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Capillary electrophoresis–mass spectrometry as a new approach to analyze neonicotinoid insecticides

Laura Sánchez-Hernández[a,b], Deamelys Hernández-Domínguez[a], José Bernal[a], Christian Neusüß[b], María T. Martín[a], José L. Bernal[a,*]

[a] I.U. CINQUIMA, Analytical Chemistry Group, University of Valladolid, 47011 Valladolid, Spain
[b] Faculty of Chemistry, Aalen University, Beethovenstraße 1, 73430 Aalen, Germany

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ABSTRACT

This paper represents the first report of a capillary electrophoresis (CE) method compatible with mass spectrometry (MS) detection for simultaneously analyzing seven neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam). Different variables affecting CE separation (buffer concentration, pH, applied voltage and injection time) and MS detection (electrospray parameters) were studied. Low limits of detection (LOD) and quantification (LOQ) were achieved for all analytes, ranging from 1.0 to 2.3 μg/L, and from 3.5 to 7.2 μg/L, respectively. In addition, the proposed method showed itself to be linear in the range from LOQ to 1000 μg/L and to be precise, as the relative standard deviations of the migration times were lower than 4% in all cases. Finally, the proposed CE–MS method was applied to assess the efficacy of a beeswax cleaning treatment with oxalic acid to remove residues of three of the most commonly used neonicotinoids (clothianidin, imidacloprid and thiamethoxam), use of which has recently been restricted by the European Union.

1. Introduction

Neonicotinoids (imidacloprid, acetamiprid, clothianidin, thiacloprid, thiamethoxam, dinotefuran and nitenpyram, see structures in Table 1) are a class of insecticides deriving from the nicotine moiety. Their use has increased considerably since the early 1990s, representing one of the fastest growing types of insecticides, employed extensively for the control of agricultural pests by spraying and also widely used in seed dressings and soil additions [1]. However, concerns regarding the side effects on health and the environment of synthetic chemical pesticides such as neonicotinoids continue to increase, since the parent compounds and their metabolites can be transferred to the environment and the food chain, with potential adverse consequences for biodiversity. Therefore, it is necessary to monitor and analyze neonicotinoid residues.

The separation and analysis of neonicotinoid insecticides has been accomplished mainly by chromatographic techniques such as gas chromatography (GC) [2–4] or liquid chromatography (LC) with UV/diode array [5–8], fluorescence [9], electrochemical [10] or mass spectrometry (MS) [1,11–18] detectors; coupled LC–MS has been the most common method. However, in the last few years certain capillary electrophoresis (CE) approaches have also been proposed to analyze this group of insecticides [19–26]. All of these CE publications were based on the micellar electrokinetic chromatographic mode (MEKC) using UV [19–25] or indirect laser-induced fluorescence (LIF) [26] detection (see Table 2). It must be pointed out that previous sample treatments such as solid phase extraction (SPE) [20,21,24,25] or dispersive liquid–liquid microextraction (DLLME) [22,24] have been employed in most of these articles in order to improve the limits of detection (LODs) when UV-absorbance detectors were used in CE applications. However, it should be highlighted that, to our knowledge, no specific CE method with mass spectrometry (MS) detection for analyzing neonicotinoids has yet been reported. This coupling combines the advantages of CE techniques (high separation efficiency, speed of analysis and low consumption of sample and reagents) with the high sensitivity, selectivity and capacity for identifying unknown compounds that is offered by MS detection. In addition, previous derivatization (LIF) or sample treatments (UV to improve sensitivity) are not strictly required for the detection of these compounds when MS detectors are used. Thus, due to the keen interest in developing methodologies enabling the analysis of these insecticides at trace levels, the aim of this study has been to present a CE methodology compatible with MS detection for the simultaneous analysis of seven neonicotinoids.

* Corresponding author. Tel.: +34 983 423280; fax: +34 983 186347.
E-mail address: jlbernal@qa.uva.es (J.L. Bernal).

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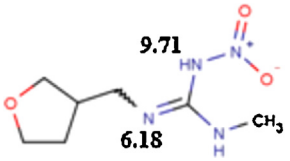
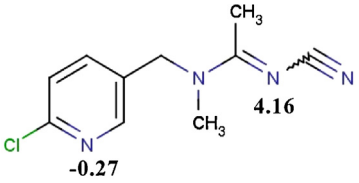
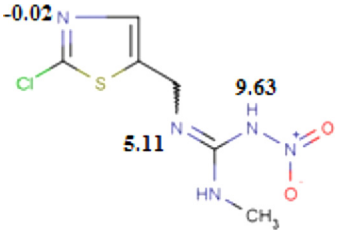
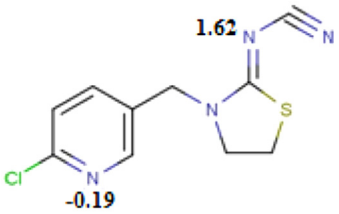
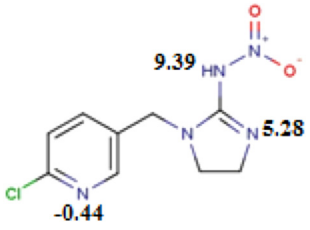
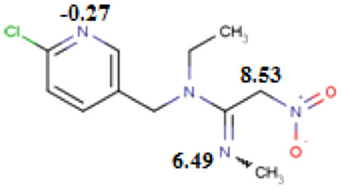
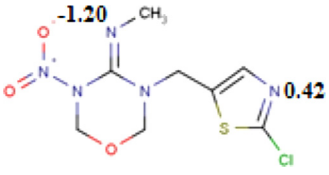
Table 1
Structures, pKa values and estimated charge of the studied neonicotinoids.

Neonicotinoid	pKa	Estimated charge	
		Acid medium (pH 1.0–6.0)	Basic medium (pH 8.0–14.0)
Dinotefuran		Positive (NH—C(—NH)=NH^+)	Negative (NH—C(—N^-)=N)
Acetamiprid		Positive (CH_3—C(—NH)=NH^+)	Neutral
Clothianidin		Positive (NH—C(—NH)=NH^+)	Negative (NH—C(—N^-)=N)
Thiacloprid		Neutral	Neutral
Imidacloprid		Positive (N—C(—NH)=NH^+)	Negative (N—C(—N^-)=N)
Nitenpyram		Positive (CH_2—C(—N)=NH^+)	Negative (CH^-—C(—NH)=N)
Thiamethoxam		Neutral	Neutral

In addition, the applicability of the method was demonstrated by the analysis of neonicotinoids in beeswax samples. The choice of beeswax as matrix is due to [1,18]: (i) beeswax could be considered a contaminant reservoir, and the pesticides present in wax could directly affect the bee colony or be transmitted to other bee products; (ii) there are concerns regarding potential adverse effects caused by such insecticides on non-target organisms, particularly pollinators; (iii) it is a complex matrix. The European Union has recently adopted a proposal (Regulation (EU) 485/2013) [27] to restrict the use of three of these (clothianidin, imidacloprid and thiamethoxam) which have been recognized as representing severe risks for honeybees due to their connection with colony collapse disorder, for example, when bees have been exposed to dust, pollen and/or nectar of several crops treated with these neonicotinoids

Table 2
CE published methods for determining neonicotinoids.

Detector	Neonicotinoids	Buffer	Analysis time (min)	LOD	Sample	Sample treatment	Ref.
UV	Imidacloprid and three degradation products	50 mM borate (pH 8.5) + 22 mM SDS +100 mL/L methanol in water	35	NS	Aqueous samples	NS	[19]
UV	Imidacloprid and 6-CNA	15 mM NH_4Cl/NH_3 (pH 8.5) + 60 mM SDS	6	0.71 and 1.18 mg/L	Greenhouse air	SPE	[20]
UV	Thiamethoxam, acetamiprid, and imidacloprid and 6-CNA	5 mM borate (pH 10.4) + 40 mM SDS + 5% (v/v) methanol	7	0.01–0.07 mg/L for river water, and 0.17–0.37 mg/kg for soil	Water and soil	SPE	[21]
UV	Thiacloprid, acetamiprid, imidacloprid and imidaclothiz	50 mM boric acid (pH 2.0) + 80 mM SDS + 25% (v/v) methanol	20	0.8–1.2 μg/kg	Cucumber	DLLME	[22]
UV	Imidacloprid, acetamiprid, thiamethoxam and NA	10 mM tetraborate + 30 mM SDS + 10% (v/v) acetonitrile	9	NS	NS	NS	[23]
UV	Imidacloprid	15 mM tetraborate + 30 mM SDS + 10 mM TBAP + 10% (v/v) acetonitrile	20	0.2 μg/L by SPE and 0.4 μg/L by DLLME	Water	SPE and DLLME	[24]
UV	Imidacloprid	15 mM tetraborate + 30 mM SDS + 10 mM TBAP + 10% (v/v) acetonitrile	17	21 μg/kg	Soil	SPE	[25]
Indirect LIF	Thiamethoxam, acetamiprid, and imidacloprid	40 mM borate (pH 8.0) + 60 mM SDS + 4.4 μM cadmium telluride quantum dots	12	0.05, 0.01, 0.009 mg/kg	Vegetables	NS	[26]

6-CNA: 6-chloronicotinic acid; DLLME: dispersive liquid–liquid microextraction; LIF: laser induced fluorescence; NA: nicotinic acid; NS: not specified; SDS: sodium dodecyl sulfate; SPE: solid phase extraction; TBAP: tetrabutylammonium phosphate.

(e.g. maize, cereals, sunflower, oilseed rape, beets, potatoes, etc.). In this study, attention will be focused on evaluating the usefulness of the developed CE–MS method to assess the efficacy of a beeswax cleaning treatment with oxalic acid to remove residues of the three restricted neonicotinoids (clothianidin, imidacloprid and thiamethoxam). There are several reasons for using oxalic acid [28]: (i) it binds to a part of the iron which is responsible for wax darkening; (ii) it helps in breaking of emulsions and help the settling of impurities; (iii) European beekeepers have also used oxalic acid to deal with the varroa mite, which affects honeybees. However, it is still unclear whether this treatment with oxalic acid is able to remove the neonicotinoids from beeswax. As a result, we deemed it of interest to check the effectiveness of oxalic acid in performing this task.

Thus, we sought to develop the first CE–MS method, both efficient and sensitive, to detect the lowest amount possible of the seven most frequently employed neonicotinoid insecticides, the aim being to provide an alternative to the existing CE and LC methods. Accordingly, another goal of the present study was to apply this method, by means of a qualitative approach, to the analysis of neonicotinoid spiked beeswax samples, thereby ascertaining the efficacy of a cleaning treatment with oxalic acid to remove three of these compounds from beeswax, which has not been previously undertaken.

2. Experimental

2.1. Materials and chemicals

Neonicotinoid insecticide standards (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 2-Propanol, methanol (both LC–MS grade), acetic acid, formic acid, sodium hydroxide, and aqueous ammonia solution (30% (v/v) in water) were obtained from Carl Roth GmbH und Co. KG (Karlsruhe, Germany). Ammonium acetate and ammonium bicarbonate were obtained from Sigma–Aldrich (Steinheim, Germany). Dichloromethane, isopropanol (both LC grade), *n*-hexane (95%) and acetone (99.8%) (both Pestiscan grade) were supplied by Lab Scan Ltd. (Dublin, Ireland), while ultrapure water was obtained using Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). In addition, a "ESI-L Tuning mix" solution was obtained from Agilent Technologies (Waldbronn, Germany).

Moreover, a vibromatic mechanical shaker, a drying oven and an ultrasonic bath (Ultrasons), all of them supplied by J.P. Selecta S.A. (Barcelona, Spain), a 5810 R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany), a 12-port system of solid–liquid extraction vacuum manifold from Waters Corporation (Milford, MA, USA), Isolute® HM-N, diatomaceous earth packed (10 mL sample) cartridges from Biotage (Uppsala, Sweden), 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).

2.2. Preparation of standard solutions

Standard stock solutions were prepared by dissolving approximately 10 mg of powder of each neonicotinoid in 10 mL of Pestican-grade acetone, to a final concentration of approximately 1000 mg/L. These solutions were further diluted with a water and acetonitrile (50:50, v/v) mixture to prepare the working solutions. Blank beeswax samples (2 g) were spiked at 100 μg/L with clothianidin, imidacloprid and thiamethoxam, corresponding to 50 μg/kg according to the unit conversion, and received a previously optimized sample treatment [1]. All samples, standard stocks and working solutions were stored in glass amber containers at +4 °C, and were stable for over one month.

2.3. Sample procurement and treatment

2.3.1. Samples

Neonicotinoid-free beeswax samples from ecological apiaries were provided by the Centro Apícola Regional (CAR) of Marchamalo (Guadalajara, Spain) and were used as blank beeswax samples. The absence of neonicotinoid residues in those samples was verified by LC–MS [1] and the proposed CE–ESI-MS method. It should be also mentioned that it was not possible to obtain real beeswaxes

containing the target compounds at the time of the experiments. All the samples were stored in the dark at 4 °C until analysis.

2.3.2. Sample treatment

2.3.2.1. Cleaning process. Briefly, beeswax samples (5 g) were spiked with one neonicotinoid (clothianidin, imidacloprid or thiamethoxam) at 100 μg/L (50 μg/kg), after which 25 mL of 0.25% oxalic acid solution in water (v/v) were added; the mixture was then centrifuged for 10 min at 60 °C and 700 rpm so as to facilitate contact between phases. After 5 min of cooling time at room temperature, the beeswax was again solidified and was then ready for the extraction procedure.

2.3.2.2. Extraction procedure. Beeswax extraction conditions were determined and optimized in a previous publication [1], where it was stated that the resulting recovery ranged from 85% to 105% with relative standard deviation (%RSD, $n = 6$) values lower than 7% in all cases, indicating that the proposed sample treatment procedure was good enough. Briefly, 2 g of the homogenized beeswax sample and 15 mL of the *n*-hexane/isopropanol mixture (8:2, v/v) were transferred to a glass beaker. The beeswax was dissolved after the mixture had been heated for 3 min at 50 °C. Once the beeswax was dissolved, 10 mL of water were added, and the mixture was centrifuged for 5 min at 50 °C and 700 rpm. Following this, the aqueous phase (10 mL) was separated and loaded onto the diatomaceous earth cartridges, and after 15 min the analytes were eluted with 20 mL of acetone. The extract was placed in a 25 mL conical flask and evaporated gently until dry in a rotary evaporator. The residue was reconstituted in 1 mL of solution water/acetonitrile (50:50, v/v), and passed through a syringe filter, before its injection into the CE–MS system.

2.4. CE–MS conditions

An HP^{3D}CE instrument (Agilent Technologies, Palo Alto, CA, USA) coupled by means of an orthogonal electrospray interface (ESI, model G1607A, from Agilent Technologies) to a Q-TOF instrument (Compact QTOF, Bruker Daltronik GmbH, Bremen, Germany), was employed. CE experiments were performed using uncoated fused-silica capillaries of 50 μm id of a total length of 75 cm (Polymicro Technologies, AZ, USA), at +10 kV and 25 °C. The sample was injected hydrodynamically, at a pressure of 50 mbar for 15 s, after which the capillary was dipped into a water vial in order to prevent contamination of the background electrolyte (BGE, 0.5 M ammonia solution). Between injections, the capillary was rinsed with water for 2 min and the BGE for the same time. Regarding storage, the capillary was flushed with water for 15 min.

Electrospray ionization (ESI) was used in positive ion mode with the application of 5.0 kV (capillary voltage) on the MS inlet (sprayer on ground). Electrical contact at the electrospray needle tip was established via a sheath liquid, which consisted of isopropanol/water (50:50, v/v) containing 0.2% formic acid in water (v/v), and delivered at a flow rate of 3.3 μL/min by a syringe pump (model 100, Holliston, USA) with a 5 mL Hamilton syringe from Supelco (Bellefonte, PA, USA). The flow rate of drying gas (N_2) was 5.0 L/min at a temperature of 250 °C. The nebulizer gas (N_2) pressure was set to 5 psi, and the capillary exit offset was 150 V. Spectra were acquired in a mass range of m/z 50–700. The m/z scale of the mass spectra was calibrated daily by infusing an electrospray calibration tuning mix solution (Bruker Daltronik, Bremen, Germany). The fragmentation was carried out in multiple reaction monitoring (MRM) mode by using a window of 5 m/z and collision energies between 14 and 29 eV. Meanwhile, extracted ion electropherograms (EIE) were obtained with a different window (±0.02 m/z) in order to extract the exact mass. MS control and data analysis were carried out by means of Bruker Compass version 4.1 (Bruker Daltronik, Bremen, Germany).

3. Results and discussion

3.1. Optimization of CE–MS conditions

In this study a CE–MS method was developed for the simultaneous separation and identification of seven neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam), as an alternative approach to those previously described by MEKC (UV and indirect LIF). It should be mentioned that those methods employed conditions incompatible with MS detection due to the presence of high concentrations of the surfactant sodium dodecyl sulfate (SDS), a non-volatile salt that causes contamination of the ionization source and subsequently low detection sensitivity. Therefore, it was decided that CE should operate in the zone mode (CZE), as the working conditions allow MS detection.

3.1.1. Selection of the CE conditions

Firstly, the pKas of the neonicotinoids were estimated in order to ascertain the properties of these compounds and evaluate the pH range in which they can be charged. These values were calculated by using MarvinSketch 6.1.6 chemical editor software program (ChemAxon Ltd., Budapest, Hungary) [29]. The results presented in Table 1 showed that, in theory, at different pH values the neonicotinoids would be differently charged depending on the compound, which would affect the order of migration. Thus, acidic, neutral and basic pH values were tested to obtain optimal CE conditions. First of all, two different acids were tested (formic acid and acetic acid, pH < 3); no peaks were observed in the electropherograms (CE–ESI-MS) after 40 min of analysis time. This could be explained by the fact that no positive charges were obtained (maybe due to the nature of the nitro group beside the guanidine group), and the neutral compounds were not detected (possibly because a low electro-osmotic flow (EOF) at these pH values). Next, ammonium acetate (pH ~ 7.0) was tested. Under neutral pH conditions all the compounds were detected but they migrated at the same time, as was to be expected on account of their neutral behavior at this pH value. Basic media were also tested by the use of ammonium bicarbonate (pH 8.0–10.0) and ammonia (pHs >10.0) solutions, in order to determine their influence on neonicotinoid separation. The results showed that there was a slight separation between the seven compounds under study when ammonia was used, as three migration groups were observed; yet it was not possible to avoid coelution of the compounds using ammonium bicarbonate. One possible explanation of this behavior could be related to the low percentage of the ionized compound at these pH values according to the pKa values (data not shown). Regarding the order of migration, it could be considered that three of the neonicotinoids were neutral at basic pHs (acetamiprid, thiacloprid and thiamethoxam, see Table 1), and therefore they eluted with EOF. Meanwhile, dinotefuran, clothianidin, nitenpyram and imidacloprid would be negatively charged, migrating later than the neutral neonicotinoids. In addition, it would be expected that nitenpyram would have a different migration time than the other negatively charged compounds due to the absence of the guanidine group in its structure (see Table 1). Hence, ammonia solution was selected and subsequently the separation conditions (BGE concentration, capillary length, applied voltage and injection time) were optimized to obtain the maximum possible resolution. Firstly, different ammonia concentrations (0.20–1.00 mol/L) were tested. It was found that at low concentration values (<0.50 mol/L), the current was not enough to maintain the separation system,

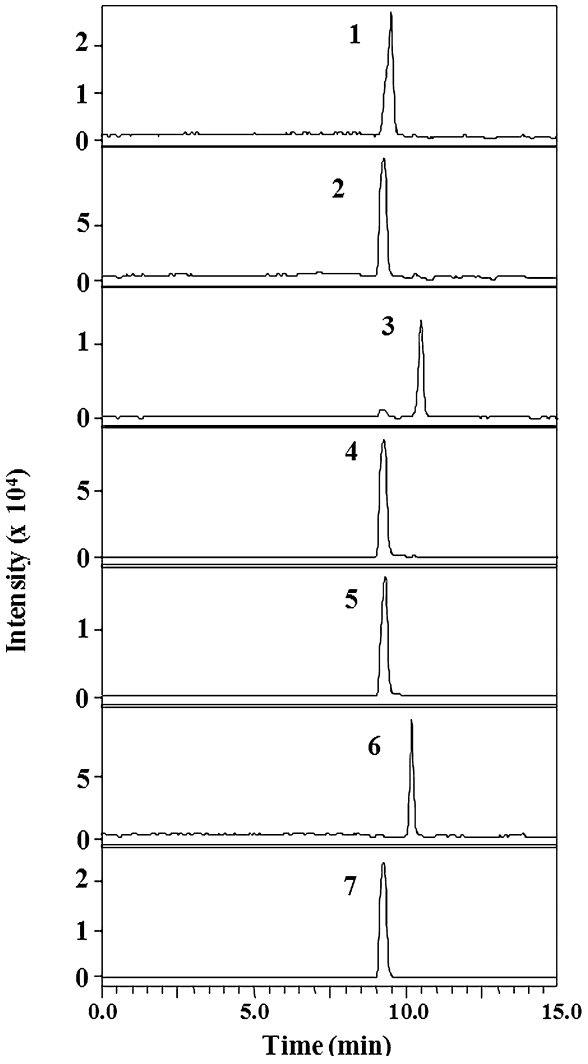


Fig. 1. CE–ESI-MS electropherograms obtained in EIE mode from a 100 μg/L standard solution mixture of (**1**) dinotefuran, (**2**) acetamiprid, (**3**) clothianidin, (**4**) thiacloprid, (**5**) imidacloprid, (**6**) nitenpyram, (**7**) thiamethoxam. The CE–ESI-MS conditions are described in Sections 2.4 and 3.1.2.

while at concentration values higher than 0.50 mol/L, the current increase was quite low in relation with 0.50 mol/L, but it might damage and even break the capillaries. Thus, ammonia concentration of 0.50 mol/L (pH 11.2) was selected in order to obtain an adequate current (5–7 μA) to maintain stable the CE–MS coupling. An examination was also made on the influence of the capillary length (55–100 cm) on CE analysis. An increase in capillary length up to 75 cm positively affected the resolution between the three groups of compounds. Capillary lengths greater than 75 cm did not improve separation but increased analysis times. For these reasons, 75 cm was selected as the optimal capillary length. Another parameter needing to be studied was the applied voltage (+5–30 kV). At high voltages (>+10 kV), no separation was obtained, whilst the use of +10 kV allowed the best separation between the groups of compounds without a broadness of peaks, as it occurred at +5 kV; consequently, +10 kV was chosen as the applied voltage. Finally, different injection times (15, 25 or 50 s) at 50 mbar were tested, and a sample injection time of 15 s was selected since the peaks were broader at higher injection times. Finally, with the CE conditions described above, the seven neonicotinoids were determined in less than 11 min (Fig. 1). As can be seen in the EIEs, acetamiprid, thiacloprid, and thiamethoxam migrated at the same time, which was in line with our expectations. In addition, imidacloprid displayed the same migration time as these three neonicotinoids. Furthermore, dinotefuran was separated from these four neonicotenoids, although full separation was not possible. Baseline separation was obtained for nitenpyram and clothianidin.

As it has been previously commented, the CE–MS analysis time required to analyze the seven compounds was lower than 11 min, which is shorter [1,11,14,15,18,19,22,24–26] or longer [12,13,16,17,20,21,23] than the previous CE-based (MECK, see Table 2 [19–26]) and LC–MS methods [1,11–18]. Although it should be remarked that in some cases, the number of studied compounds was lower than five [12,17,19–26]. Therefore, it has been demonstrated that the analysis time obtained with the proposed CZE-MS method was comparable to of the existing CE (MECK) and LC–MS methods, although it should be always taken into account that it is not a true comparison, as the separation conditions, which strongly affect to the analysis time, were quite different in all cases (e.g. BGE for MECK, flow-rate for LC).

3.1.2. Selection of MS conditions

This is the first CE–MS method developed to analyze neonicotinoid insecticides, which means that no specific conditions are described. However, as there are many papers published in which MS was coupled to other separation techniques, mainly LC, we may take into account some of the data summarized in such studies with a view to optimizing MS detection in our study. Initially, the source of ionization (ESI) was tested in both modes (positive and negative) to analyze neonicotinoids, with the result that the best performance was achieved in the positive mode. Subsequently, several analytical parameters affecting ESI-MS sensitivity were studied. First of all, different sheath-liquid compositions were tested, in all cases mixtures (50:50, v/v) of water and different organic solvents (isopropanol, methanol and acetonitrile); formic acid, bicarbonate, or ammonia was added to the sheath-liquid at low percentages (0.2%, v/v). The results showed that the water: isopropanol mixture (50:50, v/v) provided the best peak intensity values. Moreover, it should be pointed out that the separation system was more stable when the sheath-liquid was in combination with an acid (0.2% (v/v) formic acid). Once the sheath-liquid conditions were selected, the effect of several other ESI parameters on the peak intensities was investigated: drying gas (N_2) temperature (180–300 °C), capillary voltage (3.5–5.0 kV), nebulizer gas (N_2) pressure (2–10 psi) and drying gas (N_2) flow (3–10 L/min). To begin with, different drying gas (N_2) temperatures were tested, and it was observed that an increase in temperature up to 250 °C provided higher peak intensities, while no significant differences were found when working at higher temperatures. It was also observed that in the case of capillary voltage, which is the high voltage applied to the tip of the MS capillary, the peak intensity was directly related to its value, as better intensities were obtained at higher voltages; consequently, 5.0 kV was selected as the optimal value. Finally, intermediate values (5 L/min and 5 psi) were chosen as the optimal values for the drying gas (N_2) flow and nebulizer gas (N_2) pressure, respectively, due to the fact that with these conditions the highest peak intensities were obtained. Moreover, full mass spectra of the seven neonicotinoids showed a major ion m/z corresponding to the protonated molecular ion $[M+H]^+$, which was used as a quantification ion with the EIE (see Table 3). The precursor ions for MRM were chosen from the full scan mass spectrum of each compound, and for all the analytes the protonated ions were chosen as precursors. In addition, collision energies were optimized with the aim of generating several intense product ions from each precursor. For identification and confirmation of the analytes, two product ions were selected and a different collision energy was used for each one (see Table 3). In addition, the selected product ions are in good agreement with those found in the existing LC–MS/MS literature methodologies [11,13–17]. Finally, it

Table 3
Characteristic ions, migration times (MTs), linearity range (MS experiments), determination coefficients (MS experiments), limits of detection and quantification (LOD and LOQ) and collision energies (MS/MS experiments) obtained for the studied neonicotinoids in standard solutions with the optimized CE–ESI-MS methodology.

	MS experiments						MS/MS experiments			
	Precursor ions[a] (m/z)	MTs (min)	LOD (μg/L)	LOQ (μg/L)	Analytical range (μg/L)	R^2	Product ions[b] (m/z)	Collision energy (eV)	LOD (μg/L)	LOQ (μg/L)
Dinotefuran	203.1253	9.6	1.8	6.0	6.0–1000	0.9998	113.0942/129.0894	16	4.0	12.8
Acetamiprid	223.0867	9.4	1.2	4.0	4.0–1000	0.9980	126.0101/187.0392	27	2.6	8.2
Clothianidin	250.0295	10.4	2.3	7.2	7.2–1000	0.9910	131.9661/169.0738	14	4.7	15.2
Thiacloprid	253.0443	9.4	1.3	4.4	4.4–1000	0.9936	126.0199/186.1640	29	2.8	8.9
Imidacloprid	256.0718	9.4	2.0	6.5	6.5–1000	0.9919	175.1076/209.0856	20	4.0	13.2
Nitenpyram	271.1103	10.1	1.0	3.5	3.5–1000	0.9918	99.0611/225.1220	18	2.2	6.9
Thiamethoxam	292.0415	9.4	2.1	6.7	6.7–1000	0.9975	131.9764/211.0845	17	4.2	13.6

[a] Quantification ions.
[b] Confirmation ions.

should be also mentioned that any significant variation in the MS signals was not observed (<8% RSD, $n = 6$), in particular of the three compounds that coeluted with the EOF (acetamiprid, thiacloprid and thiamethoxam), when comparing the results obtained after injecting individual and mixtures of standard solutions.

3.1.3. Analytical performance of the CE–ESI-MS method

To assess the selectivity (specificity) of the method, each neonicotinoid was identified by migration time, protonated molecular ion, and two product ions. The detection (LOD) and quantification (LOQ) limits were experimentally determined by injecting a number of solvent solutions ($n = 6$), which were the same as the ones that were used to dissolve the neonicotinoid standards, and measuring the magnitude of the background analytical signal at the migration time of each analyte. The LODs and LOQs were estimated as three and ten times the signal to noise ratio (S/N), respectively, and these limits were calculated for MS (EIE) and MS/MS (MRM) experiments. As can be seen in Table 3, low LODs and LOQs could be obtained by using MS (EIE) for all analytes ranging from 1.0 to 2.3 μg/L, and from 3.5 to 7.2 μg/L, respectively, whereas these values were higher with MS/MS (~2 or 3 times depending on the analyte). This finding could be explained by the low noise obtained in the EIE electropherograms, probably due to the injection of standard solutions. Nevertheless, the expectation is that the S/N ratio would be higher in MS when analyzing complex matrices, and that in those cases the sensitivity of the MRM experiments would be better than with MS. As it was seen above, it is not possible to make a proper comparison of the sensitivity previously obtained with previous LOD and LOQ values, due to the lack of CE–MS methodologies for analyzing neonicotenoids. Moreover, in most of the published CE (MECK) approaches, concentration procedures (SPE and/or DLLME, see Table 2) were employed which significantly increased sensitivity. Therefore, as may be observed by comparing the values summarized in Tables 2 and 3, the limits obtained in the present work were better than those obtained in the previous CE studies, with, however, one exception [24]; yet in this case only one neonicotinoid was the object of study. In relation to LC methods, it could be said that the sensitivity of the proposed method was comparable to most of the LC–MS or LC–MS/MS approaches, as the LODs or LOQs obtained were in the range of the μg/L or μg/kg [1,11–18], although it should be remarked that concentration procedures (SPE, QuEChERS or DLLME) were used in most of those publications. Thus, the excellent sensitivity of the proposed method has been demonstrated. In addition, this parameter could also be improved in the analysis of specific matrices by using concentration and clean-up procedures, as was done in MECK and LC methodologies.

Calibration curves ($n = 3$), which were prepared in a range between the LOQ and 1000 μg/L, were constructed by plotting the signal against the analyte concentration. In all cases, the absence of bias was verified by means of a t-test and by studying the distribution of residuals. The coefficient of determination values (R^2) were >0.99 (Table 3) for all the linear ranges studied. Finally, the instrumental repeatability of the proposed CE–ESI-MS method was evaluated by making repeated injections of a standard solution which contained the 7 neonicotinoids at concentrations of 100 μg/L and 1000 μg/L. The %RSD ($n = 9$) values for migration times ranged from 1.4 to 3.0% in all cases, and the repeatability was also assessed by using the same standard solutions. Three replicates of these solutions were injected in triplicate the same day. In this case, the %RSD ($n = 9$) values for the migration times were between 1.7 and 3.5%.

Finally, it has been demonstrated after examining the analytical performance and the analysis time (see Section 3.1.1) the potential of the proposed CE–MS as an alternative or even a complement to the existing CE (MECK) and LC methods. It presents some advantages compared to the MECK and LC methods as higher sensitivity and the unequivocal identification of these compounds at trace levels, especially in complex matrices, that are offered by MS detection, or the low consumption (solvents, reagents and samples) and cost of the instrumentation in relation to the LC–MS proposals. On the other hand, it should be also mentioned that better separations in similar analysis times and for the same number of neonicotinoids were obtained in some LC proposals, and that MS detectors are more expensive than UV and indirect LIF, which were commonly used in MECK strategies. Finally, it should be also taken into account the importance of having orthogonal separation techniques (CZE, MECK, LC), that, according to different separation mechanism can provide different profiles, which would be useful for confirmation purposes when analyzing real samples.

3.2. Application of the CE–ESI-MS method for analyzing beeswax samples

The proposed CE–ESI-MS method was applied to monitor, from a qualitative approach, the potential presence of neonicotinoid residues (clothianidin, imidacloprid and thiamethoxam) in spiked beeswax samples, as described in Section 2.3, after cleaning with oxalic acid, efficacy of which to remove those compounds has never been assessed before. These compounds were chosen because their use has been recently restricted for plant protection, a point made previously [27]. Moreover, it should be mentioned that, to our knowledge, this is the first time that a CE method has been applied in order to analyze neonicotinoids in beeswax samples. In addition, spiked samples were used to check the efficacy of the cleaning treatments, as it is commonly done when it is not possible to obtain real samples containing the target compounds. The efficiency of the extraction procedure was previously assessed by comparing the signal (areas) of each of the three neonicotinoids in beeswax

Table 4
Evaluation of the efficiency of the sample treatment and the matrix effect. Data obtained as described in Section 3.2 ($n = 6$). All beeswax samples were spiked with individual neonicotinoid standards at 100 μg/L (~50 μg/kg). RSD: relative standard deviation.

Compounds	Evaluation of the sample treatment	Evaluation of the matrix effect
	Mean (%) ± RSD (%)	Mean (%) ± RSD (%)
Clothianidin	98 ± 8	71 ± 7
Imidacloprid	93 ± 6	63 ± 8
Thiamethoxam	88 ± 7	60 ± 8

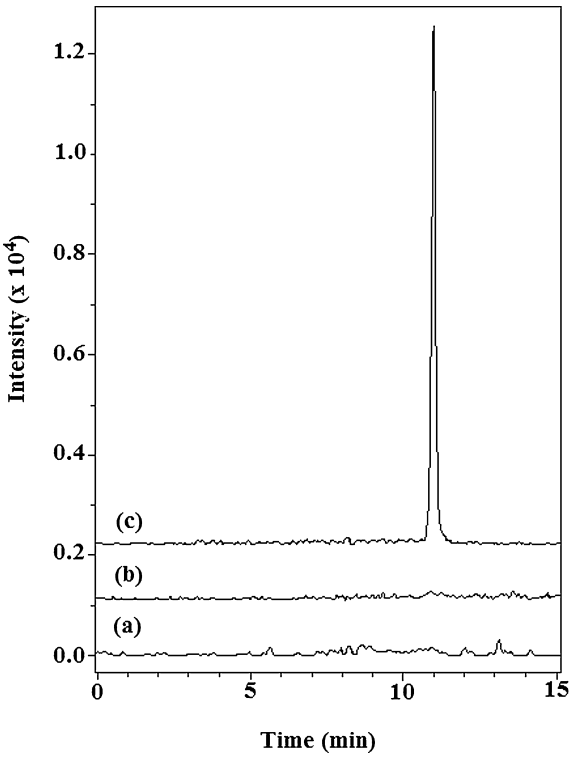


Fig. 2. CE–ESI-MS electropherograms obtained in EIE mode from (a) a blank beeswax sample, (b) a blank beeswax sample containing clothianidin at 100 μg/L (~50 μg/kg, according to the unit conversion, see Section 2.2) and cleaned with oxalic acid, and (c) a blank beeswax sample containing clothianidin at 100 μg/L (~50 μg/kg). The sample treatment and CE–ESI-MS conditions are described in Sections 2.3, 2.4 and 3.1.2, respectively.

samples, which were spiked at 100 μg/L (~50 μg/kg) before and after applying the proposed extraction procedure. The results (see Table 4) showed that the proposed procedure was good enough as the recovery percentages ranged from 88% to 98% with relative standard deviation (%RSD, $n = 6$) values lower than 8. Moreover, those values were in good agreement with the data obtained in a previous research [1] (see Section 2.3.2.2).

Once the extraction efficiency was tested, all the beeswax samples, which were processed with the sample treatment described in Section 2.3.2, were analyzed in triplicate in this study. The results demonstrated that the proposed sample treatment with oxalic acid was effective in terms of removing neonicotinoid residues, since no peak was detected at the migration time of each analyte in the EIE or MRM electropherograms. An example of this finding is provided by Fig. 2, in which it can be clearly observed that clothianidin has been totally removed from a beeswax sample spiked at 100 μg/L, which corresponded to 50 μg/kg according to the proposed sample treatment. Moreover, it could be also concluded, after an examination of Fig. 2 and the electropherograms for the other two compounds (data not shown), that the sensitivity achieved with our proposal made it possible to determine neonicotinoids at trace levels (μg/kg), as the signal obtained (peak height) for 100 μg/L (50 μg/kg) was intense enough ($>1 \times 10^4$). Furthermore, it was observed after comparing the peak of clothianidin in standard (Fig. 1) and matrix matched (Fig. 2c) solutions that the signal (area) was lower in the matrix matched than in the standard solution. To check how the matrix influenced ESI ionization, the peak areas of the three neonicotinoids in individual standard solutions (100 μg/L) were compared with those obtained in beeswax samples which were spiked at the same concentration after applying the proposed extraction procedure. The responses of all compounds were lower than 72% in all the cases (%RSD < 9), as can be observed in Table 4. Hence, it was concluded that the matrix (beeswax) affected electrospray ionization of the selected insecticides, bringing about ion suppression in this case. This finding agrees with our previous statement [1]. It could be also commented that magnitude of the matrix effect was comparable in both studies.

Finally, it should be remarked that the proposed CE–ESI-MS method was not specifically developed for determining neonicotinoids in beeswax. It was just qualitatively applied to check the efficacy of a beeswax cleaning treatment. Thus, if it is required a quantitative analysis of the samples, it would be necessary to perform further optimization and validation studies by using matrix matched standards, as matrix (beeswax) has a strong influence on the ESI-MS ionization.

4. Conclusions

This is the first time that a CE (CZE)-MS method has been developed to simultaneously identify and quantify seven neonicotinoid insecticides. Adequate analysis times with good precision (migration times) and a wide linearity range were obtained. In addition, the excellent sensitivity achieved with this method permitted determination of these compounds at trace levels. The applicability of the proposed method was verified by analyzing beeswax samples spiked with clothianidin, imidacloprid and thiamethoxam, in order to check the efficacy of an oxalic acid cleaning treatment. The proposed CE–ESI-MS was shown to be useful for monitoring the presence of low levels of neonicotinoids in a complex matrix such as beeswax, and at the same time, the suitability of the cleaning procedure to remove neonicotinoids from beeswax was proven, as no residues were detected in any treated sample.

To sum up, it can be concluded that the proposed method can be considered a promising alternative or a complement to the previously established LC and CE (MEKC) strategies. This can easily be seen in terms of the low consumption (solvents, reagents and samples) and cost of the instrumentation compared with the LC methods, or the sensitivity obtained without concentration treatment and the potential to perform an unequivocal identification of the analytes, even when they migrate or elute at the same time, in comparison with the previous LC and CE (MEKC) approaches in which MS was not used.

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