Silvia Valverde
José Luis Bernal
María Teresa Martín
María Jesús Nozal
José Bernal

IU CINQUIMA, Analytical Chemistry Group, University of Valladolid, Valladolid, Spain

Received March 18, 2016
Revised April 28, 2016
Accepted May 12, 2016

Research Article

Fast determination of neonicotinoid insecticides in bee pollen using QuEChERS and ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

In this study, a new method has been developed to determine seven neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) in bee pollen using ultra-high performance liquid chromatography coupled to a selective MS detector (qTOF). An efficient sample treatment involving an optimized quick, easy, cheap, effective, rugged and safe method was proposed. In all cases, average analyte recoveries were between 91 and 105%, and no matrix effect was observed. Chromatographic analysis (6.5 min) was performed on a core-shell technology based column (Kinetex® EVO C_{18}, 50×2.1 mm, 1.7 μm, 100 Å). The mobile phase consisted of 0.1% formic acid in water and 0.1% of formic acid in ACN, with a flow rate of 0.3 mL/min in gradient elution mode. The fully validated method was selective, linear from LOQ to 500 μg/kg, precise and accurate; relative standard deviation and relative error values were below 8%. Low limits LODs and LOQs were obtained, ranging from 0.6 to 1.3 μg/kg (LODs) and 2.1 to 4.0 μg/kg (LOQs). The method was applied to neonicotinoid analysis in several commercial bee pollen samples from different Spanish regions.

Keywords:
Bee pollen / Insecticides / Mass spectrometry / Neonicotinoids / Ultra-high performance liquid chromatography
DOI 10.1002/elps.201600146

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam; see proposed structures in Supporting Information Fig. S1), are gaining larger shares in the global crop protection market due to their broad spectrum of efficacy, their systemic and translaminar action, and their pronounced residual activity and a unique mode of action [1]. 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 [2], and for example non-target organisms, such as honeybees [3]. This is particularly relevant, if it is taken into account that exposure to neonicotinoid insecticides has been identified as one of the potential factors involved in the sudden decline in adult honeybee population, commonly known as colony collapse disorder [1, 4–6]. To tackle the colony collapse disorder problem, the European Union has recently adopted a proposal (Regulation (EU) 485/2013; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri = OJ:L:2013:139:0012:0026:en:PDF) to restrict the use of three neonicotinoids (clothianidin, imidacloprid and thiamethoxam) for a two-year period. Depending on the application procedure, some of these compounds could be

Correspondence: José Bernal, IU CINQUIMA, Analytical Chemistry Group, University of Valladolid, Valladolid 47011, Spain
E-mail: jose.bernal@qa.uva.es
Fax: +34-983-186347

Abbreviations: **AF**, samples spiked after sample treatment; **BF**, samples spiked before sample treatment; **EIC**, extracted ion chromatogram; **MRM**, multiple reaction monitoring; **PSA**, primary secondary amine; **QC**, quality control; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RE**, relative error; **UHPLC**, ultra-high performance liquid chromatography

distributed in the rest of the plant by translocation, guttation, or simply because they are sprayed over the crops [7, 8]. Consequently, neonicotinoid residues could be found in pollen from flowers or that collected from beehives, which is the main food source for honeybees [4], and it is also a widely consumed food supplement.

Some of the sample preparation procedures described in the existing literature related to the determination of neonicotinoids in pollen (solid-liquid or SPE) often involved many stages or required huge amounts of solvents [7, 9–12]. However, the current trend in sample preparation techniques is focused on the simplification of those procedures to reduce the amount of reagents and time spent on this step. Thus, in recent years, the sample preparation known as quick, easy, cheap, effective, rugged and safe (QuEChERS) has been predominately used for the extraction of neonicotinoids and other insecticides from pollen [4–6, 8, 13–18]. Different modifications of the QuEChERS methodology have been investigated in these works, such as the use of hexane [4, 5, 8] or a freezing-out step [6] to remove lipids that can interfere with mass spectrometry detection, the incorporation of isotope-labeled neonicotinoids as internal standard (IS) at the beginning of the sample treatment [4–6, 13–18], or the use of different sorbents to perform a SPE [8, 14] or a dispersive SPE (dSPE) as a final clean-up step [4–6, 17, 18]. Different analytical approaches such as ELISA [19], gas chromatography [20], capillary electrophoresis [2, 21] and supercritical fluid chromatrography [22] methods have been employed to analyze neonicotinoids insecticides, although LC with C_8 [6, 9] or C_{18} [1, 4, 5, 7, 8, 10–18] stationary phases is the preferred technique due to the physical–chemical properties of those compounds. In most of the LC based studies the couplings with mass MS [1, 7] or MS/MS [4–6,8–18] have been predominately 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 [12, 14, 17, 18].

Our aim was to develop a new, robust UHPLC-MS/MS method to determine seven neonicotinoid insecticides in bee pollen. A new commercial analytical column based on core-shell technology (Kinetex® EVO) was used for separation; this was particularly significant, as to our knowledge, there are no reports and applications regarding the use of this type of column with neonicotinoids or bee pollen. A specific and efficient extraction and determination procedures have been proposed. To perform this task, some of the published modifications performed on the QuEChERS method were evaluated, with the aim of proposing a new procedure that provided good recoveries, decreased as much as possible the potential matrix effect onto MS/MS detection, and allowed the baseline separation of the neonicotinoids in the shortest time as possible A second goal was to validate this method and apply it for analyzing bee pollen samples from local markets.

2 Materials and methods

2.1 Chemical and materials

Fluka-Pestanal analytical standards of acetamiprid (Det. Purity 99.9%), clothianidin (Det. Purity 99.9%), dinotefuran (Det. Purity 98.8%), imidacloprid (Det. Purity 99.9%), nitenpyram (Det. Purity 99.8%), thiacloprid (Det. Purity 99.9%), thiamethoxam (Det. Purity 99.6%) and thiamethoxam-d3 (Det. Purity ≥ 98%) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). An isotope-labeled standard (thiamethoxam-d3) was chosen as IS, since it has the same physical and chemical properties as the unlabeled analyte. Methanol and ACN (LC grade) were supplied by Lab Scan Ltd. (Dublin, Ireland). Formic acid (98-100% pure) and magnesium sulfate ($MgSO_4$) anhydrous were obtained from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). Sodium chloride (NaCl) and tri-sodium citrate 2-hydrate were supplied by Panreac (Barcelona, Spain), while primary secondary amine (PSA) and C_{18} were provided by Supelco (Bellefonte, PA, 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 used 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 (60:40, v/v) in order to prepare the working solutions. Bee pollen samples (1.0 g) were spiked before (BF samples) or after (AF samples) sample treatment with different amounts of the neonicotinoid insecticides and with 150 μg/kg of the IS to prepare the matrix-matched standards; this is 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 1.0 g of bee pollen spiked with three different concentrations of neonicotinoids within the linear range. These were as follows: low QC—5 μg/kg; medium QC—63 μg/kg; high QC—500 μg/kg. 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 (data not shown).

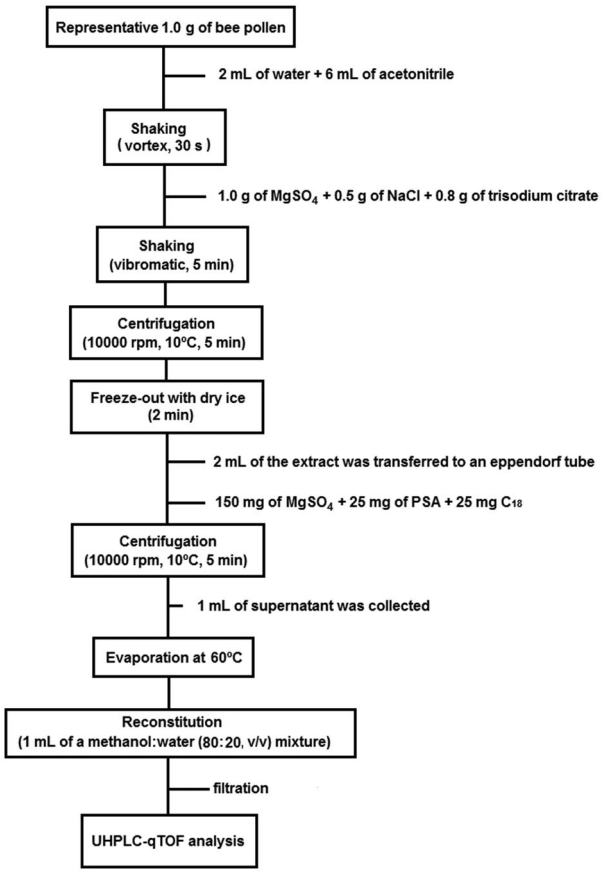

Figure 1. Analytical procedure work-up flow chart.

2.3 Sample procurement and treatment

2.3.1 Samples

Commercial bee pollen ($n = 20$) were purchased in local markets (Valladolid, Spain); these were from different Spanish regions in which an insecticide treatment with neonicotinoids has been applied. They were mixed and dried at +45ºC in an oven, ground and pooled for optimum sample homogeneity, and subsequently stored in darkness at +4ºC until analysis. All the samples underwent preliminary analysis by UHPLC-MS/MS to check for the presence of neonicotinoids. Once it was confirmed that there was no residual trace of the studied compounds, sub-samples of bee pollen were used as blanks to prepare matrix-matched standards.

2.3.2 Sample treatment

Briefly, 1.0 g of bee pollen was weighed in a 50 mL centrifuge tube, after which 2 mL of water and 6 mL of ACN were added. The tube was then shaken for 30 s in a vortex device to dissolve until a homogenous solution was obtained. Next, 1.0 g of $MgSO_4$, 0.5 g of NaCl and 0.8 g of trisodium citrate dihydrate were added and the samples were shaken in the vibromatic mechanical shaker for 5 min. The extract was then centrifuged (10 000 rpm, 10ºC) for 5 min. For the precipitation of lipids and proteins, the supernatant was transferred to different centrifuge tube, and placed in a polystyrene box filled with dry ice for 2 min. Then, 2 mL of the extract were separated for the precipitate and transferred to an eppendorf tube, in which 150 mg of $MgSO_4$, 25 mg of PSA and 25 mg of C_{18}were also added. The resulting extract was centrifuged (10 000 rpm, 10ºC) for 5 min, and 1 mL of the supernatant was taken and evaporated to dryness in a rotary evaporator (+60ºC). The dry extract was reconstituted with 1 mL of a methanol:water (80:20, v/v) mixture, and the resulting solution was passed through a nylon filter (0.45 μm). After which, a 10 μL aliquot was injected into the UHPLC-MS/MS system. Figure 1 outlines the sample treatment procedure used during the present study.

2.4 UHPLC-qTOF 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 interface (ESI). The UHPLC instrument was equipped with a vacuum degasser, a binary solvent pump, an autosampler and a thermostated column compartment. Data was acquired and processed with software Data Analysis 4.1 and Qualitative Analysis from Bruker Daltonik GmbH. 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 (Torrance, CA, USA). 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 ACN (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**) 0.0–1.8 min (**A–B**, 6:94, v/v); (**ii**) 1.8–3.0 min (**A–B**, 15:85, v/v); (**iii**) 3.0–3.5 min (**A–B**, 30:70, v/v); (**iv**) 3.5–4.5 min (**A–B**, 15:85, v/v); (**v**) 4.5–5.0 min (A–B, 6:94, v/v); (**vi**) 5.0–6.5 min (A–B, 6:94, v/v). Injection volume and column temperature were set at 10 μL and +30ºC, respectively. The ESI source operated in the positive mode ionization mode because it provided the greatest sensitivity for all the studied neonicotinoid insecticides. 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 in multiple reaction monitoring (MRM) mode by using an isolation width of 5 m/z and a collision energy ramp from 10 to 30 eV (see Table 1).

Table 1. UHPLC-MS/MS data and retention times for each neonicotinoid insecticide

Compound	RT (min)	Precursor ion[a)] (*m/z*)	Product ion[b)] (*m/z*)	CE (eV)
Dinotefuran	1.2	203.1163	113.1033	15
			129.0904	15
Nitenpyram	1.6	271.0988	99.0925	15
			225.1056	15
Thiamethoxam	3.2	292.0296	131.9678	15
			211.0677	15
Clothianidin	3.8	250.0187	131.9685	15
			169.0566	15
Imidacloprid	4.0	256.0623	175.0996	25
			209.0618	25
Acetamiprid	4.1	223.0780	56.1009	30
			126.0114	25
Thiacloprid	4.5	253.0342	126.0113	20
			186.0156	20
Thiamethoxam-d3	3.2	295.0396	134.9677	15
			214.0687	15

RT, retention time;
CE, collision energy;
a) Quantification ion
b) Confirmation ion.

3 Results and discussion

3.1 Optimization of the sample treatment

In view of the recent studies determining neonicotinoids in pollen, it was decided that a QuEChERS extraction should initially be tested for sample treatment because of its simplicity, relatively low cost and promising results [4–6, 8, 13–18]. Firstly, consideration was given to the amount of bee pollen to be analyzed. After several tests (0.1–1.5 g), 1.0 g bee pollen were selected as the maximum amount to be used. Recoveries were adequate with this weight, and good S/N ratios were achieved in order to obtain the lowest possible LODs and LOQs. For the extraction step, 2 mL of water were added to hydrate the 1 g portion of pollen as well as 6 mL of ACN. Those volumes were selected after performing an optimization procedure. The use of ACN has the advantage of being able to precipitate proteins and limit lipid solubility. Moreover, proteins are denatured in pure ACN or aqueous-ACN mixtures, and this provoked an increase of the insecticide extraction efficiencies [6, 23]. Afterwards, it was optimized the amount of salts that should be employed in the partitioning step of the QuEChERS procedure. It must be stated that this optimization was not performed in any of the previous publications. $MgSO_4$ (1 g) served to partition the water from the sample, NaCl (0.5 g) was used to reduced polar co-extractives, and trisodium citrate dihydrate (0.8 g) to was employed to buffer the liquid-liquid extraction and provide an adequate media for the further extraction [4, 6]. Moreover, the influence of ceramic homogenizers in the extraction efficiency was also tested, but there were not obtained significant improvements when using those homogenizers. Once the solvents and the salts were selected, the influence of certain extraction parameters, such as extraction time (2–10 min), and centrifugation time (2–10 min), was sequentially tested in order to obtain optimal conditions. Optimal extraction (recovery percentages >90%) was achieved with 5 min of shaking and centrifuging time, respectively. Afterwards, it was optimized the clean-up step 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. Several experiments were conducted, and the optimal amounts of $MgSO_4$ (150 mg), PSA (25 mg) and C_{18} (25 mg) were selected. Although the proposed clean-up is indicated to remove some matrix co-extractives, the analysis of the pollen extracts revealed elevated amounts of matrix constituents (see Supporting Information Fig. S2). Afterwards, it was decided to employ the freeze-out process to remove lipids as a decrease has been previously reported in the recovery percentages when using hexane [6]. Experiments were performed at different freezing times (1–3 min). The results revealed that 2 min was the shortest time period required to achieve an homogeneous fat and protein precipitate, and to obtain much cleaner chromatograms (see Supporting Information Fig. S2). Following this, the mixture was centrifuged for 5 min, and 1 mL of the supernatant was collected, transferred to a 15 mL glass flask and gently evaporated to dryness in a rotary evaporator at +60ºC. Then, the solvent used to reconstitute the sample was also studied. Different methanol:water and ACN:water mixtures (0:100, 20:80, 50:50, 80:20, 0:100; v/v) were tested, and the best results in terms of extraction efficiency were obtained with a 80:20 (v/v) methanol:water mixture. It was also found that 1 mL of this solvent was enough to obtain satisfactory results.

In order to check the effectiveness of the proposed sample treatment, neonicotinoid responses were compared: these 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. Recovery values ranged from 91 to 105% in all cases (see Table 2); this indicated that the sample treatment was both appropriate and effective. These recovery values are similar or better than the obtained with previous proposals (see Supporting Information Table S1), but with the advantages that the matrix effect has been minimized in such a way, that standard calibration curves could be used to quantify the neonicotinoid insectides. This is particular relevant, if it is taken into account that matrix effect was not minimized in most of previous publications, and it was necessary to perform a second extraction when it was achieved (see Supporting Information Table S1). Finally, the results showed that the selected freeze-out step is an efficient and simple alternative to the clean-up of the sample in contrast with the use of hexane [4, 5, 8] or a second extraction [17, 18].

Table 2. Evaluation of the efficiency of the sample treatment and the matrix effect. Data obtained described in Sections 3.1 and 3.3 ($n = 6$)

	Evaluation of the sample treatment			Evaluation of the matrix effect		
	Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
Quality control (QC) sample	Low	Medium	High	Low	Medium	High
Dinotefuran	91 ± 4	99 ± 2	93 ± 3	91 ± 5	97 ± 4	90 ± 5
Nitenpyram	99 ± 2	93 ± 2	99 ± 3	100 ± 3	94 ± 2	99 ± 3
Thiamethoxam	98 ± 3	104 ± 4	99 ± 3	104 ± 5	97 ± 3	99 ± 3
Clothianidin	92 ± 3	98 ± 3	103 ± 5	94 ± 5	102 ± 4	98 ± 4
Imidacloprid	95 ± 4	97 ± 3	105 ± 5	95 ± 2	93 ± 3	101 ± 5
Acetamiprid	96 ± 3	101 ± 3	104 ± 4	92 ± 2	98 ± 3	96 ± 5
Thiacloprid	92 ± 2	94 ± 4	97 ± 3	96 ± 2	104 ± 3	102 ± 4

Low **QC**— 5 μg/kg; Medium QC—63 μg/kg; High QC—500 μg/kg.

3.2 UHPLC optimization

We have recently published some papers about an analysis of neonicotinoids in bee pollen [7, 12]. In both works, a core-shell technology based column (Kinetex® C_{18}, 150×4.6 mm, 2.6 μm, 100 Å) was employed, and satisfactory results were obtained. These core-shell particles have increasingly been used in the last years in order to obtain highly efficient separation with relatively low back pressure [24]. A recently commercialized core-shell technology based column, Kinetex® EVO was employed in the present study. This column provides the additional benefit of better peak shape for bases, wide pH 1 to 12 stability, and the potential signal suppression caused by the presence of polar (basic compounds) is decreased, as those compounds are more retained in those columns. We therefore decided to optimize the separation with the Kinetex® EVO column and similar mobile phase components to those employed in our previous studies (0.1% (v/v) formic acid in ACN and 0.1% (v/v) formic acid in water). Several experiments were conducted with diverse mobile phases and flow rates to separate the neonicotinoid insecticides in the shortest possible time. The shortest analysis times were obtained with the chromatographic conditions described in Section 2.4. With such conditions the overall run time was 6.5 min, eluting the last of the insecticides at 4.5 min (see Fig. 2), which, to our knowledge, is the fastest proposal that has been published in relation to neonicotinoid analysis in pollen (see Supporting Information Table S1). In addition, it should be also remarked that this is the first time that a Kinetex® EVO column has been employed to analyze neonicotinoids. Finally, it is also interesting to mention that the proposed UHPLC method could be used with other detectors (diode array or ultraviolet detectors) as the seven neonicotinoids were baseline separated, and that the number of studied neonicotinoids was higher than in most of previous publications (see Supporting Information Table S1).

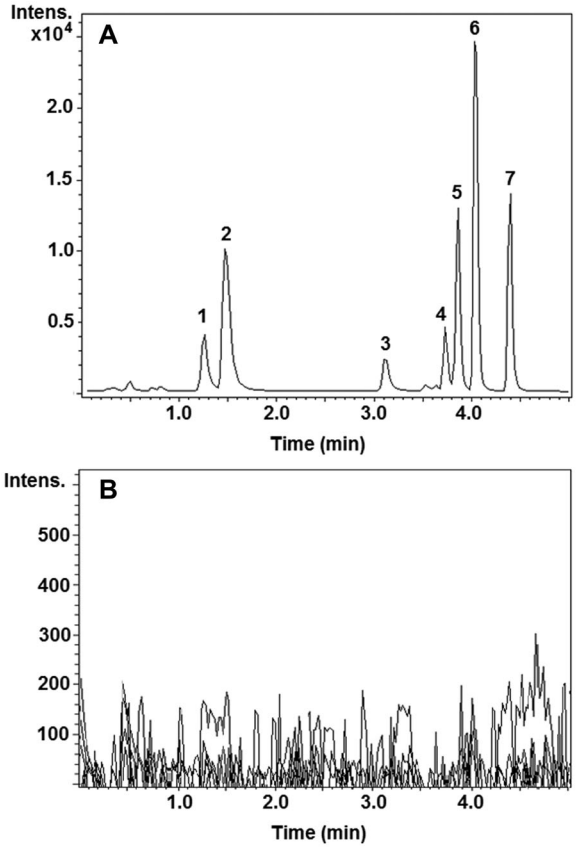

Figure 2. Representative UHPLC-ESI-MS chromatograms (EIC in positive mode using the precursor ions, see Table 1) obtained from: (**A**) a spiked (63 μg/kg) bee pollen sample in: dinotefuran (1), nitenpyram (2), thiamethoxam (3), clothianidin (4), imidacloprid (5), acetamiprid (6), and thiacloprid (7); (**B**) a blank bee pollen sample. The UHPLC-ESI-MS conditions are described in Section 2.4.

3.3 Mass spectrometry optimization

To establish the optimal MS and MS/MS conditions, several experiments (flow injection analysis) were conducted in order to choose the optimum parameters (see Section 2.4 and Table 1) and achieve the maximum sensitivity by the infusion mode (80 μL/min) of standard (500 μg/L) and matrix matched solutions (500 μg/kg). Neonicotinoids showed an intense $[M+H]^+$ (precursor ions) on their full-scan spectra,

which were used by generating extracted ion chromatograms (EIC) for each neonicotinoid to obtain the maximum sensitivity for quantitative analysis (the mass-to-charge (m/z) values are summarized in Table 1). Moreover, significant fragments (product ions) obtained of the precursor ions for each neonicotinoid were selected for MS/MS analyses in MRM mode to confirm their presence in bee pollen (see Table 1; proposed structures are shown in the Supporting Information Fig. S1).

To check how the matrix influenced ESI ionization, a comparison was made of the results (analyte peak area/IS area) with standard working solutions and blank samples spiked at three different concentrations (**QC** levels) following sample treatment (**AF** samples). The responses of all compounds at the three concentrations assayed were comprised between 90 and 104% in all cases, as can be seen in Table 2. Thus, it was concluded that the matrix did not significantly affect ESI ionization of the analytes. This is an important result, as a significant matrix effect have been reported in most of the existing literature dedicated to analyze those compounds in bee pollen (see Supporting Information Table S1).

3.4 Validation of the method

Validation was carried out following different International guidelines (http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/qualcontrol_en.pdf; http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri = CELEX:32002D0657&from = ES) and previous works [1, 7, 12] determining selectivity, LODs and LOQs, as well as linearity, precision and accuracy of the proposed method.

3.4.1 Selectivity

This was verified by a set of blank samples ($n = 6$) being injected into the chromatographic system and the results being compared with those obtained for standard and matrix-matched solutions. No chromatographic interference was observed at analytes retention times in any of the blank samples analyzed (see Fig. 2). For the 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 mass spectra. However, slight differences in the intensity of several ions were observed and certain low intensity ions appeared in a few cases (see Supporting Information Fig. S3).

3.4.2 Limits of detection and quantification

The LODs and LOQs were experimentally determined by the S/N method; as baseline noise was produced and it was possible to obtain bee pollen containing no neonicotinoids. A number of blank samples ($n = 6$) were injected, and the peak to peak noise around analyte retention time was measured. The LODs and LOQs were estimated as three and ten times the S/N ratio, respectively, and those limits were calculated for MS (EIC) and MS/MS (MRM) experiments. As can be seen in Table 3, low LODs and LOQs could be obtained by using MS (EIC) for all analytes ranging from 0.6 to 1.3 μg/kg (LOD) and 2.1 to 4.0 μg/kg (LOQ), whereas these values were slightly higher with MS/MS. Finally, it must be also emphasized that the LODs and LOQs obtained with the proposed method are very useful if attention is paid to the maximum residue limits established by the European Union in honey and other apicultural products (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event = pesticide.residue.selection&language = EN; acetamiprid 50 μg/kg; clothianidin 10 μg/kg; imidacloprid 50 μg/kg; thiacloprid 50 μg/kg; thiamethoxam 10 μg/kg-the sum of thiamethoxam and clothianidin; no data for dinotefuran and nitenpyram), and they are also similar to those reported in the existing literature (see Supporting Information, Table S1).

3.4.3 Linearity studies

The lack of influence of the matrix effect in the analyte signals was corroborated by the fact that the slopes of the standard and matrix-matched calibration curves overlapped at the confidence intervals (see Table 3). Thus, standard calibration curves from LOQ to 500 μg/L (calibration levels of LOQ, 5, 10, 20, 40, 63, 125, 250, 500 μg/L) were used to quantify neonicotinoids in bee pollen. These, in accordance with unit conversion and the sample treatment proposed, corresponded to a concentration in matrix matched solutions between LOQ and 500 μg/kg. 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; the coefficient of the determination values (R^2) was above 0.99 in all cases (Table 3).

3.4.4 Precision and accuracy studies

Intra-day precision and accuracy experiments were assessed by repeated analysis of blank samples spiked with three concentrations of the neonicotinoids (low, medium and high QC levels) on the same day ($n = 6$). Inter-day precision and accuracy were evaluated by an examination of blank samples spiked with three concentrations of this (low, medium and high QC levels) over three consecutive days ($n = 6$). Precision was expressed as the percentage of relative standard deviation (% RSD) at the three concentrations for each analyte. Accuracy was calculated by means of relative error (% RE). Intra- and inter-day precision (% RSD values) was at all times lower than 7% (see Supporting Information Table S2). Accuracy (% RE values) ranged from 1 to 6% for the intra-day readings, and from 1 to 7% for the inter-day values (see Supporting Information Table S2).

Table 3. Calibration curve data ($n = 6$), LOD and LOQ values

Compound	Calibration curve	Analytical range[a)]	Slope confidence intervals	R^2	LOD[b)]		LOQ[b)]	
					MS	MS/MS	MS	MS/MS
Dinotefuran	Standard	4.0–500	$4.5\times10^6 \pm 3.8\times10^4$	0.999	1.3	2.7	4.0	9.0
	Matrix-matched		$4.2\times10^6 \pm 5.3\times10^4$	0.997				
Nitenpyram	Standard	3.6–500	$3.6\times10^6 \pm 1.0\times10^4$	0.999	1.1	1.8	3.6	6.0
	Matrix-matched		$3.5\times10^6 \pm 6.6\times10^4$	0.998				
Thiamethoxam	Standard	2.1–500	$1.7\times10^6 \pm 4.2\times10^4$	0.997	0.6	1.6	2.1	5.3
	Matrix-matched		$1.8\times10^6 \pm 2.1\times10^4$	0.999				
Clothianidin	Standard	3.9–500	$4.9\times10^5 \pm 1.8\times10^4$	0.999	1.2	1.9	3.9	6.2
	Matrix-matched		$4.8\times10^5 \pm 5.8\times10^4$	0.996				
Imidacloprid	Standard	2.1–500	$2.1\times10^6 \pm 2.5\times10^4$	0.999	0.6	3.6	2.1	12.0
	Matrix-matched		$2.0\times10^6 \pm 1.6\times10^4$	0.998				
Acetamiprid	Standard	3.6–500	$2.9\times10^6 \pm 4.4\times10^4$	0.998	1.1	3.3	3.6	11.0
	Matrix-matched		$2.8\times10^6 \pm 1.1\times10^5$	0.993				
Thiacloprid	Standard	3.8–500	$1.4\times10^6 \pm 1.1\times10^4$	0.999	1.2	2.7	3.8	9.0
	Matrix-matched		$1.5\times10^6 \pm 5.0\times10^4$	0.995				

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 matrix (bee pollen, μg/kg).

3.5 Application of the method

The validated method was applied to determine potential residues of the studied neonicotinoids in twenty commercial bee pollen samples. All of these were analyzed in triplicate. No residues of the insecticides under study were detected in any of samples. This does not, however, mean that it was a wasted effort to develop a method for screening compounds that did not exist in these samples, since residues of spinosad in pollen have already been reported [4, 6, 7, 13], and sensitive and exclusive methods, as the presented in this manuscript, are required to detect neonicotinoids in this matrix because of the low concentrations expected.

4 Concluding remarks

A new and fast UHPLC-qTOF method to separate and quantify seven neonicotinoids in bee pollen has been developed. The usefulness of a recently commercialized core-shell technology based column (Kinetex® EVO) to analyze neonicotinoids in a shorter time to that obtained with other C_{18} columns has been demonstrated for the first time. In addition, the developed UHPLC method could be used with more economical detectors (diode array or ultraviolet detectors) as the seven neonicotinoids were baseline separated, which was not possible with most of previous proposals. The consistency and reliability of this method has been shown, as it was fully validated, and optimization of all the steps of the sample treatment (QuEChERS), which was not usually done, produced excellent recoveries at different concentration levels, and minimize the matrix effect. Thus, standard calibration curves could be used to quantify neonicotinoids in bee pollen. This is quite remarkable, as matrix effect was not minimized in most of previous publications devoted to analyze those insecticides in bee pollen, and it was not necessary to perform a second extraction to achieve this goal. Finally, several samples of bee pollen were analyzed, and neonicotinoid residues were not found in any of the samples.

Authors gratefully acknowledge funding from the Spanish Ministry "Economía y Competitividad" and INIA (RTA2013-00042-C10-03 and 06). In addition, authors thank the "Laboratorio de Técnicas Instrumentales" (University of Vallladolid) for using their UHPLC-qTOF system, and Jesús Hernández Jiménez (Centro de Idiomas, University of Valladolid) for performing the English revision.

The authors of this manuscript declare no conflict of interest.

5 References

[1] Yáñez, K. P., Bernal, J. L., Nozal, M. J., Martín, M. T., Bernal, J., *J. Chromatogr. A* 2013, *1285*, 110–117.

[2] Sánchez-Hernández, L., Hernández-Domínguez, D., Bernal, J., Neusüβ, C., Martín, M. T., Bernal, J. L., *J. Chromatogr. A* 2014, *1359*, 317–324.

[3] Bonmatin, J. M., Giorio, C., Girolami, V., Kreutzweiser, D. P., Krupke, C., Liess, M., Marzaro, M., Mitchell, E. A. D., Noome, D. A., Simon-Delso, N., Tapparo, A., *Environ. Sci. Pollut. Res.* 2015, *22*, 35–67.

[4] Chen, M., Collins, E. M., Tao, L., Lu, C., *Anal. Bioanal. Chem.* 2013, *405*, 9251–9264.

[5] Chen, M., Tao, L., McLean, J., Lu, C., *J. Agric. Food Chem.* 2014, *62*, 6082–6090.

[6] Parrilla Vázquez, P., Lozano, A., Uclés, S., Gómez Ramos, M. M., Fernández-Alba, A. R., *J. Chromatogr. A* 2015, *1426*, 161–173.

[7] Yáñez, K. P., Martín, M. T., Bernal, J. L., Nozal, M. J., Bernal, J., *Food Anal. Methods* 2014, *7*, 490–499.

[8] Kasiotis, K. M., Anagnostopoulos, C., Anastasiadou, P., Machera, K., *Sci. Total Environ.* 2014, *485–486*, 633–642.

[9] Bonmatin, J. M., Moineau, I., Charvet, R., Flechet, C., Colin, M. E., Bengsch, E. R. *Anal. Chem.* 2003, *75*, 2027–2033.

[10] Schöning, R., Schmuck, R., *B. Insectol.* 2003, *56*, 41–50.

[11] Cutler, G. C., Scott-Dupree, C. D., *J. Econ. Entomol.* 2007, *100*, 765–772.

[12] Sánchez-Hernández, L., Hernández-Domínguez, D., Martín Nozal, M. J., Higes, M., M. T., Bernal, J. L., *J. Chromatogr. A* 2016, *1428*, 220–227.

[13] Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., vanEngelsdorp, D., Pettis, J. S., *PLoS ONE* 2010, *5*, e9754.

[14] Kamel, A., *J. Agric. Food Chem.* 2010, *58*, 5926–5931.

[15] Stoner, K. A., Eitzer, B. D., *PLoS ONE* 2012, *7*, e39114.

[16] Codling, G., Al Naggar, Y., Giesy, J. P., Robertson, A. J., *Chemosphere* 2016, *144*, 2321–2328.

[17] David, A., Botías, C., Abdul-Sada, A., Goulson, D., *Anal. Bioanal. Chem.* 2015, *407*, 8151–8162.

[18] David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M., Goulson, D., *Environ. Int.* 2016, *88*, 169–178.

[19] Wang, R., Wang, Z., Yang, H., Wang, Y., Deng, A., *J. Sci. Food Agric.* 2012, *92*, 1253–1260.

[20] Nomura, H., Ueyama, J., Kondo, T., Saito, I., Murata, K., Iwata, T., Wakusawa, S., Kamijima, M., *J. Chromatogr. B* 2013, *941*,109–115.

[21] Ettiene, G., Bauza, R., Plata, M. R., Contento, A. M., Ríos, A., *Electrophoresis* 2012, *33*, 2969–2977.

[22] Chen, Z., Dong, F., Li, S., Zheng, Z., Xu, Y., Xu, J., Liu, X., Zheng, Y., *J. Chromatogr. A* 2015, *1410*, 181–189.

[23] Li, H., Yin, J., Liu, Y., Shang, J., *J. Agric. Food Chem.* 2012, *60*, 1722–1727.

[24] Hayes, R., Ahmed, A., Edge, T., Zhang, H., *J. Chromatogr. A* 2014, *1357*, 36–62.

