FAST DETERMINATION OF NEONICOTINOID INSECTICIDES IN BEESWAX BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY USING AN ENHANCED MATRIX REMOVAL-LIPID SORBENT FOR CLEAN-UP

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Abstract

This study is concerned with the development of a simple, rapid analytical method for simultaneously determining seven neonicotinoid insecticides (dinotefuran, nitenpyram, thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid) in beeswax, by means of ultra-high performance liquid chromatography coupled to a quadrupole-time-of-flight mass detector. An efficient sample treatment is proposed (with average analyte recoveries between 93% and 106%) involving solvent extraction with a methanol and ethyl acetate mixture (70:30, v/v), centrifugation, freezing with dry ice, a dispersive solid phase extraction (enhanced matrix removal-lipid) followed by evaporation. Chromatographic analysis (10.5 min) was performed on a core-shell technology-based column (Kinetex® EVO C_{18}). The method was fully validated in terms of selectivity, ruggedness, limits of detection (LODs) and quantification (LOQs), matrix effect, linearity, uncertainty, trueness and precision. Low LOQs, ranging from 1.2 to 4.9 µg/kg, were obtained in all cases. Finally, the proposed methods were applied to neonicotinoid analysis of twenty one beeswax samples from different Spanish regions, only one of the pesticides (imidacloprid) being detected at low levels (< LOQ).

Keywords: Beeswax; Core-shell column; Dispersive solid-phase extraction; EMR-Lipid sorbent; Neonicotinoid insecticides; UHPLC-MS/MS.

1. Introduction

Agrochemical products, including pesticides, are used in current agriculture in order to improve the efficiency and quality of crops by acting on various diseases and pests [1,2]. Unfortunately, several studies have shown that some of these compounds can have a harmful effect on the environment as well as on human and animal health [3]. Indeed, pesticides have been said to be one of the causes of the disappearance of bees, which are fundamental for pollination; other factors include pathogens, invasive species, or the incorrect use of phytosanitary products. [4-9]. In particular, neonicotinoids, the most widely used insecticides in the world, as a result of their broad spectrum of efficacy [3,10-12], are currently considered one of the pesticide families most related to the disappearance of bees [3,10]. Indeed, the European Food Safety Authority (EFSA) has recently published an assessment in which it has been stated that most uses of three neonicotinoid insecticides (clothianidin-CLO, imidacloprid-IMI and thiamethoxam-TMX) represent a risk to bees [13]. Neonicotinoids can enter the hive either directly or indirectly [14], and residues of these pesticides may ultimately be found in bee products [9]. The analysis of these compounds in beeswax could be of great interest in view of its potentially being considered a contaminant reservoir, and subsequently they could be transmitted to other bee products; in addition, as beeswax is recycled when a new hive is established, this might lead to a problem involving the progressive accumulation of insecticides inside it [9,14]. Therefore, development of new methods particularly for sample preparation is still important because neonicotinoid residues could be present at trace levels.

Only a few methods are described in the literature for determining neonicotinoids in beeswax (see Table 1), and in most cases the methodology was the multi-residue type in

which pesticides belonging to different families were simultaneously determined. The sample treatments employed in the studies summarized in Table 1 mainly involved a melting or dissolution of the beeswax followed by a solvent extraction and a dispersive solid phase extraction (dSPE) with primary secondary amine (PSA) and C_{18} or conventional SPE with diatomaceous earth sorbents. Some of the sample preparation procedures often described (solid-liquid extraction and SPE) required huge amounts of solvents or long times [4,6,8,9]. It is not, then, surprising that the sample preparation known as quick, easy, cheap, effective, rugged and safe (QuEChERS), or related methods, has generally been used for extracting neonicotinoids in beeswax in order to reduce the number/amount of reagents and time spent on this step [7,14-17]. In all QuEChERS methods, the dSPE was performed by using PSA and C_{18} sorbents. High-performance liquid chromatography (HPLC) with C_{18} (see Table 1) stationary phases is the preferred technique due to the physical–chemical properties of neonicotinoids. In all the studies the couplings with mass MS [8,9] or MS/MS [14-17] have been used as they offer enough sensitivity and an unambiguous identification and quantification of the insecticides. In addition, it must be also stated that ultra-high performance liquid chromatography (UHPLC) has been used in some of these works [4,6].

The aim of this study as to develop a new, robust UHPLC-MS/MS method to determine seven of the most commonly employed neonicotinoid insecticides (dinotefuran-DN, nitenpyram-NT, TMX, CLO, IMI, acetamiprid-ACET and thiacloprid-THIA) in beeswax. A recently commercialized dSPE sorbent (enhanced matrix removal-lipid; EMR-Lipid), which has shown promising results when removing lipids in a complex matrix (honeybee; [1]), together with an analytical column based on core shell technology (Kinetex® EVO) were selected for sample clean-up and separation,

respectively; this was particularly significant, as to our knowledge, there are no reports and applications regarding the use of this sorbent or type of column with neonicotinoids in beeswax. Further aims of the study involved validating the proposed method in accordance with current European legislation [18] and internationally recognized guidelines [19] and analyzing several beeswax samples from different Spanish regions.

2. Material and methods

2.1. Chemical and materials

FLUKA-PESTANAL analytical standards of the seven neonicotinoids (Det. Purity > 99.5% in all cases) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Ethyl acetate, acetone, methanol, ethanol and acetonitrile (HPLC grade) were supplied by Lab Scan Ltd. (Dublin, Ireland). Formic acid (98-100% pure) and magnesium sulfate anhydrous were obtained from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). QuEChERS dSPE EMR-Lipid tubes (15 mL, 1 g) were supplied by Agilent Technologies (Palo Alto, CA, USA). A vibromatic mechanical shaker 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), a Moulinette chopper device from Moulinex (Paris, France) and an 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

Individual standard stock solutions of each pesticide at a concentration of 1000 mg/L were prepared in methanol. These solutions were further diluted with a water and methanol (20:80, v/v) mixture in order to prepare the working solutions. Beeswax samples were spiked before (BF samples) or after (AF samples) sample treatment, with different amounts of the standards to prepare the matrix-matched standards for validation (QC samples calibration curves), matrix-effect, and sample treatment studies this is described in subsection 3.3. It must be pointed out that it was necessary to heat the beeswax at 60ºC when spiking with the neonicotinoids to obtain homogenous BF samples. This procedure has proven to be effective in previously published studies [9]. Each QC sample was prepared with 1.0 g of beeswax spiked with three different concentrations of neonicotinoids within the linear range. These were as follows: low QC- LOQ (see Table 2); medium QC- 32 µg/kg; high QC- 250 µg/kg. All standard solutions were stored in glass containers in darkness at -20ºC; working matrix-matched solutions were stored in glass containers and kept in the dark at -20ºC.

2.3. Sample procurement and treatment

2.3.1. Samples

Beeswax samples (n = 21) were collected from apiaries in different Spanish regions in which an insecticide treatment with neonicotinoids has been applied. In this study, all beeswax samples were examined in triplicate, and also underwent a preliminary analysis by UHPLC-MS/MS in order to check for the presence of neonicotinoids. Once it was confirmed that there was no residual trace of the studied compounds, subsamples of beeswax were used as blanks to prepare matrix-matched standards. Finally, beeswax samples collected were mixed and grounded with dry ice to keep them cool for

obtaining optimum sample homogeneity; subsequently, they were stored in the dark at -20ºC until analysis.

2.3.2. Sample preparation

Briefly, 1.0 g of beeswax was weighed in a 50 mL centrifuge tube, after which 10 mL of a methanol and ethyl acetate mixture (70:30, v/v) were added. The mixture was mechanically shaken for 5 min at 960 oscillations per min in a vibromatic mechanical shaker and then centrifuged for 3 min at 5°C and 10000 rpm. Then, centrifugation tubes were left to cool in a polystyrene box filled with dry ice for 3 min. Afterwards, supernatant was collected and transferred to a 15 mL dSPE EMR-Lipid tube. It was immediately shaken for 30 s in a vortex device and centrifuged for 3 min at 5ºC and 10000 rpm. As the final step, the supernatant was transferred to a 25 mL conical flask and evaporated to dryness in a rotary evaporator at 60°C. The dry extract was reconstituted with 1 mL of a methanol and water (80:20, v/v) mixture, and the resulting solution was passed through a 0.45 μm nylon filter before injection into the UHPLC–MS/MS system. Figure 1 outlines the steps of the extraction procedure used during the present study.

2.4. UHPLC-MS/MS system

An UHPLC system (ACQUITY, Waters, Milford, MA, USA) and a quadrupole-time-of-flight (QTOF) mass spectrometer (maXis impact, Bruker Daltonik GmbH, Bremen, Germany) were coupled through and electrospray (ESI) interface, which was operated in the positive mode ionization mode. Data were acquired and processed with software Data Analysis 4.1 and Qualitative Analysis from Bruker Daltonik. A Kinetex® EVO fused-core type column (C18, 50 x 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 (Torrance, CA, USA). After an optimization study, the mobile phase was selected; this was 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, 5:95, v/v); (ii) 1.5-3.0 min (A–B, 33:67, v/v); (iii) 3.0-4.5 min (A–B, 70:30, v/v); (iv) 4.5–7.5 min (A–B, 90:10, v/v); (v) 7.5–8.5 min (A–B, 70:30, v/v); (vi) 8.5–9.0 min (A–B, 33:67, v/v); (vii) 9.0–10.5 min (A–B, 5:95, v/v). Injection volume and column temperature were set at 5 µL and 35ºC, respectively. Optimal detection conditions were set as follows: capillary voltage, 3500 V; drying gas (N_2) flow, 12 L/min; drying gas (N_2) temperature, 220ºC; 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. MS/MS fragmentation was carried out by using a collision energy ramp from 10 to 30 eV (see Table 3). A window of 0.01 *m/z* for the extracted ion chromatograms (EIC) was used in order to extract the exact mass. Quantification was performed with the product ion that provided the highest signal; meanwhile, a second product ion was used for confirmation purposes.

3. Results and discussion

3.1. UHPLC-MS/MS optimization

3.1.1. UHPLC

We recently published a paper concerning the analysis of the seven neonicotinoids in bee pollen [10], we optimized the 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 x 2.1 mm, 1.7 µm, 100 Å) column due to their good performance. We therefore decided to optimize the separation of the insecticides in beeswax with the

Kinetex® EVO column and the same mobile phase components. 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). Tests were also carried out to study the influence of the column temperature (between 20 and 50°C) and the injection volume (between 2 and 10 μL) on the S/N ratio. Results showed an increase in the S/N when up to 5 μL was injected, and a loss of symmetry was observed at temperatures over 35ºC (data not shown); consequently, 5 μL and 35ºC were selected as optimal values. The shortest analysis time was obtained with the chromatographic conditions described in subsection 2.4. With such conditions the overall run time was 10.5 min, eluting the last of the insecticides in less than 5.5 min (see Figure 2), which, to our knowledge, is the fastest proposal that has been published in which several neonicotinoids were simultaneously determined in beeswax.

3.1.2. 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 [10]. 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 and Table 3). 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 3), 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 3).

3.2. Optimization of the sample treatment

Firstly, it was decided that a solvent extraction should initially be tested for sample treatment because of its simplicity and relatively low cost in comparison with some of the procedures that are summarized in Table 1. The extraction procedure was optimized on beeswax samples spiked with different amounts of the selected neonicotinoids. Firstly, a study was made of the amount of beeswax that would be used in the experiments (0.5-1.5 g). It was found that 1.0 g of beeswax was the maximum weight that could be used, as recoveries were adequate and the S/N ratios showed an improvement in relation to lower weights, which were quite similar to those obtained for greater amounts of beeswax. Several tests were made with 10 mL of diverse solvent mixtures, which were chosen on the strength of several preliminary experiments (methanol and ethyl acetate; acetonitrile and ethyl acetate; methanol and water; ethanol and water; acetonitrile and water), in order to extract neonicotinoids. The best results in terms of recoveries (> 80%) were obtained when a methanol and ethyl acetate mixture (70:30, v/v) was used (see Supplementary Material, Figure 1S); as with the other solvents, the recoveries were lower than 80% in most cases. Once the extractant mixture was selected, the influence of certain extraction parameters such as volume (5-15 mL), extraction time (1-10 min) and centrifugation time (1-10 min) was sequentially tested in order to obtain optimal conditions. The best extraction efficiencies (recovery percentages > 80%) were achieved when using 5 mL of extractant mixture (see Supplementary Material, Figure 2S) over 5 min and 3 min for extraction and centrifugation times (data not shown), respectively. Following this, and so as to precipitate some of the beeswax components to increase the sample clean-up [14,16,17], the mixture was left to cool in a polystyrene box filled with dry ice for 3 min. As, however, beeswax is a complex matrix that contains several substances, this freezing step was not enough to remove certain matrix components which affected ionization of

the neonicotinoids, like lipids, causing a significant signal suppression, especially for NT and DN (> 25%; data not shown). Consequently, an additional clean-up step was introduced by using a recently commercialized sorbent (enhanced matrix removal-lipid; EMR-lipid), which was chosen as it has shown promising results when analyzing pesticides, including neonicotinoids, in a complex matrix (honeybee; [1]). This novel sorbent contains C_{18} and some special kind of polymers, although specific details of the structure of EMR-Lipid are still a trade secret [1]. The EMR-Lipid material is a sorbent with pores that selectively bind long unbranched hydrocarbon chains. Polar headgroups remain on the solvent-exposed side, while the analytes do not interact with the sorbent and remain in solution [20]. Thus, it is possible to remove lipids without losing the analytes. Once, then, the mixture had cooled, the supernatant was collected, transferred to a 15 mL dSPE EMR-Lipid tube containing 1 g of sorbent, and centrifuged for 3 min. It was found that in all cases recoveries were over 80%, whilst a significant improvement was observed in the matrix effect (< 20% in all cases) and in the removal of interferences (data not shown). Following this last clean-up step, the supernatant was collected, transferred to a 25 mL conical flask and gently evaporated to dryness in a rotary evaporator at 60ºC. It is worth mentioning that no loss of neonicotinoids was observed during the evaporation step. Next, reconstitution was deemed important so as to improve extraction efficacy. Different volumes (0.5-2.0 mL) of a methanol and water mixture (80:20, v/v), which were selected on the basis of the good results obtained in previous research [10], were assayed. Since it was observed that amounts of solvent in excess of 1 mL did not improve the recovery percentages (data not shown), 1 mL of the mixture was deemed appropriate to reconstitute the dry residue. In order to ascertain the effectiveness of the proposed sample treatment, neonicotinoid responses were compared. These were the peak areas obtained from blank samples spiked at three

different neonicotinoid concentrations (QC levels), either prior to (BF samples) or following (AF samples) sample treatment. Recovery values ranged from 93-106% in all cases (see Table 4); this indicated that the sample treatment was both appropriate and effective. These recovery values are similar to or better than those obtained with previous proposals (see Table 1), but with the advantage that the matrix effect was minimized in such a way that solvent-based calibration standards could be used to quantify the neonicotinoid insecticides, which could be related with the use in the clean-up step of the novel EMR-lipid sorbent. This is particular relevant, since the matrix effect was neither minimized nor even studied in several of the previous publications (see Table 1). Finally, these results have demonstrated that the proposed procedure is an efficient, shorter, and greener alternative to the existing procedures for analyzing these pesticides in beeswax. The recovery values (93-106%), overall procedure time (20 minutes), and volume/amount of reagents, especially organic solvents (11 mL), are generally better to reported values (see Table 1).

3.3. Method validation

Validation of the method was based on the Eurachem Guidelines [18], the current European legislation for pesticide residues analysis in foods [19] and recent studies [10,21]. Reference standards were prepared in solvent as well as in matrix (i.e. matrix-matched calibration) and treated with the selected procedure.

3.3.1. Selectivity

To evaluate the selectivity of the proposed method, a set of unspiked blank beeswax samples (n = 10) was injected onto the chromatographic system and the results were compared with those obtained for spiked beeswax samples. As can be seen in Figure 4, no chromatographic interference was observed at analyte retention times in any of the blank

samples. Meanwhile, for identification of neonicotinoid peaks in the samples (spiked and with endogenous neonicotinoid content), a comparison was made of the mass spectra of each of the neonicotinoid peaks in standard solutions and beeswax samples with endogenous or spiked pesticide content; concentrations were comparable and measurements were taken under the same conditions. In fact, both mass spectra were quite similar, as can be seen for IMI (see Supplementary Material, Figure 3S), although some minor differences in ion intensity were observed. Nevertheless, the relative intensities of the ions/transitions in the matrix-matched samples concurred with the corresponding standard solutions to within ± 10% (data not shown); this is lower than the maximum permitted rates (± 30%; [18]).

3.3.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) were experimentally determined by injection of blank beeswax samples (n = 10), in which the absence of insecticide residues was previously confirmed, and measurement of the magnitude of background analytical response at the elution time. The LODs and LOQs were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively, and those limits were calculated for MS/MS experiments. Low LODs and LOQs were obtained for all neonicotinoids, ranging from 0.4 to 1.4 µg/kg (LODs) or from 1.2 and 4.9 µg/kg (LOQs). The LOQs we obtained are comparable to those of previous publications (see Table 2), and they are also much lower than the maximum residue limits (MRLs) established by the European Commission for several of these pesticides in honey and other apiculture products (50-200 µg/kg; [22]).

3.3.3. Matrix effect

To ascertain how the matrix influenced ESI ionization for QTOF, a comparison was made of the responses (analyte peak area) of standard working solutions in solvent and blank

beeswax extracts (AF samples) spiked at three different concentrations (QC levels). Responses at the different concentrations assayed (QC levels) ranged from 81% to 106% in all cases (see Supplementary Material, Table 1S). As can be observed, the values were slightly lower for DN and NT, but generally speaking there was not a great disparity between compounds or QC levels. These results complied with the criteria of the European Commission for pesticide residue analysis (± 20% of the response from standard solutions; [18]). To confirm this finding, the slopes of the standard and matrix-matched calibration curves were compared (see Table 2), and it was found that in all cases overlapping occurred at the confidence intervals. Therefore, it was concluded that the matrix did not significantly affect ESI ionization of the analytes.

3.3.4. Working range

It was possible to measure neonicotinoid insecticides with solvent-based calibration standards, as the matrix did not significantly affect analyte ionization (see subsection 3.3). Regarding the reference standards in solvent, the analytical ranges were between LOQ and 250 µg/L (calibration levels of LOQ, 10, 25, 50, 75, 150, 250 µg/L). Meanwhile, blank beeswax was treated according to the proposed procedure (see subsection 2.3) and spiked with variable amounts of the seven neonicotinoids over an analytical range of between LOQ and 250 µg/kg (calibration levels of LOQ, 10, 25, 50, 75, 150, 250 µg/kg). As may be appreciated, 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) against the analyte concentration on the *x*-axis. Linearity was evaluated by visual analysis of the plots; this involved calculating the determination coefficients (R^2) and making 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 0.99 in all cases (see Table 2). The deviation of the back-calculated standard concentrations was at all times equal to or less than 5% from the nominal values. Absence of bias was confirmed by a *t* test and by studying the distribution of residuals (data not shown).

3.3.5. Precision

Precision experiments were performed concurrently; these involved repeated analysis using blank beeswax samples spiked at three different concentrations (low, medium and high QC levels) on the same day (n=6) (intra-day precision [18], repeatability [19]) experiments, experiments over three consecutive days (n=6) (inter-day precision [18], or partial reproducibility [19]. Results, expressed as the percentage of relative standard deviation (%RSD), were at all times less than 10% (see Supplementary Material, Table 2S). Moreover, these values displayed no significant differences depending on the neonicotinoid or QC level. The results indicate that the proposed methods are precise enough according to existing European norms (%RSD ≤ 20;[18]), and for this reason no internal standard was required.

3.3.6. Trueness

It was evaluated by means of recovery experiments (as a measure of trueness), by comparing the results (analyte peak area) obtained from blank beeswax 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 93% to 106% with %RSD values lower than 10% in all cases (see Table 4). Those values, which are similar or better than the obtained in previous works (see Table 1), fulfilled the requirements established by the European Commission [18] for pesticide residue analysis (recovery percentages between 70% and 120%; %RSD ≤ 20).

3.3.7. Ruggedness

A ruggedness study was conducted using the Youden procedure [19,21,23]. Seven variables were chosen and deliberately altered: the percentage of formic acid in the mobile phase (0.10 and 0.09%), volume injected (5.0 and 5.2 μL), column oven temperature (35 and 33ºC), flow rate (0.30 and 0.29 mL/min), percentage (20:80 and 22:78, v/v) and volume (1.0 and 0.95 mL) of the reconstitution solution (water and methanol) and volume (10.0 and 10.5 mL) of the extraction mixture (methanol and ethyl acetate mixture; 70:30, v/v). Eight experiments were conducted to evaluate the seven factors, by spiking a beeswax sample with the selected neonicotinoids at medium QC level and performing three independent replicates. The effect of a particular variable was estimated by subtracting the mean result obtained with the "nominal" value of the variable from that obtained with the "alternative" value. An examination of the results of the experiments showed that the proposed method was robust when subjected to small variations of the seven parameters analysed (< ± 2; data not shown).

3.3.8. Uncertainty

Combined method uncertainty (%U) was determined in all cases with bias uncertainty ($\%U_{bias}$) together with precision uncertainty ($\%U_P$) based on the equations summarized in Table 3S (see Supplementary Material; [24]. Due to the absence of certified specific reference material or of an official analysis method for determining neonicotinoids in beeswax, 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 guideline [19]. After examining the results (see Supplementary Material, Table 4S) it can be concluded that there was no substantial variation in the $\%U_{bias}$ and $\%U_P$ values, and consequently of the %U (< 18% in all cases) depending on the neonicotinoid and spiking level. Therefore, no common major contributor to method uncertainty (%U) can be identified.

3.4. Application of the method

The validated methodology was applied to determine neonicotinoid residues in twenty-one beeswax samples from different Spanish regions. All analyses were made in triplicate. No residues of the insecticides studied were detected in nineteen of the samples, whilst residues of IMI were detected in two samples; however, this could not be measured due to low concentration (< LOQ; see Figure 3). The lack of detection of neonicotinoid residues in most of the samples does not, however, mean that it was a wasted effort to develop a method for screening compounds that did not exist in those samples, since residues of neonicotinoids have already been reported (see Table 1).

4. Conclusions

In this work, a new and fast analytical methodology to simultaneously identify and measure seven neonicotinoid insecticides in beeswax has been developed and validated. 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 publications. The proposed extraction method has proven to be rapid, efficient and to require low consumption of organic solvents, in line with the principles of green analytical chemistry and in comparison, with previous studies. Moreover, this is the first time the suitability of a recently commercialized sorbent (EMR-Lipid) for effective clean-up in beeswax has been demonstrated. Neonicotinoid insecticides can be quantified with solvent-based calibration standards, as the matrix did not significantly affect analyte ionization; this is a significant advantage compared with some of the previous proposals. Additionally, the excellent sensitivity reached led to LOQs much lower than the MRLs established by the European Commission for honey and other apiculture products, as well as being comparable with the best values reported in the literature. Analysis of beeswax samples

from different Spanish regions revealed the absence of residues of the insecticides under study in most cases, although it must be remarked that residues of IMI (< LOQ) were found in two samples.

Acknowledgements

Authors thank David Rixham (White Rose English School, Valladolid, Spain) for performing the English revision. In addition, authors thank the "*Laboratorio de Técnicas Instrumentales*" (University of Valladolid) for using their UHPLC-QTOF system.

Funding

This work was supported by the Spanish Ministry "*Economía, Industria y Competitividad*" and INIA-FEDER (grant numbers RTA2013-00042-C10-03 and 06);

Conflict of interest statement

The authors of this manuscript declare no conflict of interest.

Abbreviations:

ACET, acetamiprid**; AF**, samples spiked after sample treatment; **BF**, samples spiked before sample treatment; **CLO**, clothianidin; **DN**, dinotefuran; **EIC**, extracted ion chromatogram; **EMR**, enhanced matrix removal-lipid; **IMI**, imidacloprid; **MRM**, multiple reaction monitoring; **NT**, nitenpyram; **PSA**, primary secondary amine; **QC**, quality control; **QTOF**, quadrupole-time-of-flight; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RSD**, relative standard deviation; **THIA**, thiacloprid; **TMX**, thiamethoxam; **%U**, uncertainty of the method; **%U_{bias}**, uncertainty of the bias;

UHPLC, ultra-high performance liquid chromatography; **$\%U_P$**, uncertainty of the precision.

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Figure captions

Figure 1.- Analytical procedure work-up flow chart.

Figure 2.- Representative UHPLC-ESI-MS chromatograms (EIC in positive mode using the quantification ions, see Table 3) obtained from: (**A**) a blank beeswax sample; (**B**) a spiked (32 µg/kg) beeswax sample in: DN (1), NT (2), TMX (3), CLO (4), IMI (5), ACET (6), and THIA (7). The UHPLC-ESI-MS conditions are described in subsection 2.4 and Table 3.

Figure 3.- Representative UHPLC-ESI-MS chromatograms (EIC in positive mode using the quantification ions, see Table 3) obtained from a beeswax sample with endogenous IMI content (< LOQ). The UHPLC-ESI-MS conditions are described in subsection 2.4 and Table 3.

Figure 1

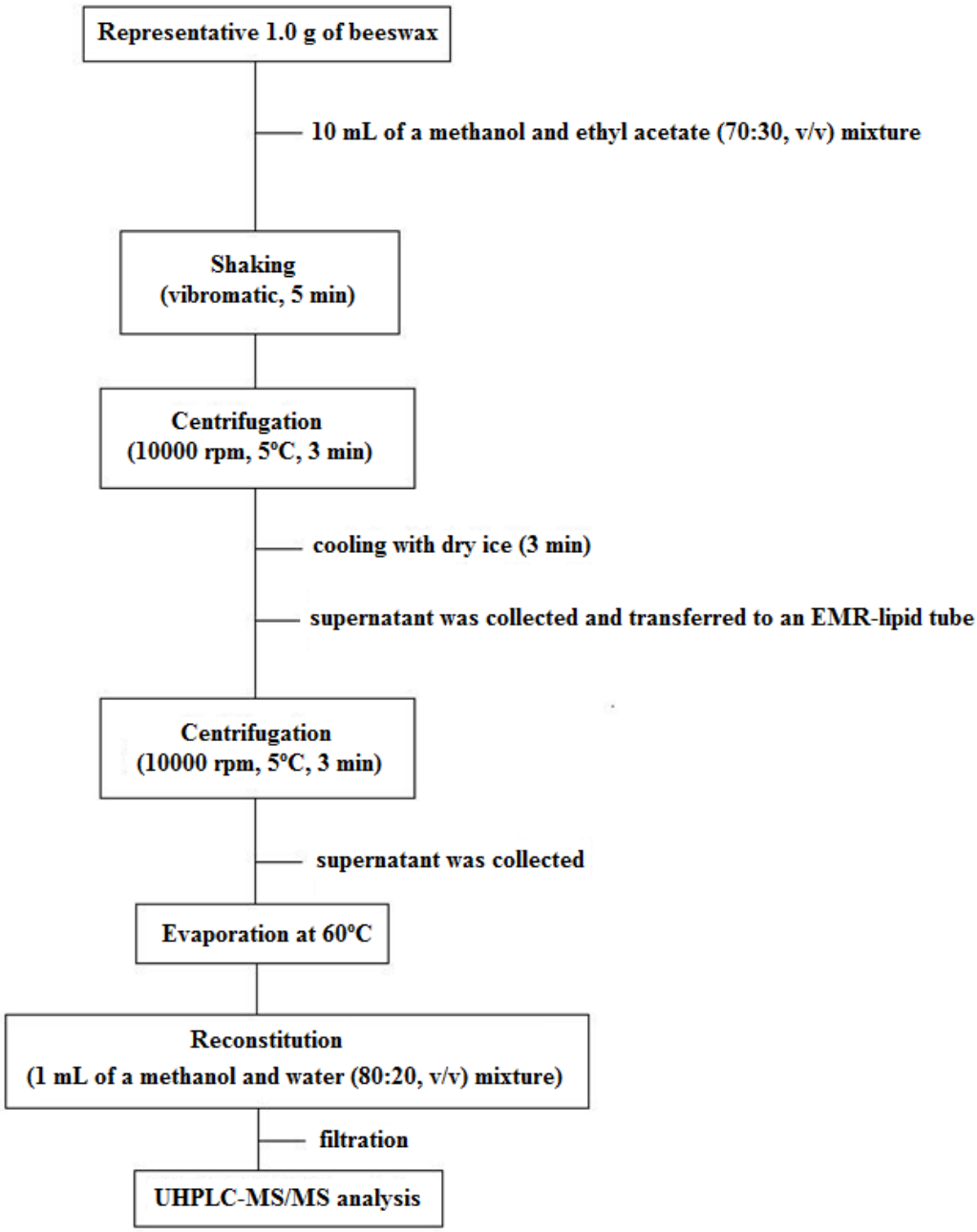
Representative 1.0 g of beeswax
10 mL of a methanol and ethyl acetate (70:30, v/v) mixture
Shaking
(vibromatic, 5 min)
Centrifugation
(10000 rpm, 5°C, 3 min)
cooling with dry ice (3 min)
supernatant was collected and transferred to an EMR-lipid tube
Centrifugation
(10000 rpm, 5°C, 3 min)
supernatant was collected
Evaporation at 60°C
Reconstitution
(1 mL of a methanol and water (80:20, v/v) mixture)
filtration
UHPLC-MS/MS analysis

Figure 2

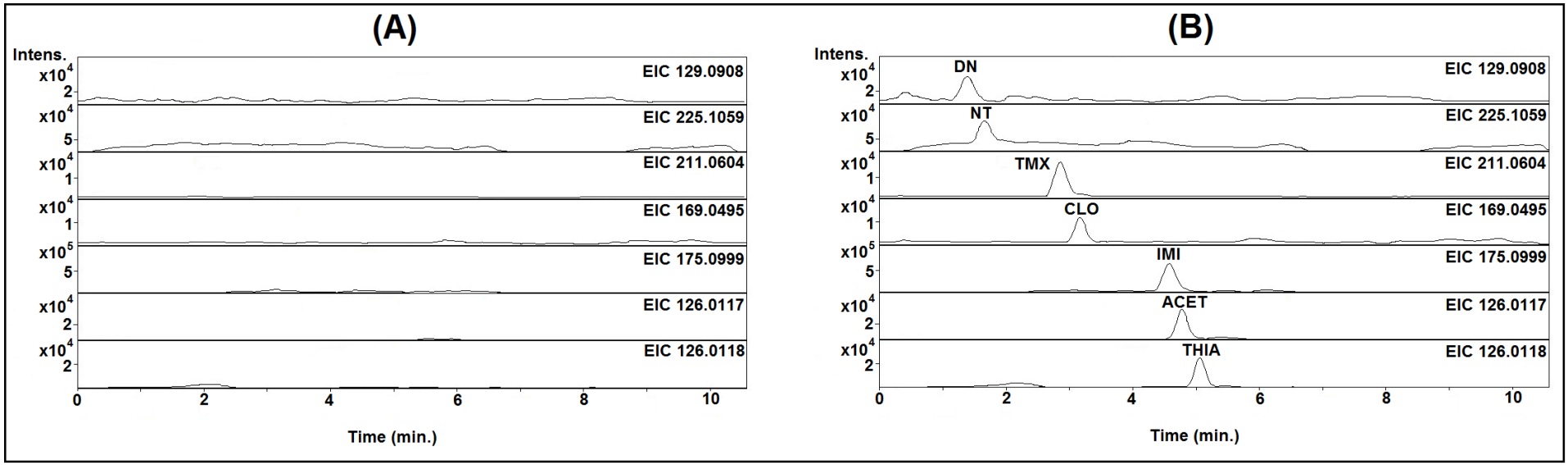
(A)
Intens.
x10^4
2
EIC 129.0908
x10^4
5
EIC 225.1059
x10^4
1
EIC 211.0604
x10^4
1
EIC 169.0495
x10^5
5
EIC 175.0999
x10^4
2
EIC 126.0117
x10^4
2
EIC 126.0118
0
2
4
6
8
10
Time (min.)
(B)
Intens.
DN
NT
TMX
CLO
IMI
ACET
THIA
EIC 129.0908
EIC 225.1059
EIC 211.0604
EIC 169.0495
EIC 175.0999
EIC 126.0117
EIC 126.0118
Time (min.)

Figure 3

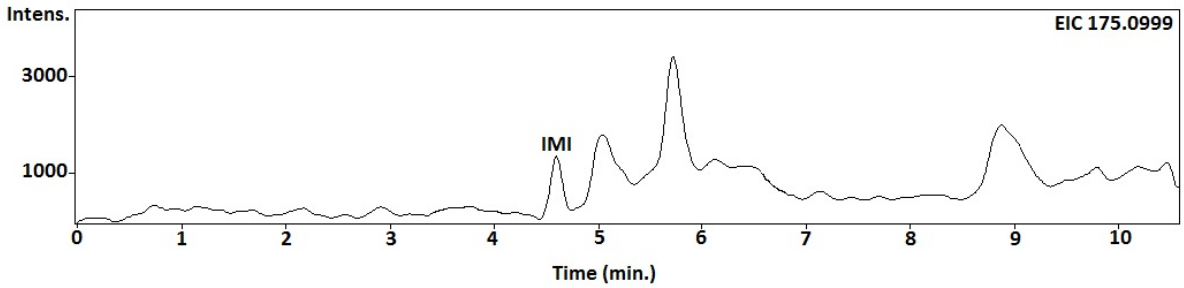

Table 1.- HPLC published methods for determining neonicotinoids in beeswax.

Neonicotinoid (overall pesticide number)	Sample treatment (overall time, reagents consumption)	Matrix Effect[A]	Recoveries (%)[A]	LOQs (µg/Kg)[A]	LC system (min, SP)	Reference
3-7 (13)	SLE + dSPE (DE)+ EV (> 15 h, 34 mL, 2.4 g)	NS	92-97	1	UHPLC-MS/MS (12, phenyl-hexyl)	**[4]**
3-7 (13)	SLE + dSPE (DE)+ EV (> 15 h, 38 mL, NS)	No	72-97	1	UHPLC-MS/MS (12, phenyl-hexyl)	**[6]**
3,5-7 (> 100)	QuEChERS (PSA+C_{18}) (25 min, 27 mL, 7.75 g)	NS	NS	1-2 (LOD)	HPLC-MS/MS (NS, C_{18})	**[7]**
5 (1)	SPE (DE)+ EV (25 min, 46 mL, NS)	Yes	95-103	0.5	HPLC-MS (5, C_{18})	**[8]**
1-7 (7)	SPE (DE) + EV (25 min, 46 mL, NS)	Yes	85-105	1.5-7.0	HPLC-MS (25, C_{18})	**[9]**
3,5,7 (51)	[QBM]SLE + freezing + dSPE (PSA+C_{18}) (> 2h, 10 mL, 50 mg)	No	100-120	10	HPLC-MS/MS (> 30, C_{18})	**[14]**
3,5,6 (58)	QuEChERS (PSA+C_{18}) (> 12 h, 10 mL, 100 mg)	Yes	70-120	2.5-10	HPLC-MS/MS (> 30, C_{18})	**[15]**
3-7 (30)	[QBM]SLE + freezing + dSPE (PSA+C_{18}) (> 2h, 10 mL, 50 mg)	NS	NS	1-10	HPLC-MS/MS (35, C_{18})	**[16]**
3,6,7 (3)	[QBM]SLE + freezing + dSPE (PSA+C_{18}) (> 2h, 10 mL, 50 mg)	NS	NS	1-10	HPLC-MS/MS (> 30, C_{18})	**[17]**
1-7 (7)	SLE + freezing + dSPE (EMR-lipid) + EV (20 min, 11 mL, 1 g)	No	89-116%	1.2-4.9	UHPLC-MS/MS (10.5, C_{18})	**Present study**

[A]data related only to neonicotinoids; [QBM]QuEChERS based method; **1**-DN; **2**-NT; **3**-TMX; **4**-CLO; **5**-IMI; **6**-ACET; **7**-THIA.
DE; diatomaceous earth**; dSPE,** dispersive SPE; **EV,** evaporation; **HPLC,** high performance liquid chromatography; **MS,** mass spectrometry; **MS/MS,** tandem mass spectrometry; **NS**, not specified; **PSA,** primary secondary amine; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **SLE**, solid-liquid extraction; **SP,** stationary phase**; SPE,** solid-phase extraction; **UHPLC,** ultra-high performance liquid chromatography.

Table 2.- Calibration curve data, LOD and LOQ values (n =6).

Compound	Calibration curve	Analytical range[A]	Slope confidence intervals	R^2	LOD	LOQ (Mean ± SD)
DN	Standard	3.2-250	$2.9 \times 10^4 \pm 3.5 \times 10^3$	0.9990	0.9	3.1 ± 0.18
	Matrix-matched		$2.4 \times 10^4 \pm 3.1 \times 10^3$	0.9879		
NT	Standard	3.5-250	$3.7 \times 10^4 \pm 3.3 \times 10^3$	0.9957	1.0	3.6 ± 0.22
	Matrix-matched		$3.1 \times 10^4 \pm 4.7 \times 10^3$	0.9899		
TMX	Standard	4.1-250	$2.1 \times 10^4 \pm 4.1 \times 10^2$	0.9990	1.2	4.3 ± 0.14
	Matrix-matched		$2.1 \times 10^4 \pm 2.5 \times 10^2$	0.9996		
CLO	Standard	4.9-250	$1.9 \times 10^4 \pm 1.3 \times 10^3$	0.9999	1.4	5.1 ± 0.27
	Matrix-matched		$2.0 \times 10^4 \pm 9.5 \times 10^2$	0.9996		
IMI	Standard	3.8-250	$1.9 \times 10^4 \pm 1.6 \times 10^3$	0.9998	1.1	3.7 ± 0.11
	Matrix-matched		$2.0 \times 10^4 \pm 8.9 \times 10^2$	0.9995		
ACET	Standard	2.5-250	$3.2 \times 10^4 \pm 3.9 \times 10^2$	0.9996	0.7	2.7 ± 0.33
	Matrix-matched		$3.3 \times 10^4 \pm 8.6 \times 10^2$	0.9997		
THIA	Standard	1.2-250	$4.8 \times 10^4 \pm 1.8 \times 10^3$	0.9990	0.4	1.5 ± 0.26
	Matrix-matched		$4.6 \times 10^4 \pm 9.6 \times 10^2$	0.9988		

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

Table 3.- 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
CLO	250.0166[A]	131.9622[A]	15
		169.0495[B]	15
IMI	256.0623[A]	175.0999[B]	25
		209.0614[A]	25
ACET	223.0780[A]	56.1002[A]	30
		126.0117[B]	30
THIA	253.0342[A]	126.0118[B]	20
		186.0154 [A]	20

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

Table 4.- Evaluation of the efficiency (recoveries) of the sample treatment. Data obtained as described in subsections 3.2 and 3.3 (n = 6).

	Evaluation of the sample treatment		
	Mean (%) ± RSD (%)		
Quality control (QC) sample	**Low**	**Medium**	**High**
DN	95 ± 3	98 ± 5	99 ± 5
NT	93 ± 4	95 ± 3	97 ± 6
TMX	99 ± 4	97 ± 4	105 ± 5
CLO	101 ± 2	104 ± 3	106 ± 6
IMI	106 ± 4	103 ± 7	101 ± 4
ACET	102 ± 4	96 ± 3	101 ± 6
THIA	97 ± 7	94 ± 6	96 ± 5

Low QC- LOQs (see Table 2); **Medium QC**-32 µg/kg; **High QC**-250 µg/kg.

FAST DETERMINATION OF NEONICOTINOID INSECTICIDES IN BEESWAX BY ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY USING AN ENHANCED MATRIX REMOVAL-LIPID SORBENT FOR CLEAN-UP

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Table 1S.- Evaluation of the matrix effect (comparison of responses). Data obtained as described in subsection 3.3 (n = 6).

Quality control (QC) sample	Evaluation of the matrix effect		
	Mean (%) ± RSD (%)		
	Low	Medium	High
DN	85 ± 6	83 ± 7	81 ± 7
NT	81 ± 5	84 ± 7	82 ± 6
TMX	105 ± 4	97 ± 6	98 ± 5
CLO	101 ± 7	102 ± 6	104 ± 5
IMI	106 ± 4	99 ± 5	101 ± 7
ACET	102 ± 7	97 ± 6	101 ± 6
THIA	89 ± 5	91 ± 7	92 ± 7

Low QC- LOQs (see Table 2); **Medium QC**-32 µg/kg; **High QC**-250 µg/kg.

Table 2S.- Summary of precision studies for the neonicotinoid determination in spiked beeswax samples (n=6).

Validation parameter		DN	NT	TMX	CLO	IMI	ACET	THIA
Intraday precision (%RSD)	Low QC	5	6	7	7	3	4	4
	Medium QC	5	7	5	6	5	5	5
	High QC	5	6	7	5	7	4	7
Interday precision (%RSD)	Low QC	9	8	7	5	7	8	6
	Medium QC	8	5	8	5	7	7	5
	High QC	8	7	6	8	5	7	7

low QC-LOQs (see Table 2); **medium QC**-32 µg/kg; **high QC**-250 µg/kg.

Table 3S.- Equations employed for measurement uncertainty.

Uncertainty of repeatability (U_{rep})	$U_{rep}=\frac{S_{rep}}{\sqrt{n}}$
Uncertainty of repeatability (%U_{rep})	$\%U_{rep} = 100 \times U_{rep}$
Uncertainty inter-days ($U_{inter\text{-}days}$)	$U_{inter-days} = \sqrt{\frac{\bar{x} - RV}{n}}$
Uncertainty of precision (%U_P)	$\%U_P = \sqrt{\%U_{rep}^2 + \%U_{inter-days}^2}$
Relative error (%RE)	$\%RE = 100\ (\bar{x} - RV)$
Uncertainty typical of the measure (U_m)	$Um_= \sqrt{\sum_{i=1}^{N} u_i^2}$
Uncertainty of reference value (U_{RV})	$U_{RV} = \frac{Um}{k}$
Uncertainty of bias (U_{bias})	$U_{bias} = \sqrt{U_{RV}^2 + \frac{U_{rep}^2}{n}}$
Uncertainty of bias (%U_{bias})	$\%U_{Bias} = 100 \times U_{Bias}$
Uncertainty of the method (%U)	$\%U = \sqrt{\%U_P^2 + \%U_{bias}^2}$

k, coverage factor (= 2; 95% of confidence); **n,** number of replicates (n = 6); **RV,** reference value (QC levels); **u_i,** uncertainty typical of the different contributions; **S_{rep},** standard deviation of repeatability; **$\bar{x}$,** average recoveries.

Table 4S.- Measurement uncertainty.

	DN			NT			TMX			CLO			IMI			ACET			THIA		
	Low QC	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**	**Low QC**	**Medium QC**	**High QC**
%U_P	9.25	8.27	8.26	8.42	5.84	7.44	7.57	8.27	6.59	5.75	5.53	8.23	7.09	7.28	5.75	8.16	7.32	7.18	6.23	5.45	7.61
%U_{bias}	11.4	3.41	1.23	6.30	6.27	1.72	3.19	3.41	1.61	4.52	2.80	0.860	5.57	4.53	3.30	8.65	9.58	7.50	16.2	5.25	4.26
%U	14.7	8.95	8.35	10.51	8.55	7.64	8.21	8.95	6.78	7.31	6.19	8.27	9.02	8.57	6.63	11.9	12.1	10.4	17.4	7.55	8.72

Low QC-LOQs (see Table 2); **Medium QC**-32 µg/kg; **High QC**-250 µg/kg.

%U_P, uncertainty of precision (repeatability and inter-day precision); **%U_{bias}**. uncertainty of the bias; **%U**, combined uncertainty of the method.

Figure 1S.- Evaluation of the extraction efficiency (recoveries) obtained for blank beeswax samples spiked with the selected neonicotinoids at medium QC (32 µg/kg) after testing with the extractant mixtures that provided the best results. Data represent the mean of three replicates ± the relative standard deviation of the mean (narrow bars) obtained with 10 mL of the selected solvent mixtures.

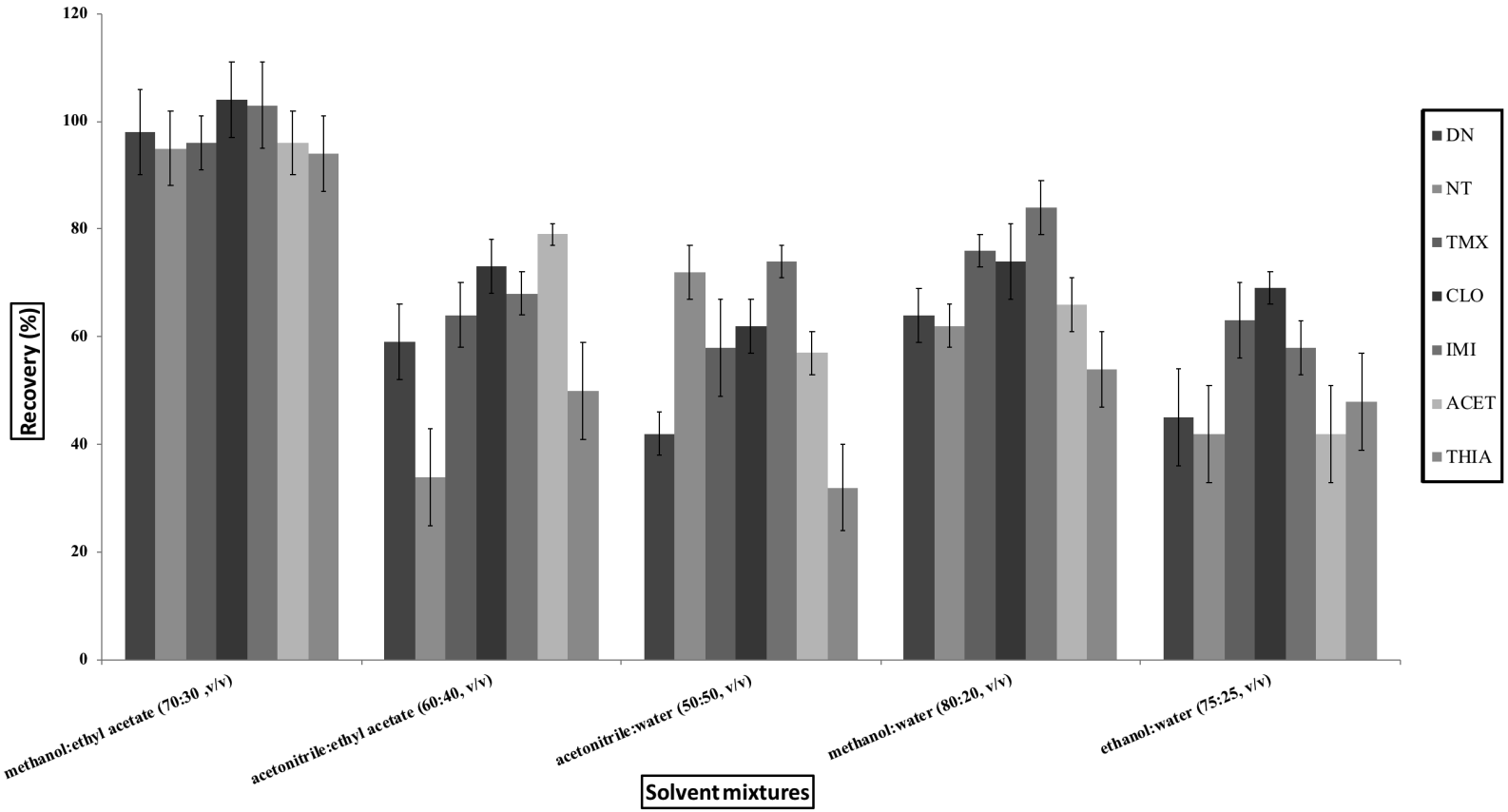

Figure 2S.- Evaluation of the extraction efficiency (recoveries) obtained from blank beeswax samples spiked with the selected neonicotinoids at medium QC (32 µg/kg) after testing with the different volumes (5-15 mL) of selected extractant mixture (methanol and ethyl acetate, 70:30, v/v). Data represent the mean of three replicates ± the relative standard deviation of the mean (narrow bars).

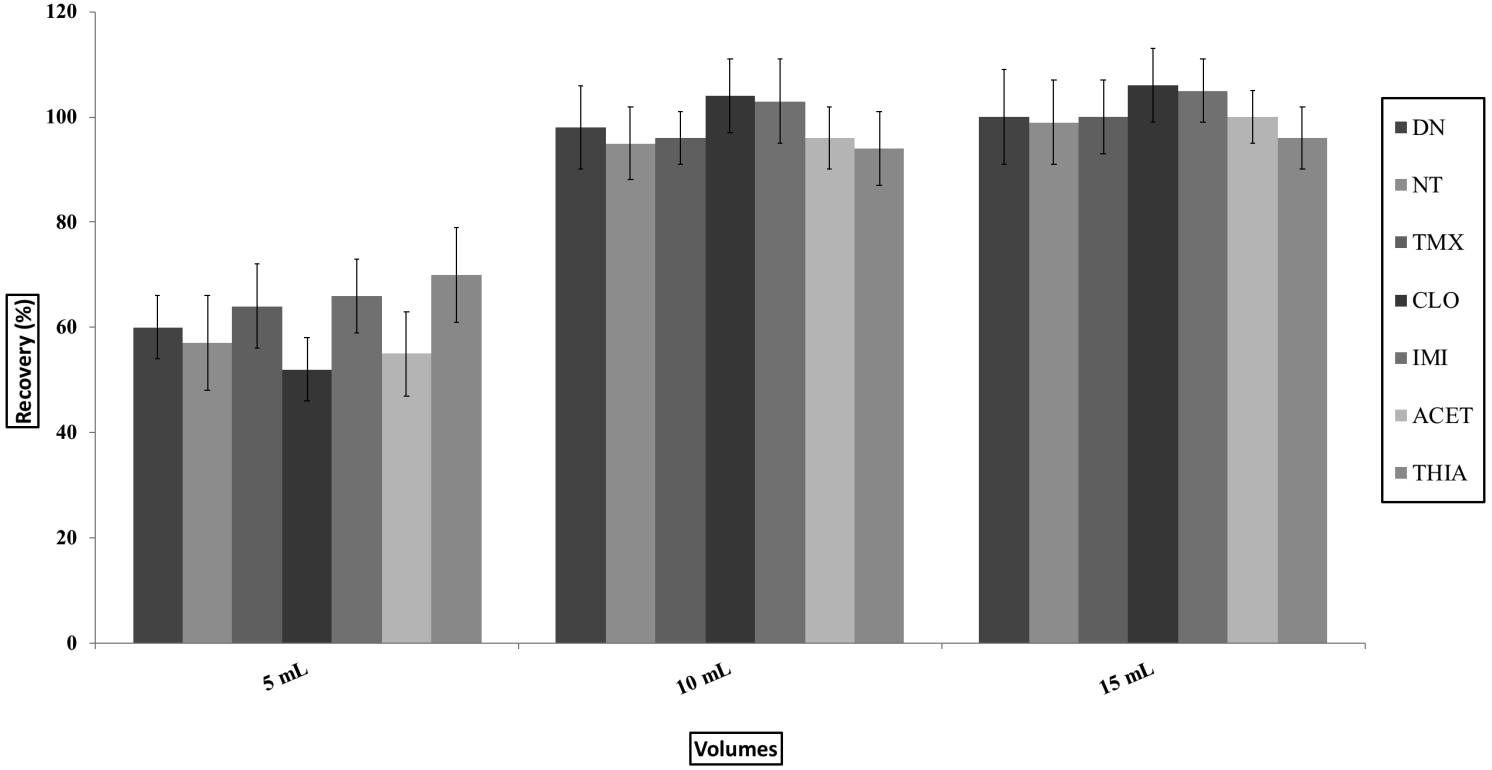

Figure 3S.- Full scan ESI-MS/MS spectra of IMI in: (**A**) standard solution (32 µg/L); (**B**) a beeswax sample with endogenous IMI content (< LOQ). The ESI-MS/MS conditions are summarized in subsection 2.4 and Table 3.