Sweeping-micellar electrokinetic chromatography with tandem mass spectrometry as an alternative methodology to determine neonicotinoid and boscalid residues in pollen and honeybee samples

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ARTICLE INFO

ABSTRACT

In this work, it is proposed for the first time an electrophoretic approach based on micellar electrokinetic chromatography coupled with tandem mass spectrometry (MEKC-MS/MS) for the simultaneous determination of nine neonicotinoids (NNIs) together with the fungicide boscalid in pollen and honeybee samples. The separation was performed using ammonium perfluorooctanoate (50 mM, pH 9) as both volatile surfactant and electrophoretic buffer compatible with MS detection. A stacking strategy for accomplishing the on-line pre-concentration of the target compounds, known as sweeping, was carried out in order to improve separation efficiency and sensitivity. Furthermore, a scaled-down QuEChERS was developed as sample treatment, involving a lower organic solvent consumption and using Z-Sep+ as dispersive sorbent in the clean-up step. Regarding the detection mode, a triple quadrupole mass spectrometer was operating in positive ion electrospray mode (ESI+) under multiple reaction monitoring (MRM). The main parameters affecting MS/MS detection as well as the composition of the sheath-liquid (ethanol/ultrapure water/formic acid, 50:49.5:0.5 v/v/v) and other electrospray variables were optimized in order to achieve satisfactory sensitivity and repeatability. Procedural calibration curves were established in pollen and honeybee samples with LOQs below 11.6 µg kg^{-1} and 12.5 µg kg^{-1}, respectively. Precision, expressed as RSD, lower than 15.2% and recoveries higher than 70% were obtained in both samples. Two positive samples of pollen were found, containing imidacloprid and thiamethoxam. Imidacloprid was also found in a sample of honeybees. The obtained results highlight the applicability of the proposed method, being an environmentally friendly, efficient, sensitive and useful alternative for the determination of NNIs and boscalid in pollen and honeybee samples.

1. Introduction

In the last years, several studies have demonstrated the potential toxic effects of pesticides, especially of systemic insecticides such as neonicotinoids (NNIs), on pollinators and their close relation with the colony collapse disorder (CCD) in honeybees [1–4]. CCD is a phenomenon characterized by the abrupt loss and death of adult worker bees due to, among other factors, their contamination with insecticides. NNIs are broad-spectrum insecticides that act as antagonists of the nicotinic acetylcholine receptors mainly present in insects, provoking the paralysis and subsequent death of the organism [5,6]. Currently, NNIs are the most widely used family of insecticides worldwide for plant protection replacing traditional insecticides and representing the 27% of the global insecticide market [6]. The most predominant NNIs, which can be seen in Fig. S1, are imidacloprid, thiacloprid, clothianidin, thiamethoxam, acetamiprid, nitenpyram, dinotefuran, and flonicamid, while others, such as imidaclothiz, are relatively new [7]. Due to their high solubility in water, systemic nature and persistence, neonicotinoid residues can remain in plant pollen and nectar for a long time, being easily available for pollinators. Moreover, as a result of their long-lasting persistence and the variability in their application modes in agriculture, it is common to find them in all environmental compartments (i.e., air, soil, surface water), entailing a risk for beneficial insects and even other non-target

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organisms [8–10]. In 2013, the European Commission restricted the use of plant protection products and treated seeds containing clothianidin, imidacloprid, and thiamethoxam to protect honeybees [11] based on a risk assessment of the European Food Safety Authority (EFSA). These NNIs were banned in bee-attractive crops (including maize, oilseed rape and sunflower) except for uses in greenhouses, the treatment of some crops after flowering and winter cereals. However, considering the worrying exposure of pollinators to NNIs and its consequences, in May 2018 the European Commission restricted the application of imidacloprid, clothianidin, and thiamethoxam to greenhouse uses only [12]. Also, on February 2020, the approval of thiacloprid was not renewed following the scientific advice of EFSA that the substance presents health and environmental concerns [13]. However, some EU countries have repeatedly granted emergency authorizations for their use in different crops, such as sugar beets. In this sense, maximum residues levels (MRLs) for different commodities or lower limit of analytical determination (in such matrixes for which their use is forbidden, including apiculture products) have been established [14]. In addition, due to their toxicity, the Worldwide Integrated Assessment (WIA) has recently reported alternatives to systemic insecticides such as NNIs in pest control [15].

On the other hand, recent works have demonstrated that certain fungicides, such as boscalid (Fig. S1), in the presence of NNIs, are able to reduce the lethal time and median lethal dose (LD_{50}) for honeybees, increasing the harmful effects of NNIs in agricultural areas [16,17]. Boscalid belongs to the carboxamide family and acts via decreasing the ATP concentration, pollen consumption, and protein digestion in bees, so it has also been related to the CCD [18]. For that reason, it is of great interest to consider this fungicide together with NNIs for their monitoring. However, these compounds have been rarely determined simultaneously so far [19].

Usually, liquid chromatographic (LC) methods have been mostly used for the determination of NNIs as it has been compiled in some reviews concerning the analysis of these compounds [20,21]. LC coupled to tandem mass spectrometry (LC-MS/MS) is the technique of choice for most recent applications [22–25]. On the contrary, the use of capillary electrophoresis (CE) has been scarcely investigated despite of presenting numerous advantages. These include low solvent consumption, low sample volume, high separation efficiency, and short separation time, being also in compliance with green analytical chemistry [26]. Considering that most of NNIs are neutral in a wide range of pH, the determination of NNIs by capillary zone electrophoresis (CZE) leads to poor separations [27]. Instead, micellar electrokinetic chromatography (MEKC) is more suitable to determine these compounds. Some CE-based methods have been developed for the determination of NNIs in vegetables [28,29], soil and environmental waters [30,31] mainly using MEKC coupled to UV detection, however, CE has been rarely applied to honeybee products [27]. In some cases, sweeping-MEKC-UV using sodium dodecyl sulfate (SDS) as micellar medium has been reported to provide an on-line pre-concentration of the analytes [28,30]. Nevertheless, the coupling with tandem mass spectrometry (MS/MS) is the most suitable technique to improve selectivity and sensitivity, allowing the unequivocal identification of target compounds at trace levels; a key point in food safety. However, commonly used surfactants such as SDS are non-volatile and can contaminate the ion-source of the mass spectrometer, leading to an analyte signal suppression and a marked decrease of sensitivity. To overcome this shortcoming, several studies have recently revealed the potential of using a volatile surfactant such as ammonium perfluorooctanoate (APFO), which can also act as background electrolyte. This is a robust alternative to common surfactants and allows the direct coupling of MEKC to MS without negatively affecting both, the electrophoretic separation nor the MS detection [32–35].

Regarding sample treatments to determine NNIs by LC, the QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure and solid-phase extraction (SPE) appear as the most-often used techniques. They have been applied to numerous environmental and food samples, including honeybee products such as beeswax, pollen, honey, and royal jelly [36]. However, QuEChERS is not usually applied in CE methods as it involves a sample dilution, which can compromise sensitivity.

In light of the environmental problem associated to the above-mentioned pesticides and the lack of studies reported using CE-MS for their determination, the main aim of this work is to demonstrate the potential of MEKC-MS/MS for the simultaneous determination of NNIs and boscalid in complex samples. In addition, we have modified a traditional QuEChERS procedure to avoid sample dilution and decrease of sensitivity, being compatible with the CE method for the analysis of pollen and honeybee samples.

2. Materials and methods

2.1. Materials and reagents

Unless otherwise specified, analytical grade reagents and HPLC grade solvents were used in this work. Acetonitrile (MeCN), formic acid (FA), propan-2-ol and methanol (MeOH) were supplied by VWR International (West Chester, PA, USA), while ethanol (EtOH) and ammonia solution, (NH_3 (aq), 30% (m/m)) were obtained from Merk (Darmstadt, Germany). Sodium hydroxide (NaOH) as well as salts such as magnesium sulfate anhydrous ($MgSO_4$), sodium sulfate (Na_2SO_4), and sodium chloride (NaCl) were obtained from PanReac-Química (Madrid, Spain) while ammonium sulfate (($NH_4)_2SO_4$) was obtained from VWR Chemicals (Barcelona, Spain). Dispersive sorbents such as Primary Secondary Amine (PSA) and C18 end-capped sorbent were supplied by Agilent Technologies (Waldbronn, Germany) while activated carbon and Z-Sep+ were obtained from Sigma-Aldrich (St. Louis, MO, USA) as well as the perfluorooctanoic acid (PFOA) (96% m/m).

Analytical standards of dinotefuran (DNT), thiamethoxam (TMT), clothianidin (CLT), nitenpyram (NTP), imidacloprid (IMD), thiacloprid (TCP), acetamiprid (ACT), imidaclothiz (IMZ), flonicamid (FNC), and boscalid (BCL) were supplied by Sigma Aldrich.

Individual standard solutions were obtained by dissolving the appropriate amount of each compound in MeOH, reaching a final concentration of 500 μg mL^{-1}, which were kept in dark at -20 °C. Intermediate stock standard solution containing 50 μg mL^{-1} of each compound were obtained by mixing individual stock standard solutions, followed by a solvent evaporation step under a current of N_2, and subsequent dilution with ultrapure water. Working standard solutions were freshly prepared by dilution of the intermediate stock standard solutions with ultrapure water at the required concentration. Both, intermediate and working solutions were stored at 4 °C avoiding exposure to direct light.

Solutions of 50 mM APFO at pH 9 used as background electrolyte (BGE) were prepared weekly dissolving the necessary amount of PFOA in ultrapure water and adjusting the pH with 5 M NH_3 (aq).

Polytetrafluoroethylene (PTFE) syringe filters (0.22 μm x 13 mm) such as CLARIFY-PTFE (hydrophilic) obtained from Phenomenex (California, USA) were used for sample filtration, and PTFE from VWR international (West Chester, PA, USA) were employed for filtration of NaOH and BGE.

2.2. Instrumentation

MEKC experiments were performed with an Agilent 7100 CE system coupled to a triple quadrupole 6495C mass spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with

an electrospray ionization source operating in positive ionization mode (ESI$^+$). Sheath liquid was supplied with a 1260 Infinity II Iso Pump. MS data were collected and processed by MassHunter software (version 10.0).

Separations were carried out in bare fused-silica capillaries (70 cm of total length, 50 μm I.D., 375 O.D.) from Polymicro Technologies (Phoenix, AZ, USA).

A pH meter (Crison model pH 2000, Barcelona, Spain), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a multi-tube vortexer BenchMixer XL (Sigma-Aldrich, St. Louis, MO, USA), and a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany) were also employed.

2.3. Sample treatment

2.3.1. Sample collection and preparation

Commercially available pollen from a local market (Granada, Spain) was used for method optimization. The pollen was kept in its original packaging at room temperature until further handling. Natural pollen samples used to monitor the presence of the target compounds were gathered from almond blossoms at three different farmlands located in Fuente Vera (Granada, Spain), and immediately stored at -20 °C until their use. Flowers were defrosted and dried at 30 °C for 24 hours to extract the pollen from the anthers. Afterwards, flowers were carefully sieved through a 0.2 mm mesh to separate pollen from them. Lab tweezers were also needed to release the pollen in some cases. The obtained natural pollen samples from each farmland were kept in a dry place until their analysis.

In order to characterize the method in blank honeybee samples, approximately 500 specimens were carefully collected from an organic farmland in which the use of beehives is common. In addition, about 200 honeybees were collected in an area located close to the almond fields above mentioned. This sampling point was selected because hundreds of dead adult worker bees were found there, so the analysis of these samples was particularly interesting in order to prove the usefulness of this method. All samples were rapidly stored at -20 °C until their use. Then, honeybees were lyophilized at -109 °C in order to guarantee the proper crushing and homogenization of the sample.

2.3.2. Scaled-down QuEChERS procedure

The sample treatment for pollen and honeybee samples was carried out as follows: a representative 200 mg portion of each sample was placed into a 15 mL centrifuge tube and 1 mL of ultrapure water was added to hydrate the samples, which were subsequently vortexed for 1 min. Then, 2.5 mL of MeCN were added as well as 200 mg of MgSO$_4$ to favor salting-out effect. The tube was mechanically shaken for 2 min and centrifuged for 5 min at 8487 g and 4°C. Then, the whole supernatant was transferred to another 15 mL centrifuge tube containing 30 mg of Z-Sep+ as dispersive sorbent and 100 mg of MgSO$_4$. The tube was stirred in vortex for 2 min and centrifuged for 5 min at 9000 rpm (8487 g) and 4°C. Afterwards, an aliquot of 2 mL of supernatant was transferred to a glass vial and dried under a gentle N$_2$ stream at 35°C. Finally, the dried residue was reconstituted with 200 μL of ultrapure water, shaken in an ultrasonic bath and filtered through a 0.22 μm hydrophilic-PTFE filter before its injection into the CE-MS/MS system.

2.4. Micellar electrokinetic chromatography separation

New capillaries were conditioned with 1 M NaOH for 15 min, followed by ultrapure water for 10 min and then, with the running BGE for 15 min at 1 bar and 25°C. At the beginning of each day, this procedure was repeated but using 0.1 M NaOH. In order to obtain an adequate repeatability between runs, capillary was rinsed with the BGE for 3 min at 1 bar and 25°C at the beginning of each run. At the end of the working day, capillary was cleaned with ultrapure water for 5 min, followed by MeOH for 2 min, and finally dried with air for 1 min at 1 bar and 25°C.

MEKC separation was performed using a BGE consisted of an aqueous solution of 50 mM PFOA at pH 9, which gave a stable electric current of 25 μA. The temperature of the capillary was kept at 25°C and a constant separation voltage of 25 kV (normal polarity) was applied. Samples were hydrodynamically injected for 50 s at 50 mbar using ultrapure water as injection solvent in order to induce sweeping.

2.5. MS/MS conditions

Sheath-liquid consisting of a mixture 50:50 (v/v) EtOH/ultrapure water containing 0.05% (v/v) formic acid was provided at a flow rate of 5 μL min^{-1} (0.5 mL min^{-1} with a 1:100 splitter). The mass spectrometer was operated in positive ionization mode (ESI$^+$) under multiple reaction monitoring (MRM) conditions. 2000 V were applied in both capillary and nozzle. Other electrospray parameters at optimum conditions were: nebulizer pressure, 69 kPa, dry gas flow rate, 11 L min^{-1}; and dry gas temperature, 200 °C. MS/MS experiments were performed by fragmentation of the molecular ions [M+H]$^+$, which were selected as the precursor ions in all cases. Collision energies (V) were set between 9 and 25, depending on the analyte, and product ions were analyzed in the range of 114-307 m/z. Optimized MS/MS parameters are summarized in Table 1.

3. Results and discussion

3.1. Optimization of electrophoretic conditions

CE separations were performed using MEKC mode, in which neutral analytes can be separated due to their different interaction with the micelles. Optimization of the main variables affecting the separation of the target compounds by MEKC were carried out considering different response variables such as S/N ratio, migration time and peak resolution. In addition, the generated current was kept lower than 30 μA to minimize the Joule effect.

As stated before, surfactants such as the commonly used SDS are not recommended when MS detection is used. Therefore, the use of a volatile surfactant such as APFO was considered as both, BGE and micellar medium. Firstly, basic pH conditions are needed to dissolve PFOA in ultrapure water. In addition, target compounds are neutral at basic conditions. Therefore, the effect of pH in the separation was investigated between 8 and 10 using 75 mM PFOA. There were no significant differences in this pH range, so a pH of 9 was selected.

Subsequently, taking into consideration that the critical micelle concentration (CMC) of APFO is 25 mM, different concentrations of APFO between 50 and 100 mM were investigated at pH 9. As the concentration increases so does the resolution between peaks as well as the migration time. However, the intensity of the signal for most analytes was higher at concentrations lower than 50 mM, and the migration time was significantly shorter (11 min). Thus, a concentration of 50 mM APFO was selected as a compromise between migration time, signal intensity and resolution. In order to reduce the total analysis time, capillary was shortened from 80 to 70 cm. Separation efficiency, particularly for ACT, was slightly better and the total analysis time was reduced in 2 min when this capillary was used, so a length of 70 cm was selected as optimum for further experiments.

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Table 1
MS/MS parameters for target compounds.

	Migration time (min)	Precursor ion (*m/z*)	Molecular ion	Product ions[a]	CE[b]	Dwell time (ms)
DNT	5.29	203.1	$[M+H]^+$	129.2 (Q)	9	50
				114.0 (I)	9	50
TMT	5.25	292	$[M+H]^+$	210.9 (Q)	10	50
				131.7 (I)	10	50
FCM	5.4	230.1	$[M+H]^+$	202.8 (Q)	15	40
				173.9 (I)	15	40
CLT	5.42	250	$[M+H]^+$	168.9 (Q)	10	80
				132.0 (I)	10	80
NTP	5.88	271.1	$[M+H]^+$	189.0 (Q)	15	50
				237.3 (I)	15	50
IMZ	6	262	$[M+H]^+$	180.9 (Q)	15	50
				131.7 (I)	15	50
IMD	6.47	256.1	$[M+H]^+$	209.1 (Q)	15	50
				175.0 (I)	15	50
TCP	6.52	253	$[M+H]^+$	125.9 (Q)	25	50
				90.0 (I)	25	50
ACT	6.77	223.1	$[M+H]^+$	126.0 (Q)	15	80
				56.1 (I)	15	80
BCL	7.18	343	$[M+H]^+$	307.0 (Q)	20	60
				140.0 (I)	20	60

[a] Product ions: (Q) Transition used for quantification, (I) Transition employed to confirm the identification.
[b] Collision Energy (CE) expressed in volts (V).

Afterwards, the effect of the temperature on the MEKC separation was studied in the range of 20-35 °C. It was observed that the total analysis time decreased when the temperature increased up to 30 °C. Nevertheless, the electrophoretic current increased with the temperature, so in order to avoid this, a temperature of 25 °C was selected. Moreover, the separation voltage was also studied in the range of 20-30 kV. The best results as a compromise between the analysis time and the electrophoretic current were obtained when 25 kV was used, so it was selected for further analysis.

In order to improve sensitivity, an on-line pre-concentration of the analytes was performed using a solvent devoid of micelles as injection solvent. This approach, known as "sweeping" is designed to focus the analytes into a narrow band within the capillary, thereby increasing the sample volume that can be injected without any loss of separation efficiency. It is based on the interactions between an additive (i.e. a pseudostationary phase or micellar media) in the separation buffer and the sample in a matrix that is free of the used additive. It involves the accumulation of charged and neutral analytes by the pseudostationary phase that penetrates the sample zone and "sweeps" the analytes, producing a focusing effect. In this case, ultrapure water was used as injection solvent, since it allowed the stacking of the analytes when they were swept by the BGE containing APFO micelles [37,38]. The use of an organic solvent as injection solvent was discarded since this negatively affected the formation of micelles and had an adverse impact on peak shapes as it was also previously reported [35]. Finally, the effect of the hydrodynamic injection time on peak height was checked in the range from 20 to 60 s at 50 mbar. There was an increase in the peak height up to 50 s without significantly affecting separation efficiency. In this regard, an injection time of 50 s was set. This injection time corresponds to a sample volume of 55 nL approximately (4% of the total capillary volume).

Sensitivity enhancement factors (SEFs) for NNIs and boscalid were estimated comparing peak heights of standard solutions dissolved in water (sweeping) with standard solutions of the same concentration dissolved in BGE (no sweeping):

$$SEF_{height} = \frac{\text{Analyte peak height using sweeping}}{\text{Analyte peak height without using sweeping}}$$

SEFs ranging from 1.6 to 5.6 were achieved for the studied analytes using sweeping as can be seen in Table S1. In addition, peak efficiencies (theoretical plate number) with and without sweeping

were checked for each analyte. Significantly better results were obtained when ultrapure water was employed as injection solvent (Table S2). In view of these results, the use of sweeping as on-line pre-concentration led to an improvement in sensitivity as well as in separation efficiency for the studied compounds.

3.2. Optimization of MEKC-ESI-MS/MS conditions

The sheath-liquid must be carefully selected in order to have a stable electrospray and good sensitivity. Thus, different parameters affecting the electrospray such as composition and flow of the sheath-liquid, dry gas flow and temperature, and nebulizer pressure have been optimized considering the S/N ratio obtained as response variable.

At the beginning, the composition of the sheath-liquid was evaluated considering different organic solvents such as MeOH, EtOH, propan-2-ol and MeCN. The sheath-liquid in all cases consisted of a 50:50 organic solvent/ultrapure water mixture containing 0.5% (v/v) of formic acid. For most compounds, similar S/N ratios were obtained when MeOH and EtOH were used, except in the case of TCP and ACT that showed an increase in the S/N ratio when EtOH was employed. With MeCN and propan-2-ol the S/N was lower in all cases (Fig. 1). Considering also that EtOH is more environmentally friendly, it was selected as the organic solvent for the sheath-liquid. Subsequently, the percentage of EtOH was studied from 40 to 60%. An increase in the S/N ratio was achieved using 50%, so it was chosen as optimum. Formic acid was added to ensure the adequate positive ionization of the analytes. The percentage added was checked from 0.1 to 1%. It was observed that percentages higher than 0.5 did not improve the S/N ratio, therefore, this value was selected as optimum. Because of these results, sheath-liquid composition was 50:49.5:0.5 (v/v/v), EtOH/ultrapure water/formic acid.

Sheath-liquid flow rate plays an important role to ensure electrospray stability and therefore, it has an influence in the analysis repeatability. Consequently, it was studied in the range 2.5-15 µL min⁻¹ (Fig. S2). A reduction of the S/N ratio was observed when the flow rate increased, which may be explained because of the dilution of the CE effluent. A flow rate below 5 µL min⁻¹ led to an unstable electrospray, so it was discarded. Ergo, 5 µL min⁻¹ was selected as optimum for further analysis.
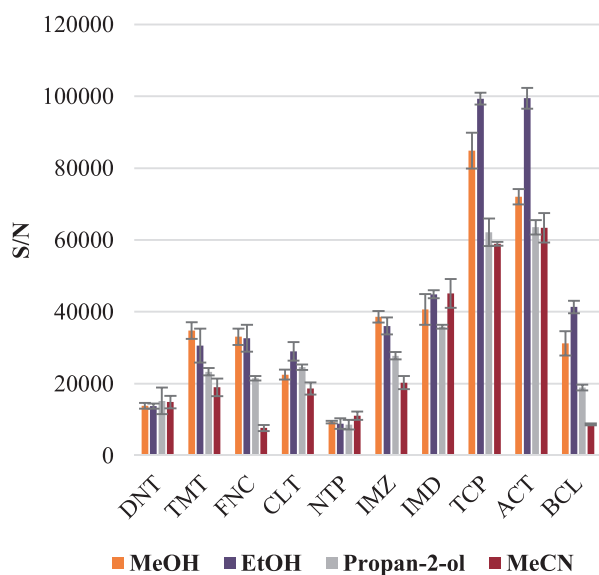
Fig. 1. Effect of the sheath-liquid composition on the signal-to-noise (S/N) ratio. Error bars represent standard error (n=4).

After optimizing the sheath-liquid, the nebulizer pressure was evaluated between 6 and 12 psi. Above 10 psi, the spray stability decreased inducing poor repeatability in the migration. The best compromise between repeatability and S/N ratio was obtained when a nebulizer pressure of 10 psi was used. Regarding the dry gas, temperature and flow were evaluated. Firstly, the dry gas temperature was tested from 200-300 °C taking into consideration that APFO can be used as volatile surfactant at temperatures above 150 °C at which this surfactant decomposes. An increase in the temperature did not improve the S/N ratio, so 200 °C was selected. Then, the dry gas flow rate was studied from 11 to 20 L min^{-1}, obtaining the best S/N ratio when 11 L min^{-1} was employed.

Finally, the ESI voltage which affects the sensitivity of MS detection was also studied. The voltage was varied from 1000 to 3000 V keeping the nozzle at 2000 V. With a voltage of 1000 V a significant reduction of the S/N ratio was observed, however, for the rest of the tested voltages no significant differences were noticed. Thus, 2000 V was chosen as ESI voltage.

In order to get optimum selectivity, the main MS/MS parameters were studied. First of all, using the SCAN mode, it was observed that the protonated molecules $[M+H]^+$ were the most abundant for all analytes. Once the precursor ion was fixed for each compound, the main fragments were investigated by collision induced dissociations selecting the optimum collision energy to be applied in order to obtain the highest signal in each case. Finally, an MRM method was developed taking into consideration the data mentioned before as well as the migration time of the target analytes. In this method, dwell time for each transition was also optimized varying from 40 to 80 ms depending on the analyte to guarantee a minimum data acquisition of 10 points per peak.

3.3. Optimization of the sample treatment

In this work, a scaled-down QuEChERS procedure has been developed for the extraction and clean-up of nine NNIs and boscalid from pollen and honeybee samples. In a scaled-down QuEChERS, the amount of sample is reduced as well as the volume of MeCN required for the extraction of the analytes, reducing the organic solvent consumption and avoiding the dilution of the analyte concentration.

No satisfactory recoveries were obtained when a previously published protocol for determination of NNIs by LC-MS was ap-

plied [39], probably due to a higher matrix effect (ME) in CE-MS. In consequence, the main variables affecting the scaled-down QuEChERS were optimized to achieve the highest extraction recoveries.

To begin with, a representative pollen sample (200 mg) was placed in a 15 mL centrifuge tube and spiked with the desired concentration of the target analytes. Then, the sample was hydrated with 1 mL of ultrapure water and vortexed for proper homogenization. Subsequently, 2.5 mL of MeCN were added, which was the minimum volume able to extract the studied compounds with acceptable recoveries from this amount of sample.

The ionic strength was studied because the addition of salts to the aqueous phase may have a salting-out effect decreasing the analyte solubility in water and favoring their transference to the organic phase. In this sense, several salts such as $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$, and NaCl were evaluated. Thus, after adding the extraction solvent to the aqueous sample, 200 mg of each salt were also added, and the tube was shaken for 2 min and centrifuged for 5 min at 8487 g and 4 °C. It must be mentioned that NaCl quite often led to electrophoretic current disruptions, therefore, it was discarded. The best results in terms of recoveries (above 75% in all cases) were obtained when $MgSO_4$ was employed, so it was selected as salting-out agent. Subsequently, the amount of this salt was also tested from 100 to 400 mg. It was observed that 100 mg was not enough to obtain a well-defined phase separation, leading to poor recoveries. On the other hand, above 200 mg, recoveries decreased in all cases. Therefore, 200 mg of $MgSO_4$ was selected as salting-out agent.

Afterwards, to improve the extraction efficiency and to reduce the matrix effect, different dispersive sorbents were evaluated in the d-SPE step such as Z-Sep+, EMR lipids, PSA, C18 and a mixture of PSA/C18 (1:1) as it is shown in Fig. 2. In all cases an amount of 80 mg of sorbent was used together with 100 mg of $MgSO_4$ anhydrous to remove possible traces of ultrapure water in the organic extraction solvent. In general, recoveries were above 70% in most cases except when the EMR lipids sorbent was used. In addition, the recovery for NTP significantly decreased when Z-Sep$^+$ was employed, being around 40% (Fig. 2A). On the other hand, this sorbent provided the best results in terms of ME (Fig. 2B). The amount of Z-Sep$^+$ was reduced to improve NTP recovery. As can be seen in Fig. S3, reducing the amount of this sorbent to 30 mg, recoveries around 70% for NTP were achieved. Decreasing the amount of sorbent led to ME slightly higher for all analytes, but still better than those obtained with the other sorbents. This sorbent, despite its high potential to clean the complex extract, has not been explored in d-SPE of honeybee products and NNIs determination where PSA sorbent has been traditionally used [3,40].

Finally, different syringe filters were tested through the filtration of a standard solution with each one. Then, the results obtained were compared with a standard solution without filtering at the same concentration. The best results, in terms of recoveries, for most analytes were obtained with hydrophilic-PTFE filter. Unfortunately, even with this filter, around 50% of boscalid was lost during filtration (Fig. S4).

An electropherogram of a pollen sample spiked with the studied analytes submitted to the optimized sample treatment and analyses by the proposed MEKC-MS/MS method is shown in Fig. 3.

3.4. Method characterization

The optimized scaled-down QuEChERS-MEKC-MS/MS method was evaluated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), extraction recovery, matrix effect, and precision (i.e., repeatability and intermediate precision) in pollen and honeybee samples. Both samples were previously analyzed using the proposed method and neither analytes nor interferences were found.
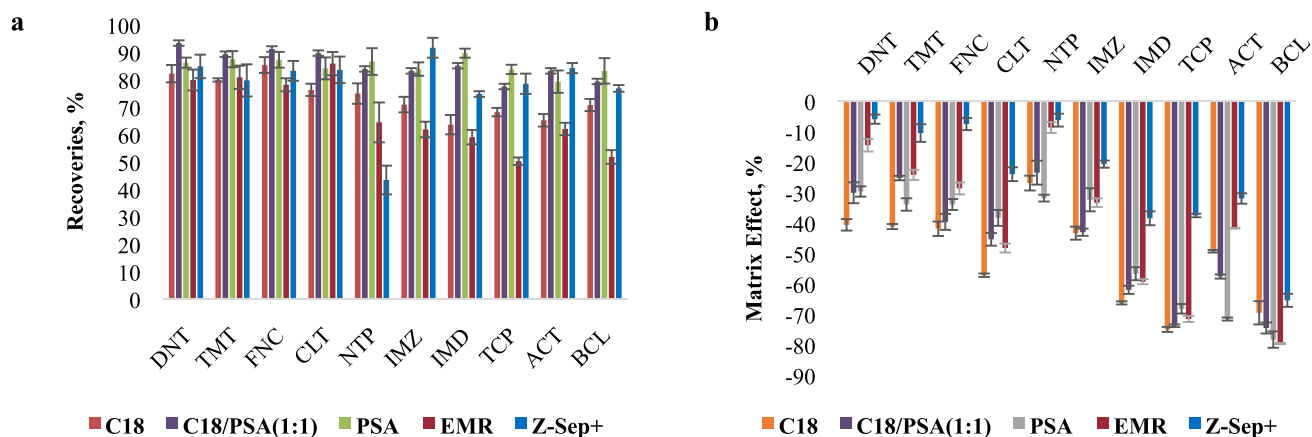
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Fig. 2. Optimization of dispersive sorbents in the d-SPE step of the sample treatment procedure for the extraction of the analytes from a spiked pollen sample. a) Effect on the extraction recoveries; b) Effect on the matrix effect. Error bars represent standard error (n=4).

Table 2
Statistical and performance characteristics of the proposed method for the determination of NNIs and boscalid in commercial pollen samples by MEKC-MS/MS.

Analyte	Linear regression equation	Linear range (μg kg^{-1})	Linearity (R^2)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	MRL (μg kg^{-1})
DNT	y = 16.902x + 75.7	9.7-200	0.9915	2.9	9.7	♦
TMT	y = 22.533x − 39.225	6.5-200	0.9904	1.9	6.5	50*
FCM	y = 13.244x − 25.013	3.8-200	0.9915	1.1	3.8	50*
CLT	y = 13.38x + 8.885	9.7-200	0.9902	2.9	9.7	50*
NTP	y = 2.458x + 7.149	9.0-200	0.9906	2.7	9.0	♦
IMZ	y = 35.417x − 23.187	8.0-200	0.9900	2.4	8.0	♦
IMD	y = 10.372x − 8.522	6.1-200	0.9906	1.8	6.1	50*
TCP	y = 25.305x − 45.832	5.7-200	0.9911	1.8	5.7	200
ACT	y = 19.975x + 32.224	6.0-200	0.9930	1.8	6.0	50*
BCL	y = 5.303x − 28.086	11.6-200	0.9923	3.5	11.6	150

♦MRL non-established. Default value of 10 μg kg^{-1}.
* Indicates lower limit of analytical determination.

3.4.1. Calibration curves and analytical performance characteristics

Procedural calibration curves for pollen and honeybee samples were performed at different concentration levels; 5, 10, 25, 50, 100, and 200 μg kg^{-1} for pollen samples and 2, 5, 10, 25, 50, 100, and 200 μg kg^{-1} for honeybee samples. Procedural calibration involves the analysis of samples fortified before the sample treatment. Two samples were spiked at each concentration level, treated according to the scaled-down QuEChERS procedure, and analyzed in triplicate by the proposed MEKC-MS/MS method. Peak area was selected as analytical response and considered as a function of the analyte concentration on the sample. LODs and LOQs were calculated as the minimum analyte concentrations yielding a S/N ratio equal to three and ten times, respectively. As shown in Table 2, a satisfactory linearity was confirmed at the concentration range studied (R^2 > 0.9900) with LODs and LOQs below 3.5 μg kg^{-1} and 11.6 μg kg^{-1} respectively, for pollen samples, and below 4.0 μg kg^{-1} and 12.5 μg kg^{-1}, respectively, for honeybee samples (Table S3). These results highlight that the proposed method allows the determination of NNIs and boscalid in pollen samples at levels below their MRLs established in apiculture products by the European Legislation [14].

3.4.2. Precision

Precision of the proposed method was evaluated in terms of repeatability (i.e., intra-day precision) and intermediate precision (i.e., inter-day precision) by the application of the method to pollen and honeybee samples spiked at two concentration levels in the linear range (10 and 50 μg kg^{-1}). For repeatability, three samples were submitted to the sample procedure (experimental replicates) and injected in triplicate (instrumental replicates) the same day

Table 3
Precision of the proposed method for the determination of NNIs and boscalid in commercial pollen samples.

Analyte	Repeatability, %RSD (n=9)		Intermediate precision, %RSD (n=9)	
	10 μg kg^{-1}	50 μg kg^{-1}	10 μg kg^{-1}	50 μg kg^{-1}
DNT	8.3	5.7	12.9	9.6
TMT	10.0	10.4	14.4	13.8
FCM	9.4	8.2	13.6	8.7
CLT	10.3	8.5	13.9	9.8
NTP	10.1	9.0	14.8	12.7
IMZ	8.3	8.9	14.2	9.2
IMD	10.6	8.3	13.6	8.6
TCP	10.8	9.6	13.7	12.2
ACT	9.0	7.5	12.0	11.4
BCL	11.3	9.3	15.5	13.5

under the same conditions (n=9). In the case of intermediate precision, it was evaluated with a similar procedure, but analyzing one sample prepared each day during three different days (n=9). The obtained results, expressed as RSD (%) of peak areas, for pollen samples are summarized in Table 3 while the corresponding results for honeybee samples are in Table S4. Satisfactory RSD were achieved for both samples, being lower than 10.6% and 15.2% for repeatability and intermediate precision, fulfilling the EU recommendations concerning the performance of analytical methods for the determination of contaminants, which set an upper limit for RSD of 20% [41].

3.4.3. Recovery studies

In order to evaluate the efficiency of the proposed scaled-down QuEChERS, recovery experiments were carried out. Three blank
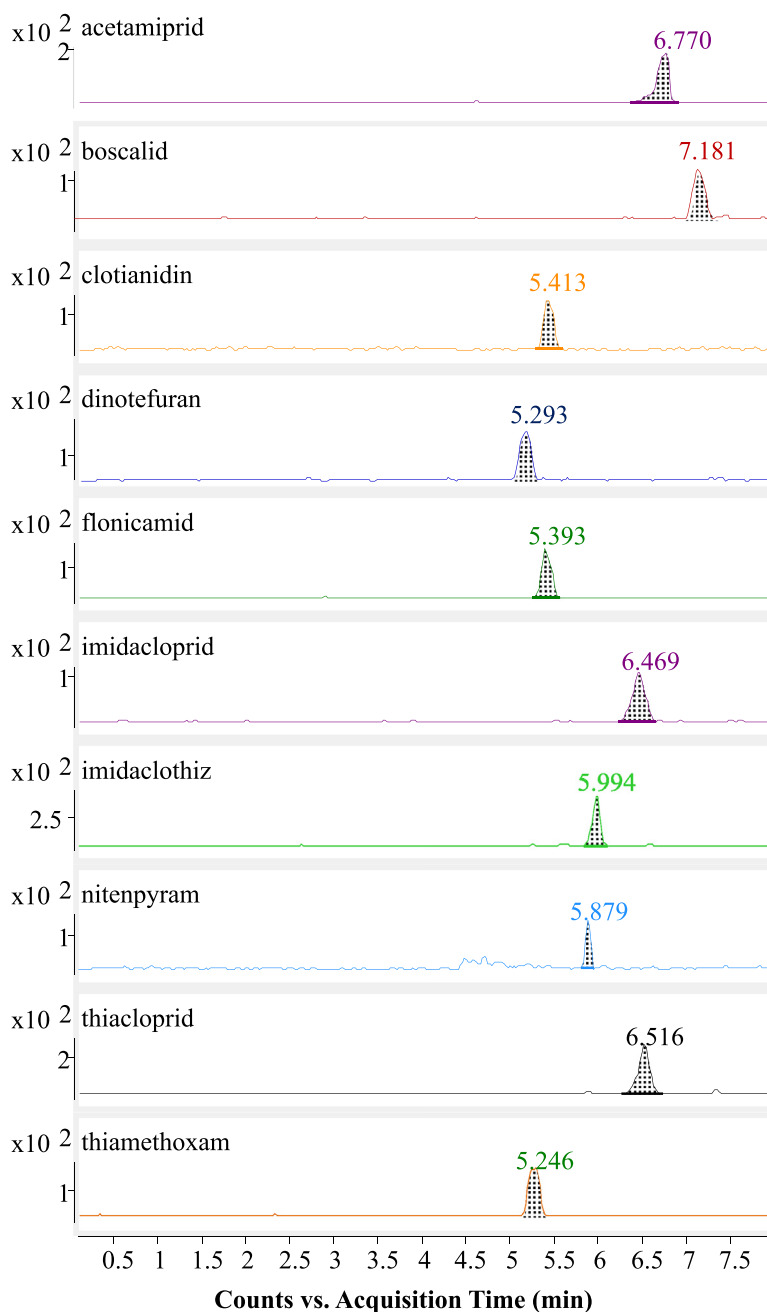
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Fig. 3. Electrophoretic separation of a blank pollen sample spiked with the standard mixture solution of NNIs and boscalid at a concentration of 200 µg kg^{-1}.

samples of each matrix were fortified at two different concentration levels (10 and 50 µg kg^{-1}), treated following the sample treatment procedure and analyzed in triplicate by MEKC-MS/MS. The data, in terms of peak area, were compared with those obtained by analyzing extracts of blank samples submitted to the sample treatment and fortified at the same concentration levels just before the injection. Generally, recoveries over 80% were obtained except for nitenpyram and boscalid in pollen samples, which showed recovery values above 70% (Table 4). The results for honeybee samples are shown in Table S5. In any case, these results suggest that the

proposed sample treatment method could be satisfactorily applied to determine NNIs and boscalid in these matrixes.

3.4.4. Evaluation of matrix effect

Matrix effect (ME) can be attributed to many factors, affecting analyte ionization in MS and, therefore, resulting in ion suppression or signal enhancement. ME can be estimated by comparing the analytical response provided by blank extracts spiked after the sample treatment with the response that results from a standard solution at the same concentration. The following equation is used for this comparison:

$$ME(\%) = \frac{\text{signal of extract spiked after extraction} - \text{signal of standard solution}}{\text{signal of standard solution}} \times 100$$
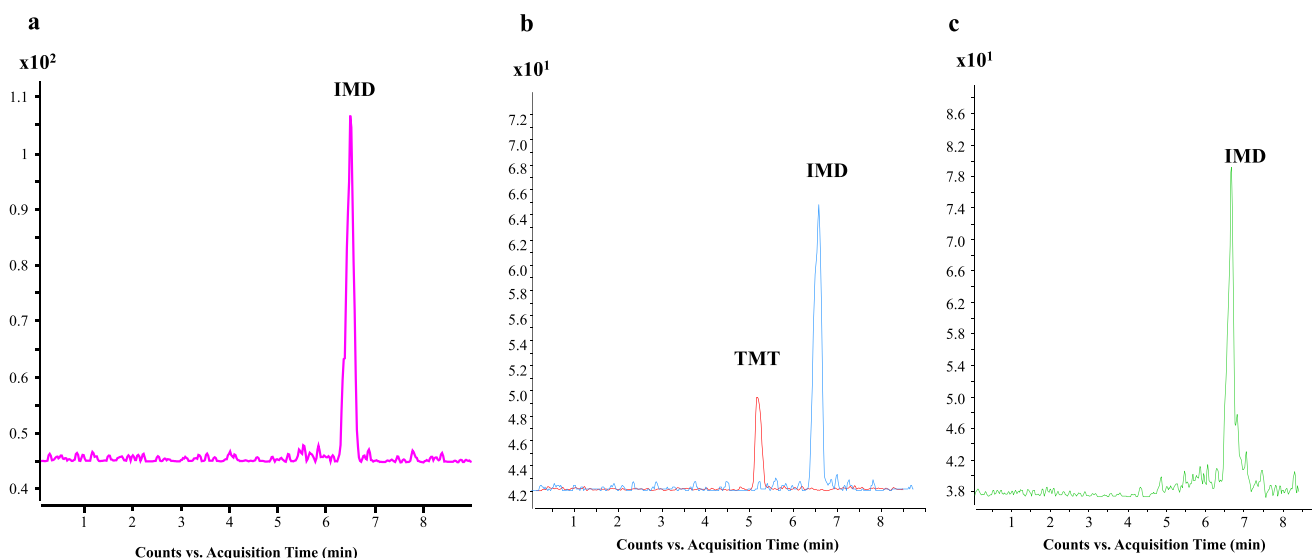
Fig. 4. Electropherograms of naturally contaminated samples of pollen: a) IMD (61.2 µg kg^1); b) IMD (20.1 µg kg^{-1}) and TMT (10.7 µg kg^{-1}), and honeybees C) IMD (8.4 µg kg^{-1}).

Table 4
Matrix effect and recovery studies of the proposed method for the determination of NNIs and boscalid in commercial pollen samples.

Analyte	Matrix Effect (%)		Recovery (%)	
	10 µg kg^{-1}	50 µg kg^{-1}	10 µg kg^{-1}	50 µg kg^{-1}
DNT	-15.4	-11.3	80.1	85.5
TMT	-21.4	-19.6	87.3	90.1
FCM	-22.0	-18.7	86.1	88.2
CLT	-33.7	-30.1	80.8	83.9
NTP	-17.9	-16.7	70.6	74.2
IMZ	-16.8	-16.2	85.4	86.4
IMD	-41.9	-38.4	91.5	94.2
TCP	-42.8	-37.2	80.5	85.9
ACT	-37.6	-34.7	92.6	95.2
BCL	-70.1	-66.1	75.2	79.4

The ME was evaluated in pollen and honeybee samples at two concentration levels (10 and 50 µg kg^{-1}). A ME of 0% indicates the absence of the matrix effect, a ME below 0% involves signal suppression while a ME above 0% reveals signal enhancement from interferences. As shown in Table S5, most of the analytes presented a negligible ME ($<|20\%|$) in honeybee samples. However, higher signal suppression was observed for most analytes in pollen samples (Table 4). Nevertheless, procedural calibration curves were established for both matrices to compensate both, ME and losses due to the sample treatment procedure.

3.5. Analysis of real samples

Three pollen samples collected from almond blossoms in three different locations and one sample of honeybee bodies were analyzed in triplicate in order to demonstrate the applicability of the validated method. The honeybees were found dead under suspicious circumstances since hundreds of these specimens died suddenly in the same area. Both sampling points (pollen and honeybees) were less than 100 m apart from each other.

The criteria set for the positive identification of NNIs in the samples was that a peak should have a S/N ratio of at least 3 and the relative ion intensities for detection and quantification ions must correspond to those of these ions in the solutions of standards. Thereby, samples which met these requirements and also exceeded the corresponding LOQs, were considered as positives.

Hence, the results revealed that imidacloprid was found in two of the three analyzed pollen samples, in concentrations of 61.2 µg kg^{-1} (1.7% RSD, n=3) and 20.1 µg kg^{-1} (0.9% RSD, n=3), respectively. The first sample exceeded the "limit of analytical determination" established for this compound in honey and other apiculture products (50 µg kg^{-1}), considering that no MRL is established because of its prohibition. In addition, thiamethoxam was also found in the second sample with a concentration of 10.7 µg kg^{-1} (1.1% RSD, n=3) (Fig. 4).

The results also indicated that honeybees were contaminated with 8.4 µg kg^{-1} of imidacloprid (0.7% RSD). These results suggest that some NNIs could have been applied as a control insecticide in near agricultural fields leading to the presence of residues in the pollen of almond tree's flowers. Additionally, the presence of imidacloprid in honeybee samples could suggest that honeybees could have been in contact with this insecticide despite of being banned for foliar uses. This fact suggests a possible causal link between the presence of this insecticide and the death of the honeybees analyzed in this study.

4. Conclusions

To the best of our knowledge, this is the first time that MEKC coupled to tandem MS detection has been applied for monitoring NNIs and boscalid. A volatile surfactant such as APFO, which acts simultaneously as BGE and micellar medium compatible with MS, has been employed. The proposed MEKC-MS/MS method offers shorter analysis time, higher resolution, and higher selectivity and sensitivity than the only one previous method for the control of NNIs in beeswax using CZE-MS [27]. Furthermore, MEKC enables an on-line pre-concentration strategy such as sweeping that made possible to achieve SEFs between 1.6 and 5.6 for the studied compounds. Regarding sample treatment, a scaled-down QuEChERS has been optimized. Different dispersive sorbents were evaluated and Z-Sep+, although less commonly employed than C18 and PSA, provided better results in terms of matrix effect. In addition, unlike traditional QuEChERS methods, sample is not diluted, which improves method sensitivity. LOQs in the range of low µg kg^{-1} were obtained for all target pesticides in pollen and honeybee samples which demonstrated for the first time the potential of using MEKC-MS/MS for their quantification. In addition, this method is in compliance with the principles of green analytical chemistry. It com-

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bines the low solvent consumption during sample treatment with the reduced volume of BGE used in CE and the lower waste production. Moreover, this method involves a low amount of sample and lower cost than LC methods. The usefulness of the developed method was proved by its application to natural pollen and honeybee samples suspected of being contaminated. Results suggest that the use of these pesticides could be the reason of the sudden death of hundreds of honeybees close to a field of almond trees. To conclude, the proposed scaled-down QuEChERS-MEKC-MS/MS method can be a real alternative to LC methods to monitor NNIs and boscalid in pollen and honeybee samples.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Laura Carbonell-Rozas: Conceptualization, Investigation, Methodology, Writing – review & editing. **Burkhard Horstkotte:** Formal analysis, Methodology. **Ana M. García-Campaña:** Supervision, Project administration. **Francisco J. Lara:** Conceptualization, Supervision, Writing – review & editing.

Acknowledgments

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463023.

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