPE) work-up flow charts.

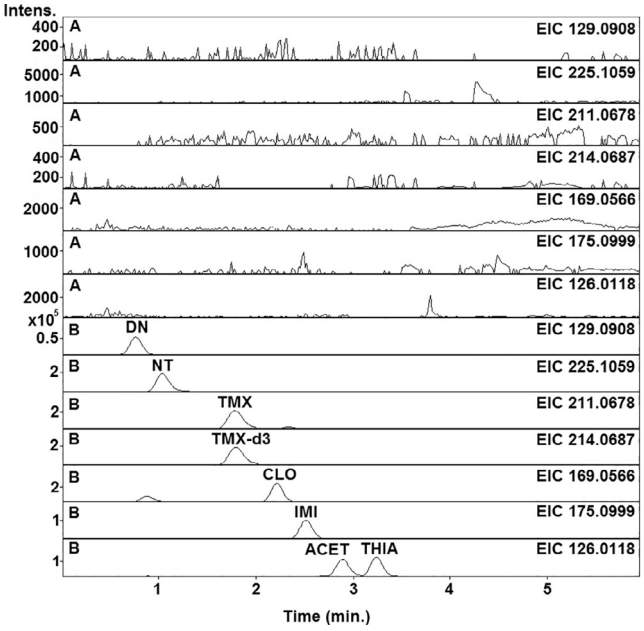


**Fig. 2.** Representative UHPLC-QTOF chromatograms (EIC in positive mode using the quantification ions; see Supplementary Material, Table S2) obtained from: (A) non spiked rosemary honey sample; (B) spiked (50 µg/kg) rosemary honey sample. The UHPLC-QTOF conditions are summarized in Section 2.4 and Supplementary Material (see Table S2).

degasser, a binary solvent pump, an autosampler and a thermostated column compartment. As we recently published a paper concerning the analysis of the seven neonicotinoids in bee pollen (Valverde et al., 2016), we therefore decided to begin the optimization studies with the same chromatographic conditions; selecting as mobile phase components 0.1% (v/v) formic acid in ACN and 0.1% (v/v) formic acid in water, and a Kinetex® EVO ($C_{18}$, 50 × 2.1 mm, 1.7 µm, 100 Å) column protected with a Kinetex® EVO $C_{18}$ guard column, both from Phenomenex (Torrance, CA, USA). After those 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) at a flow rate of 0.3 mL/min in the following gradient mode: (**i**) 0.0–1.0 min (A–B, 10:90, v/v); (**ii**) 1.0–1.5 min (A–B, 60:40, v/v); (**iii**) 1.5–2.5 min (A–B, 90:10, v/v); (**iv**) 2.5–3.5 min (A–B, 90:10, v/v); (**v**) 3.5–4.0 min (A–B, 60:40, v/v); (**vi**) 4.0–4.5 min (A–B, 10:90, v/v); (**vii**) 4.5–6.0 min (A–B, 10:90, v/v). Injection volume and column temperature were set at 5 µL and 30 °C, respectively. With such conditions, the overall run time was 6.0 min (see Figs. 2 and 3), which, to our knowledge, is the fastest proposal that has been published in relation to neonicotinoid analysis in honey (see Supplementary Material, Table 1S).

### 2.4.2. QTOF conditions

A QTOF mass spectrometer (maXis impact, Bruker Daltonik GmbH, Bremen, Germany) were coupled through and electrospray (ESI) interface operated in the positive ionization, which was chosen to conduct the experiments as a result of our previous experience; (Valverde et al., 2016), to the UHPLC system. The optimal conditions were set as follows: capillary voltage, 3500 V; drying gas (nitrogen) flow, 12 L/min; drying gas (nitrogen) temperature, 220 °C; nebulizer pressure, 2 bar. Spectra were acquired in a mass range of mass/charge ($m/z$) 50–400. The $m/z$ scale of the mass spectra was calibrated daily by infusing a 0.01 mol/L sodium formate solution. Compounds showed an intense $[M+H]^+$ on their full-scan spectra, which were selected as precursor ions for MS/MS experiments, which were carried out by using an isolation width of 10 $m/z$ and variable collision energies (10–30 eV). 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 Supplementary Material, Table S2). A mass window of ± 0.01 $m/z$ for the extracted ion chromatograms (EIC) was used in order to extract the exact mass.

### 2.4.3. QqQ conditions

A Xevo TQ-S (QqQ) mass spectrometer (Waters) equipped with an orthogonal Z-spray ESI, operating in positive ion mode, was coupled to the UHPLC system. Cone gas as well as desolvation gas used was nitrogen (Praxair, Valencia, Spain) setup at 250 L/h and 1200 L/h, respectively. For operation in the MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) with a pressure of $4 \times 10^{-3}$ mbar in

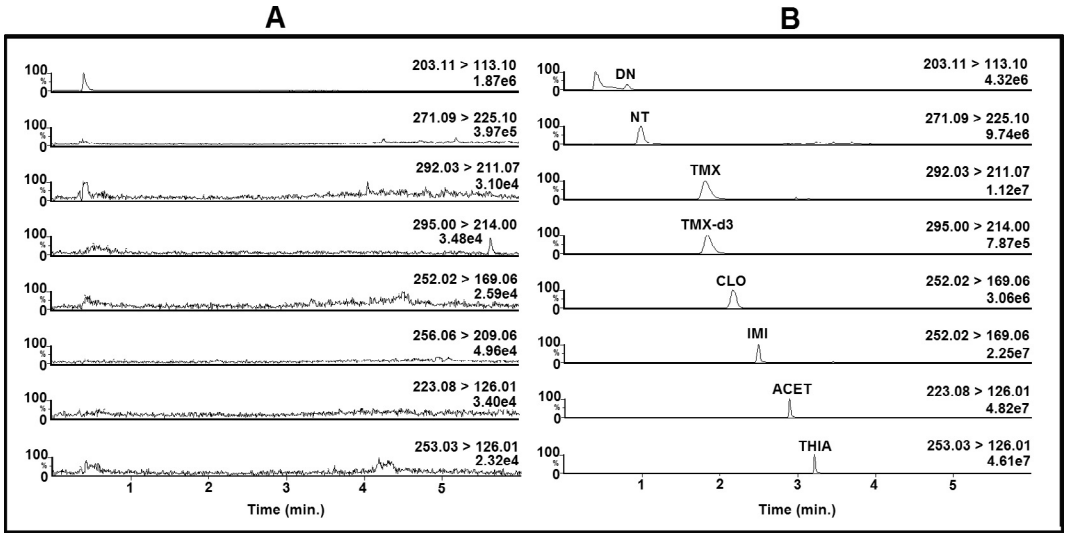


**Fig. 3.** Representative UHPLC-QqQ chromatograms (MRM in positive mode using the quantification transitions; see Supplementary Material, Table S3) obtained from: (A) non spiked rosemary honey sample; (B) spiked (50 µg/kg) rosemary honey sample. The UHPLC-QqQ conditions are summarized in Section 2.4 and Supplementary Material (see Table S3).

the collision cell (0.15 mL/min). Other parameters optimized were capillary voltage 3.5 kV; source temperature 150 °C and desolvation temperature 650 °C. Acquisition was performed in multiple reaction monitoring (MRM) mode, with the protonated molecular ion ($[M+H]^+$) of each compound chosen as precursor ion. The most abundant product ion of each target neonicotinoid was used for quantification and an additional product ion was used for confirmation. More specific MS/MS parameters (MRM transitions, cone voltages and collision energies) are summarized in the Supplementary Material (see Supplementary Material, Table S3). Finally, it must be commented that dwell times were automatically selected in order to obtain enough points per peak.

## 3. Results and discussion

### 3.1. Optimization of sample treatment

#### 3.1.1. Optimization of the QuEChERS procedure

The first consideration was given to the amount of honey (1–10 g) to be analyzed and the most suitable volume of water (5–20 mL), which was chosen according to scientific literature (see Supplementary Material, Table S1). It should be remarked that honey samples from the three different botanical origins were used for optimization of the QuEChERS procedure. After several tests, 5.0 g of honey and 10 mL of water were selected as the optimal amounts to be used. Recoveries were adequate, and good signal to noise (S/N) ratios were achieved. Regarding the extraction step, some assays were conducted with diverse volumes (5–15) of different solvent mixtures of acetonitrile with water and ethyl acetate (100:0, 80:20, 50:50, v/v), chosen according to preliminary experiments and the existing literature (see Supplementary Material, Table S1). The best results in terms of recoveries were obtained with 10 mL of an acetonitrile and ethyl acetate (80:20, v/v) mixture. Afterwards, it was optimized the amount of salts to be employed in the partitioning step of the QuEChERS procedure, and the highest recoveries were obtained when using 2.0 g of magnesium sulfate, 1.0 g of sodium acetate, 1.5 g of trisodium citrate dehydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate. Once the solvents and the salts were selected, the influence of certain extraction parameters, such as the agitation source (vibromatic, vortex and ultrasound), extraction time (1–15 min), and centrifugation time (1–10 min), was sequentially tested. Optimal extraction (recovery percentages > 80%; see Supplementary Material, Fig. S1) was achieved with 5 min of agitation in the ultrasound, and 3 min of centrifuging at 5000 r.p.m at 5 °C. Different temperatures were also tested when performing the ultrasound agitation (20–40 °C), and the highest recoveries were obtained for 30 °C. Afterwards, it was studied if a further clean-up step would be necessary in order to reduce as much as possible the extraction of matrix-components that could affect to analyte ionization, but without affecting the extraction efficiency. Thus, the supernatant was collected and transferred to a centrifuge tube, in which PSA (70 mg), $C_{18}$ (70 mg), and a mixture of them (35 mg of each) were added in different experiments with the aim of removing sugars and fatty acids (PSA) and non-polar compounds. It must be mentioned that the amounts of PSA and $C_{18}$, which were employed in the above-mentioned experiments, were selected after performing a series of preliminary experiments, as those amounts may affect the purification of the target insecticides (Dong & Xiao, 2017). It was observed that the clean-up step did not significantly reduce the matrix effect, but it had a marked negative effect onto the recovery percentages, especially for DN and NT in heather honeys (< 60%) (see Supplementary Material, Fig. S2). Thus, no clean-up step was performed. Then, the supernatant was directly transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 60 °C. Different volumes (0.5–2.0 mL) of a methanol and water (80:20, v/v) mixture, which were selected due to the good results obtained in previous researches (Valverde et al., 2016), were assayed. Amounts of solvent over 1 mL did not improve the recovery percentages, so it was decided that 1 mL of the mixture should be employed to reconstitute the dry residue.

#### 3.1.2. Optimization of the SPE procedure

As a result of the physicochemical properties of the neonicotinoids and our research experience in honey (Sánchez-Hernández et al., 2016), we decided to check the suitability of polymeric (Strata® X) SPE sorbents to extract the insecticides. It must be specified that the optimization procedure was performed with heather honey samples, as it was the honey botanical origin that was mainly affected for the matrix effect when applying the QuEChERS procedure. Firstly, the amount of honey (1–10 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 checked. After several tests, 5.0 g of honey and 10 mL of ammonium formate (10 mM) in water were deemed the optimal values, as in this way the highest S/N ratio for securing maximum sensitivity was obtained. Some parameters were evaluated to optimize the extraction procedure. Firstly, different

volumes of methanol and water were tested in order to precondition the cartridge; 5 mL of both applied sequentially was the most suitable. Then, several water and methanol mixtures (100:0, 90:10, 80:20, 70:30, 50:50, v/v) and volumes (5–15 mL) were tested in the washing step, as they have provided good results in previous works (Gblylik-Sikorska et al., 2015; Sánchez-Hernández et al., 2016). In all cases, two of the neonicotinoids (DN and NT) were lost when performing this step, and no significant improvement in the matrix effect or in the removal of the interferences was observed. Thus, it was decided to eliminate the washing step 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-min drying period was chosen to avoid delays in the extraction procedure. Different mixtures of acetonitrile with water or ethyl acetate (100:0, 80:20, 50:50, 20:80, 0:100; v/v) to elute neonicotinoids from the cartridges were tested. The best results in terms of extraction efficiency were obtained when an acetonitrile and ethyl acetate (80:20, v/v) mixture was employed. Regarding elution volumes (ranging from 1.0 to 5.0 mL), 4 mL of the selected mixture was appropriate for procuring satisfactory recoveries (> 75%; see Supplementary Material, Fig. S1). The solution obtained was transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 60 °C. According to results obtained when optimizing the QuEChERS procedure, 1 mL of a methanol and water (80:20, v/v) mixture was employed to reconstitute the dry residue.

#### 3.1.3. Comparison of the proposed sample treatments

In order to check the effectiveness of the proposed sample treatments, neonicotinoid responses (analyte peak area/IS area) obtained from blank samples spiked at three different concentrations (QC levels), either prior to (BF samples) or following (AF samples) sample treatment were compared. Recovery values ranged from 80% to 109% when employing the QuEChERS approach; while, these values were slightly lower when using SPE (see Table 1 and Supplementary Material, Fig. S1), except for NT, ACET and THIA in some cases. In relation to the evaluation of the matrix effect, which was calculated as stated in Section 3.2.3, no significant differences were observed when comparing the responses for light honeys (see Tables 2 and 3), with the exception of ACET in multifloral honeys. On the other hand, a significant matrix effect (ion suppression) was observed for all the analytes in dark honeys when using the QuEChERS approach; while, a lower signal suppression was also observed for four of the neonicotinoids in the SPE treated samples, and in this case three of the insecticides (TMX, ACET and THIA) were not affected by this effect (see Tables 2 and 3). Thus, it can be concluded that the QuEChERS approach should be employed when analyzing light honeys, as the results were comparable to those obtained with SPE, but in a rapid and simpler way; whilst, the SPE procedure is the best option when analyzing dark honeys. These results have demonstrated that the proposed procedures are an efficient and green alternative to the existing procedures for analyzing these insecticides in honeys. The recovery values are comparable with or better than the reported values, and similar sample treatment times or volume/amount of reagents are employed (see Supplementary Material, Table S1), but with the advantage that the matrix effect has been minimized in such a way for multifloral and rosemary honey botanical origins, that standard calibration curves could be used to quantify the neonicotinoid insectides. This is particular relevant as matrix effect was not minimized in most of previous publications, requiring longer extraction times and more steps when it was achieved.

### 3.2. Method validation

The method validation was based on the Eurachem Guidelines (EURACHEM, 2014), the current European legislation for pesticides residues analysis in foods (SANTE, 2015) as well as with recent studies (Ares et al., 2017; Calatayud-Vernich et al., 2016; Idowu et al., 2018). The validation was performed using reference standards prepared in solvent as well as in matrix (i.e. matrix-matched calibration), which were treated with the procedures selected for each botanical origin (multifloral and rosemary-QuEChERS; heather-SPE). Two MS/MS systems were tested during validation: QTOF and QqQ.

#### 3.2.1. Selectivity

To evaluate the selectivity of the proposed method, a set of unspiked blank honey samples (n = 6) from three different botanical origins was injected onto the chromatographic system and the results were compared with those obtained for spiked honey samples. No chromatographic interference was observed at analytes retention times in any of the blank samples analysed of the three botanical origins for both MS/MS systems (see Figs. 2 and 3). Moreover, the relative intensities of the selected product ions/transitions in the matrix-matched samples concurred with the corresponding standard solutions to within ± 10%; this is lower than the maximum rates permitted ( ± 30%; SANTE, 2015).

#### 3.2.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) were experimentally determined by injection of blank honey samples (n = 6), in which the absence of insecticide residues was previously confirmed, and measurement of the magnitude of background analytical response at the elution time in each honey sample investigated. The LODs and LOQs were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively. Low LODs and LOQs were obtained in all cases for both MS/MS systems (see Supplementary Material, Tables S4–S6 and Figs. S3 and S4), although those values were ten times lower when using the QqQ. Moreover, the LOQs we obtained with the QqQ detector are also lower than those of the previous publications; while, the LOQs obtained with the QTOF are also comparable with most of the published data (see Supplementary Material, Table S1). However, the sensitivity achieved with both MS/MS systems is more than enough to fulfil the criteria of the European Commission in relation to the maximum residue limits (MRL) established for some of this pesticides in honey and

**Table 1**
Evaluation of the efficiency (recoveries) of the optimized and selected sample treatment for each botanical origin (heather-SPE; multifloral and rosemary-QuEChERS). Data obtained as described in Sections 3.1 and 3.2 (n = 6) using a QTOF detector.

| Quality control (QC) sample | Heather Mean (%) ± RSD (%) | | | Rosemary Mean (%) ± RSD (%) | | | Multifloral Mean (%) ± RSD (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC |
| DN | 80 ± 5 | 85 ± 4 | 81 ± 4 | 102 ± 3 | 93 ± 5 | 95 ± 6 | 87 ± 4 | 90 ± 3 | 92 ± 5 |
| NT | 108 ± 7 | 102 ± 5 | 101 ± 4 | 94 ± 4 | 92 ± 2 | 88 ± 6 | 91 ± 5 | 94 ± 3 | 101 ± 3 |
| TMX | 104 ± 3 | 97 ± 4 | 92 ± 4 | 98 ± 2 | 102 ± 5 | 99 ± 4 | 96 ± 4 | 100 ± 5 | 103 ± 3 |
| CLO | 93 ± 5 | 87 ± 6 | 85 ± 4 | 109 ± 3 | 105 ± 2 | 98 ± 6 | 95 ± 6 | 101 ± 3 | 93 ± 4 |
| IMI | 87 ± 5 | 82 ± 4 | 83 ± 6 | 97 ± 3 | 100 ± 2 | 90 ± 4 | 94 ± 4 | 90 ± 5 | 92 ± 7 |
| ACET | 97 ± 4 | 100 ± 5 | 92 ± 3 | 90 ± 7 | 87 ± 5 | 85 ± 4 | 102 ± 4 | 107 ± 5 | 99 ± 3 |
| THIA | 95 ± 3 | 94 ± 4 | 91 ± 2 | 105 ± 4 | 95 ± 5 | 97 ± 6 | 100 ± 5 | 98 ± 4 | 97 ± 6 |

**Low QC**-LOQ (see Supplementary Material, Tables S4–S6); **Medium QC**-50 μg/kg; **High QC**-300 μg/kg.

**Table 2**
Evaluation of the matrix effect (comparison of responses) with the optimal sample treatment for each botanical origin (heather-SPE; multifloral and rosemary-QuEChERS) using a QTOF. Data obtained as described in Section 3.2 (n = 6).

| Quality control (QC) sample | Heather Mean (%) ± RSD (%) | | | Rosemary Mean (%) ± RSD (%) | | | Multifloral Mean (%) ± RSD (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC |
| DN | 55 ± 4 | 57 ± 7 | 50 ± 6 | 91 ± 2 | 93 ± 4 | 86 ± 3 | 99 ± 3 | 94 ± 3 | 102 ± 5 |
| NT | 45 ± 5 | 47 ± 5 | 45 ± 3 | 81 ± 3 | 86 ± 5 | 83 ± 3 | 94 ± 4 | 97 ± 5 | 96 ± 2 |
| TMX | 94 ± 7 | 97 ± 6 | 102 ± 4 | 103 ± 5 | 100 ± 3 | 102 ± 5 | 105 ± 3 | 107 ± 3 | 101 ± 6 |
| CLO | 60 ± 6 | 63 ± 4 | 65 ± 7 | 99 ± 2 | 95 ± 4 | 96 ± 3 | 80 ± 4 | 82 ± 5 | 85 ± 3 |
| IMI | 61 ± 5 | 57 ± 6 | 55 ± 5 | 103 ± 4 | 104 ± 2 | 107 ± 5 | 85 ± 5 | 87 ± 4 | 92 ± 3 |
| ACET | 102 ± 6 | 99 ± 7 | 105 ± 3 | 100 ± 6 | 102 ± 3 | 98 ± 4 | 71 ± 6 | 73 ± 5 | 75 ± 7 |
| THIA | 105 ± 8 | 102 ± 7 | 108 ± 4 | 102 ± 4 | 101 ± 3 | 105 ± 5 | 95 ± 6 | 98 ± 5 | 92 ± 5 |

**Low QC**-LOQ (see Supplementary Material, Tables S4–S6); **Medium QC**-50 μg/kg; **High QC**-300 μg/kg.

other apiculture products (50–200 μg/kg; European Union Pesticide Database, 2017), that are much higher than the LOQs obtained with our proposals.

### 3.2.3. Matrix effect

To ascertain how the matrix influenced ESI ionization for both detectors, a comparison was made of the responses (analyte peak area/IS area) of standard working solutions in solvent and blank honey extracts of the different botanical origins (AF samples) spiked at three different concentrations (QC levels). Responses at the different concentrations (QC levels) assayed ranged from 81% to 108% for multifloral and rosemary honey samples, with the exception of ACET in multifloral honey samples for QTOF that presented a lower response (< 80%; see Tables 2 and 3). As it can be observed, the values were slightly better in most cases when using a QqQ, but in general, no great differences were observed between both MS/MS systems. When analyzing heather honey samples, significant differences between both systems were observed in the neonicotinoid responses, as four of the insecticides (DN, NT, CLO and IMI) presented responses lower than 70% in all cases with a QTOF system; for QqQ detection only two analytes were significantly affected for the matrix effect (DN and NT). To confirm these findings the slopes of the standard and matrix-matched calibration curves were contrasted (see Supplementary Material, Tables S4–S6), and it was found that for multifloral and rosemary honeys overlapping occurred at the confidence intervals, with the exception of ACET in multifloral honeys for QTOF detection; this was not the case for some of the neonicotinoids (four-QTOF; two-QqQ) in heather honey. Therefore, it was concluded that the matrix did not significantly affect ESI ionization of the analytes in multifloral (with the exception of ACET for QTOF) and rosemary honey samples for both MS/MS systems. These results 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 heather honey samples. This is an important result not only because a significant matrix effect has been reported in most of the existing literature dedicated to analyze those compounds in honey (see Supplementary Material, Table S1), but due to the demonstration that there is a need of evaluating the matrix effect for different honey botanical origins in order to avoid potential quantification errors.

### 3.2.4. Working range

Different calibration curves were used to quantify neonicotinoid insecticides in accordance to the botanical origin of the honey and the influence of the matrix effect onto the analyte ionization. When using a QTOF, matrix-matched standard calibration curves were used to quantify four (DN, NT, IMI and CLO) and one (ACET) neonicotinoid insecticides in heather and multifloral honeys, respectively; while, when employing a QqQ, only two insecticides (DN and NT) in heather honeys must be quantified with matrix-matched standard calibration curves. In contrast, neonicotinoid insecticides can be quantified with standard calibration curves prepared in solvent in all other cases. Blank honey was treated accordingly to the proposed procedure and spiked with variable amounts of the seven neonicotinoids over an analytical range between LOQ and 300 μg/kg and between LOQ and 50 μg/kg for matrix matched calibration curves using QTOF and QqQ, respectively. Regarding reference standards in solvent, the analytical ranges prepared were between LOQ and 1500 μg/L for QTOF and between LOQ and 250 μg/L for QqQ. 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. Linearity was evaluated by visual analysis of the plots, a calculation being made of the determination coefficients ($R^2$), and by our back calculation of the concentrations of the individual calibration standards. The graphs obtained in all the calibration curves were straight lines, and the coefficient of the determination values ($R^2$) was above

**Table 3**
Evaluation of the matrix effect (comparison of responses) with the optimal sample treatment for each botanical origin (heather-SPE; multifloral and rosemary-QuEChERS) using a QqQ. Data obtained as described in Section 3.2 (n = 6).

| Quality control (QC) sample | Heather Mean (%) ± RSD (%) | | | Rosemary Mean (%) ± RSD (%) | | | Multifloral Mean (%) ± RSD (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC | Low QC | Medium QC | High QC |
| DN | 60 ± 4 | 62 ± 5 | 57 ± 7 | 101 ± 3 | 104 ± 2 | 98 ± 4 | 102 ± 2 | 98 ± 2 | 101 ± 2 |
| NT | 66 ± 6 | 68 ± 7 | 62 ± 6 | 95 ± 3 | 96 ± 3 | 93 ± 2 | 99 ± 4 | 103 ± 2 | 105 ± 5 |
| TMX | 98 ± 3 | 101 ± 3 | 102 ± 5 | 102 ± 2 | 101 ± 4 | 99 ± 3 | 99 ± 2 | 98 ± 2 | 97 ± 3 |
| CLO | 84 ± 5 | 86 ± 4 | 89 ± 5 | 105 ± 2 | 102 ± 2 | 105 ± 3 | 87 ± 5 | 92 ± 4 | 90 ± 5 |
| IMI | 93 ± 7 | 100 ± 6 | 104 ± 5 | 97 ± 3 | 99 ± 5 | 101 ± 5 | 98 ± 5 | 102 ± 3 | 104 ± 2 |
| ACET | 99 ± 4 | 100 ± 4 | 101 ± 6 | 105 ± 2 | 108 ± 2 | 102 ± 5 | 81 ± 5 | 84 ± 6 | 82 ± 5 |
| THIA | 95 ± 6 | 94 ± 3 | 97 ± 2 | 104 ± 3 | 107 ± 5 | 108 ± 6 | 101 ± 3 | 103 ± 5 | 99 ± 3 |

**Low QC**-LOQ (see Supplementary Material, Tables S4–S6); **Medium QC**-10 μg/kg; **High QC**-50 μg/kg.

0.99 in all cases (see Supplementary Material, Tables S4–S6). The deviation of the back-calculated standard concentrations was equal to or less than 5% in all cases from the nominal values. Absence of bias was confirmed by a *t* test and by studying the distribution of residuals. Finally, it must be also commented that the linearity ranges were different according to the MS/MS system and their corresponding LOQ values for each insecticide. This is a relevant finding, as a dilution of the extract would be necessary prior to their UHPLC-QqQ analysis for concentrations higher that 50 μg/kg in order to provide a correct quantification; while it would not require the dilution until a highest concentration value (300 μg/kg) for QTOF detection.

#### 3.2.5. Precision

Precision experiments were performed concurrently by repeated sample analysis using blank honey samples spiked at three different concentrations (low, medium and high QC levels) on the same day (n = 6) (intra-day precision (SANTE, 2015) or repeatability (EURACHEM, 2014) experiments), or over three consecutive days (n = 6) (inter-day precision (SANTE, 2015) or partial reproducibility (EURACHEM, 2014)). Results, expressed as the percentage of relative standard deviation (%RSD), was at all times (intra and inter-day experiments) lower than 10% (see Supplementary Material, Tables S7–S9). Moreover, there were not observed significant differences of those values depending on the MS/MS system. Those results indicate that the proposed methods are precise according to existing normative (%RSD ≤ 20; SANTE, 2015).

#### 3.2.6. Trueness

It was evaluated by means of recovery experiments (as a measure of trueness), by comparing the results (analyte peak area/IS area) obtained from blank honey samples spiked at three different concentrations (low, medium and high QC levels), either prior to (BF samples) or following (AF samples) sample treatment. Mean recoveries ranged from 80% to 109% with %RSD values lower than 8% in all cases (see Table 1). Those values, which are similar or better than the obtained in previous works (see Supplementary Material, Table S1), fulfilled the requirements established by the European Commission (SANTE, 2015) for pesticide residue analysis (recovery percentages between 70% and 120%; %RSD ≤ 20).

#### 3.2.7. Uncertainty

The combined method uncertainty (%U) was determined in all cases with the uncertainty of the bias ($\%U_{bias}$) combined with the uncertainty of the precision ($\%U_P$) based on the equations summarized in Table S10 (see Supplementary Material; EALC, 2013). It must be remarked that due to the absence of specific reference certified material or an official analysis method for determining neonicotinoids in honey, recovery studies (spiking experiments at the different QC levels) were used to give an indication of the level of bias, as recommended in the EURACHEM (2015) guideline. Tables S11 and S12 (see Supplementary Material) presented an overview of the contribution of each of the two uncertainty sources ($\%U_{bias}$ and $\%U_P$) to the combined method uncertainty, which was calculated for each botanical origin and detector at the three QC levels. It can be concluded after examining the results (see Supplementary Material, Tables S12 and S13) that there was a great variation of the $\%U_{bias}$ and $\%U_P$ values, and subsequently of the %U (< 18% in all cases), depending on the neonicotinoid, spiking level and botanical origin of the honey. Thus, it cannot be identified a common major contributor to the method uncertainty (%U).

### 3.3. Application of the method

The validated methodologies were applied to the determination of neonicotinoids residues in eighteen commercial honey samples from three different botanical origins, and in ten multifloral honey samples collected from experimental apiaries (see Section 2.3). All analyses were made in triplicate, and the IS was added at the same concentration (50 μg/kg) than in the matrix-matched samples. No residues of the insecticides under study were detected in any of the commercial samples; while residues of TMX and CLO were found in some honeys from experimental apiaries (see Supplementary Material, Table S13 and Fig. S5). TMX was quantified in six samples (0.3–144 μg/kg) with QqQ, while using QTOF it was detected in the same samples but could be quantified only in one of them (141 μg/kg) due to the low concentrations found in the rest of samples (< 0.6 μg/kg). In addition, CLO was quantified in only one sample with both MS/MS systems (~45 μg/kg), although use of QqQ allowed its detection in another sample. We concluded that QqQ is more recommendable for quantifying neonicotinoids in honey due to the highest sensitivity provided.

## 4. Conclusions

In this work, analytical methodologies for the simultaneous identification and quantification of seven neonicotinoids in honey samples from three different botanical origins (multifloral, rosemary and heather) have been developed and validated. The different physico-chemical characteristics of the honey depending on its botanical origin made necessary the application of different sample treatments. The proposed extraction procedures, based on SPE (heather honeys) and QuEChERS (multifloral and rosemary honeys), have proven to be fast, efficient and to have low consumption of organic solvents, following the principles of green analytical chemistry. The QuEChERS approach, was the best choice (in terms of efficiency of sample treatment and lower matrix effects) for analyzing light honeys (multifloral and rosemary), while the SPE-based procedure provided good performance in all cases and was found the best option for dark honeys. The performance of two different MS/MS systems (QqQ and QTOF) was evaluated and the main validation parameters obtained for both MS/MS systems were compared. Our results showed that the best overall analytical performance was achieved with UHPLC–MS/MS (QqQ), mainly due to its better sensitivity (LOQs ten times lower) and the reduced influence of the matrix onto the analyte detection. The chromatographic separation of the insecticides was achieved with a core-shell technology based column (Kinetex® EVO) in a shorter time than reported in previous works for these compounds in honey. The excellent sensitivity reached by QqQ led to LOQs much lower than the MRLs established, improving the majority of values reported in the literature. Analysis of commercial honey samples revealed the absence of the insecticides under study, while several honey samples obtained from experimental apiaries presented residues of TMX and CLO.

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## Conflict of interest statement

The authors of this manuscript have declared no conflict of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the

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