JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Liquid Chromatography with Diode Array Detection and Tandem Mass Spectrometry for the Determination of Neonicotinoid Insecticides in Honey Samples Using Dispersive Liquid−Liquid Microextraction

Natalia Campillo, Pilar Viñas, Gema Férez-Melgarejo, and Manuel Hernández-Córdoba*

Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, E-30100 Murcia, Spain

ABSTRACT: The combination of solid-phase extraction with dispersive liquid−liquid microextraction (SPE-DLLME) is proposed for the determination of five neonicotinoid insecticides in honey. After a cleanup stage, the analytes were eluted using acetonitrile. DLLME was performed by injecting rapidly the acetonitrile extract into 10 mL of water containing a 10% (w/v) NaCl and 100 μL of $CHCl_3$. The settled organic phase obtained after centrifugation was evaporated, reconstituted in acetonitrile (ACN), and submitted to liquid chromatography (LC) with photodiode array detection (DAD) and atmospheric pressure chemical ionization−ion trap−tandem mass spectrometry (APCI-IT-MS/MS). The matrix effect was evaluated, and quantification was carried out using external aqueous calibrations when using DAD, the matrix-matched calibration method was applied for MS/MS. Detection limits in the 0.2−1.0 and 0.02−0.13 ng/g ranges were obtained when using DAD and MS/MS, respectively. The SPE-DLLME-LC-APCI-IT-MS/MS method was applied for the analysis of different honey samples, and none was found to contain residues.

KEYWORDS: *neonicotinoid insecticides, honey, solid-phase extraction cleanup, dispersive liquid−liquid microextraction (DLLME), LC-MS/MS*

INTRODUCTION

Neonicotinoids, also known as chloronicotinyls, are a group of insecticides with a wide range of chemical and biological properties, for which reason they are used throughout the world for crop protection and in veterinary medicine.[1] These chemicals act as agonists at the insect nicotinic acetylcholine receptor, leading to insect paralysis and death. The broad-spectrum insecticidal activity, low application rates, mode of action, and low toxicity for vertebrates mean that they account for about 15−20% the world insecticide market, imidacloprid (IMI) being the most sold insecticide worldwide.[2] Nevertheless, the results obtained in recent toxicological studies have suggested thiamethoxam (TMX) and IMI may have negative effects on human health.[3,4] Furthermore, the use of several neonicotinoid insecticides has been restricted in some countries because of a possible relationship between them and honeybee colony collapse disorder, a hypothesis lent weight by the demonstrated toxicity of neonicotoid insecticides to honeybees.[5]

The preservation of honeybee colonies is of great importance because they play an essential role in agriculture, fertilizing plants by transporting pollen grains to female floral plants. Indeed, about one-third of the European agricultural production is dependent on pollination by insects, mainly honeybees.[6] In recent years, the consumption of honey has increased considerably because of its perceived healthy properties. Therefore, an ability to monitor pesticides in honey is important not only because of possible serious risks for human health but also because their levels may reflect the risk to which agriculture in general is exposed.

Pesticide residue levels in honey have been fixed by European Union (EU) regulations,[7] maximum residues limits (MRLs) for honey (royal jelly and pollen) being 0.01 μg/g for the sum of TMX and clothianidin (CLO) (expressed as TMX), 0.2 μg/g for thiacloprid (THIA), and 0.05 μg/g for acetamiprid (ACE) and IMI. Consequently, the development of simple, rapid, robust, and economic analytical methods is a necessity. Neonicotinoid insecticides have mainly been determined by liquid chromatography (LC) coupled to different detection systems, the most common being diode array detection (DAD)[8−18] and mass spectrometry (MS).[19−33] The low volatility, thermolability, and high polarity of these compounds mean that gas chromatography (GC) has rarely been used.[34−39] On the other hand, because of the water solubility and asymmetric structure of neonicotinoids, enzyme-linked immunosorbent assay (ELISA) has proved to be a suitable method for their analysis, being applied for the determination of IMI,[2,40−43] ACE,[44] and TMX.[45] Although most LC methods have been applied to the analysis of vegetables and/or fruits,[8,9,12,14−20,25,26,28,32] few methods deal with honey samples.[23,27,29,30,33] The complexity of honey matrices and the need to achieve limits of quantification in the low nanogram per gram range make it necessary to include a sample preparation step in the analytical procedure. In this respect, solid-phase extraction (SPE)[23,27,29,33] and the QuEChERS

Received: February 13, 2013
Revised: April 20, 2013
Accepted: May 1, 2013
Published: May 1, 2013

Table 1. Summary of the Experimental Parameters

compound	molecular formula	retention time (min)	monitored wavelength (nm)	precursor ion (m/z)	product ions (m/z)
thiamethoxam (TMX)	$C_8H_{10}ClN_5O_3S$	6.48	253	292	129
				292	175
clothianidin (CLO)	$C_6H_8ClN_5O_2S$	7.38	270	250	169
				250	132
imidacloprid (IMI)	$C_9H_{10}ClN_5O_2$	8.39	270	256	209
				256	175
acetamiprid (ACE)	$C_{10}H_{11}ClN_4$	9.29	245	223	126
				223	187
thiacloprid (THIA)	$C_{10}H_9ClN_4S$	10.03	245	253	126
				253	226

methodology[30] have been applied. Dispersive liquid−liquid microextraction (DLLME) is based on a ternary component solvent system formed by an aqueous solution, a water-immiscible extraction solvent, and a disperser solvent miscible both in the aqueous phase and in the extractant solvent. A cloudy solution, consisting of very fine droplets of the extractant, is formed, leading to a rapid and efficient preconcentration technique.[46,47] DLLME has been successfully applied for the extraction and determination of mainly organic compounds in aqueous and food samples.[48] High enrichment factors are obtained with DLLME, but the complexity of honey matrices prevents low detection limits being attained without including a cleaning step in the procedure. For this reason, the aim of this work was to combine SPE and DLLME as a sample preparation method for the analysis of several neonicotinoid insecticides in honey samples by LC with DAD and tandem mass spectrometry (MS/MS). Two of the five neonicotinoids analyzed belong to the second-generation compounds, IMI and ACE, and the other three are included in the subclass of thianicotinyl compounds, TMX, TCL, and CLO.[1] As far as we know, combined SPE-DLLME has not previously been used for the analysis of honey samples.

MATERIALS AND METHODS

Instrumentation. The LC system consisted of an Agilent 1200 (Waldbronn, Germany) binary pump (G1312B) operating at a flow rate of 0.5 mL/min. The solvents were degassed using an online membrane system (Agilent 1100, G1379A). The column was maintained in a thermostated compartment (Agilent 1200, G1316B). Separation was performed on a Spherisorb ODS2 column (Teknokroma) (150 mm × 4 mm, 5 μm). The injection (20 μL) was performed using an autosampler (Agilent 1200, G1367C). Autosampler vials of 2 mL capacity provided with 250 μL microinserts with polymeric feet were used. The diode array and multiple wavelength detector was an Agilent 1100 (G1315C) operating at three wavelengths of 245, 253, and 270 nm. The mass spectrometer was an Agilent Ion Trap (VL-01036) equipped with a HP atmospheric pressure chemical ionization (APCI) source operating in positive ionization mode at 400 °C. The nebulizer (60 psi) and the drying (5 L/min) gases were heated at 400 and 350 °C, respectively. The internal source voltage was held at 86.1 V. The maximum accumulation time for the ion trap was set at 300 ms, the target count at 30000, and the product ions spectrum was collected between m/z 90 and 320. The mass spectrometer was operated in multiple reaction monitoring mode (MRM) with monitoring of two precursor/products ion transitions for each analyte (Table 1). Both transitions were used for quantification and confirmation purposes. Collision energies between 20 and 200% were applied to ensure the maximum fragmentation yield.

The SPE was performed with Discovery DSC-18Lt cartridges (500 mg) from Supelco (Bellefonte, PA, USA) by means of a Visipreb SPE vacuum manifold system (Supelco). An EBA 20 (Hettich, Tuttlingen, Germany) centrifuge was used at the maximum speed supported by the conical glass tubes, 4000 rpm.

Reagents. Acetamiprid ((*E*)-N^1-[(6-chloro 3-pyridil)methyl]-N^2-cyano-N^1-methylacetamidine) (99.9%; ACE), thiamethoxan (3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-*N*-nitro-1,3,5-oxadiazinan-4-imine) (99.7%; TMX), thiacloprid ((*Z*)-3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylidenecyanamide) (99.9%; THIA), and clothianidin ((*E*)-1-(2-xhloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine) (99.9%; CLO) were purchased from Fluka (Buchs, Switzerland). Imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine) (certified analytical standard, 99.0%; IMI) was obtained from Dr. Ehrenstorfer (Augsburg, Germany). Individual stock solutions of the compounds (1000 μg/mL) were prepared using LC grade methanol as solvent and stored in darkness at −20 °C. Working standard solutions were freshly prepared and stored at 4 °C. Sodium chloride (99.5%) and potassium dihydrogen phosphate (99%) were purchased from Merck (Rahyway, NJ, USA) and Fluka, respectively. Analytical-reagent grade methanol, acetonitrile (ACN), acetone, dichloromethane, chloroform, carbon tetrachloride, and 1,1,2,2-tetrachloroethane were obtained from Sigma (St. Louis, MO, USA). Water used was previously purified in a Milli-Q system (Millipore, Bedford, MA, USA).

Samples and Analytical Procedure. A total of 13 honey samples were obtained from different suppliers. These samples had been marketed as eucalyptus (3 samples), heather (3), orange blossom (3), rosemary (2), and thousand flowers (2), but no verification of the floral origin was made.

Solid-phase cartridges were equilibrated with 3 mL of acetonitrile and then with 2 mL of 0.1% (v/v) formic acid, following the instructions of the manufacturer. Two grams of honey was dissolved in 10 mL of water and loaded into the cartridge at a 5 mL/min flow rate. Then the cartridges were washed with 10 mL of Milli-Q water and dried under vacuum in a manifold system for 2 min. The analytes were then eluted with 1.5 mL of acetonitrile, which was used as disperser solvent in the subsequent DLLME step. For this, 10 mL of aqueous solution containing 10% (w/v) sodium chloride was placed in a 15 mL screw-cap glass centrifuge tube with conical bottom, and 100 μL of $CHCl_3$ (extraction solvent) was added. Next, the 1.5 mL of acetonitrile collected from the SPE was rapidly injected into the aqueous mixture using a syringe. A cloudy solution, resulting from the dispersion of the fine $CHCl_3$ droplets in the aqueous solution, was formed. After a few seconds of extraction, the mixture was centrifuged for 2 min at 4000 rpm, and the dispersed organic droplets were sedimented at the bottom of the conical tube. The lower phase was collected by means of a microsyringe and evaporated to dryness by using a mild nitrogen

Table 2. Slopes[a] of Standard Additions Calibration Graphs (Milliliters per Nanogram)

compd	aqueous	eucalyptus honey	heather honey	blossom honey	thousand flowers honey
			LC-DAD		
TMX	2.72 ± 0.18	2.64 ± 0.21	2.79 ± 0.27	2.81 ± 0.21	2.89 ± 0.32
CLO	3.27 ± 0.20	3.21 ± 0.44	3.14 ± 0.30	3.08 ± 0.35	3.31 ± 0.39
IMI	9.32 ± 0.62	8.99 ± 0.85	9.37 ± 0.73	9.13 ± 0.94	8.85 ± 0.78
ACE	23.9 ± 1.6	23.6 ± 1.9	24.3 ± 2.1	22.8 ± 1.9	24.1 ± 1.8
THIA	24.3 ± 1.8	23.6 ± 1.7	24.0 ± 1.9	22.9 ± 2.1	23.5 ± 1.9
			LC-MS/MS		
TMX	4739 ± 349	3975 ± 210	3915 ± 139	3862 ± 195	3840 ± 186
CLO	5679 ± 635	4297 ± 264	4278 ± 198	4380 ± 241	4199 ± 216
IMI	32531 ± 6120	23241 ± 3026	21972 ± 1661	24698 ± 2370	22667 ± 2740
ACE	19732 ± 3368	16349 ± 1984	16958 ± 1205	16277 ± 1811	15234 ± 2154
THIA	50460 ± 6202	43029 ± 3824	41290 ± 3340	44016 ± 4209	44422 ± 3983

[a]Mean value ± standard deviation (n = 4).

Table 3. Analytical Characteristics of the Methods

compd	linearity range (ng/g)	intercept[a]	detection limit (ng/g)	quantitation limit (ng/g)	RSD[b] (%)	
			LC-DAD			
TMX	2.5−7500	2.21 ± 0.18	1.0	3.3	6.4 (25)	4.1 (100)
CLO	2.5−7500	3.06 ± 0.17	0.8	2.7	6.8 (25)	5.3 (100)
IMI	1−5000	2.90 ± 0.25	0.4	1.3	5.7 (10)	4.6 (50)
ACE	1−5000	3.98 ± 0.31	0.2	0.7	4.5 (10)	3.8 (50)
THIA	1−5000	4.01 ± 0.33	0.2	0.7	3.8 (10)	3.2 (50)
			LC-MS/MS			
TMX	0.5−1000	943 ± 34	0.13	0.43	7.1 (5)	5.1 (25)
CLO	0.5−1000	791 ± 35	0.12	0.40	7.0 (5)	4.8 (25)
IMI	0.1−500	2268 ± 44	0.03	0.10	4.9 (1)	3.7 (10)
ACE	0.1−500	1567 ± 45	0.04	0.13	5.3 (1)	3.9 (10)
THIA	0.1−500	3221 ± 57	0.02	0.07	3.2 (1)	2.8 (10)

[a]Mean value ± standard deviation (n = 4). [b]n = 10. Values in parentheses correspond to concentrations in ng/g.

stream; the residue was reconstituted in the minimum volume of acetonitrile necessary to inject by means of the autosampler (50 μL), and a 20 μL aliquot was injected in the LC system.

Recovery experiments were carried out using honey samples of about 25 g spiked with a standard mixture of the insecticides at concentration levels roughly corresponding to 5- and 50-fold the quantification limit of each compound. The samples were left to equilibrate at room temperature for at least 30 min before proceeding with the optimized procedure, which was applied to aliquots of 2 g sample mass. The fortification procedure was applied to three different honey samples at two concentration levels, and three replicates were analyzed in each case.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Conditions. The mobile phase optimization was performed at a flow rate of 0.5 mL/min using mixtures of MeOH and ACN with water and 0.1% (v/v) formic acid. The highest resolution was attained for the three less retained compounds (TMX, CLO, and IMI) when using 40:60 ACN/formic acid. Nevertheless, isocratic elution was not possible because peaks were unresolved and/or compounds were strongly retained. Consequently, gradient elution was assayed, with the optimized gradient program being 9 min linear gradient elution from 20:80 to 45:55 ACN/formic acid and then a new linear gradient from 45 to 55% ACN in 2 min. The injection volume was also studied between 5 and 20 μL, and 20 μL was selected because it provided the highest sensitivity and no overlapping of the chromatographic peaks with the elution program finally selected. The elution order, the absorption wavelengths monitored for each insecticide when using DAD, and MRM precursor/product ion transitions appear in Table 1. Retention factors (k) between 1.3 and 2.51, separation factors (α) in the 1.12−1.25 range, and resolution (R_s) between 2.9 and 3.6 were obtained from LC-DAD chromatograms.

Table 4. Recoveries[a] of the Insecticides in Spiked Honey Samples Using the LC-MS/MS Method

compd	spike level (ng/g)	eucalyptus honey	heather honey	rosemary honey
TMX	2.0	94 ± 7	96 ± 4	91 ± 4
	20	103 ± 5	98 ± 4	97 ± 6
CLO	2.0	91 ± 6	91 ± 8	90 ± 6
	20	98 ± 6	95 ± 5	96 ± 6
IMI	0.5	97 ± 5	90 ± 5	97 ± 5
	5.0	98 ± 5	94 ± 8	102 ± 6
ACE	0.5	92 ± 8	99 ± 10	94 ± 7
	5.0	101 ± 1	102 ± 4	104 ± 3
THIA	0.5	94 ± 4	90 ± 5	94 ± 5
	5.0	103 ± 3	99 ± 6	99 ± 5

[a]Mean value ± standard deviation (n = 3).

SPE and DLLME Conditions. SPE was used as the isolation and purification method for the honey sample. DSC-18Lt was chosen as the stationary phase. The cartridges were first conditioned as indicated under Materials and Methods,

dx.doi.org/10.1021/jf400669b | J. Agric. Food Chem. 2013, 61, 4799−4805

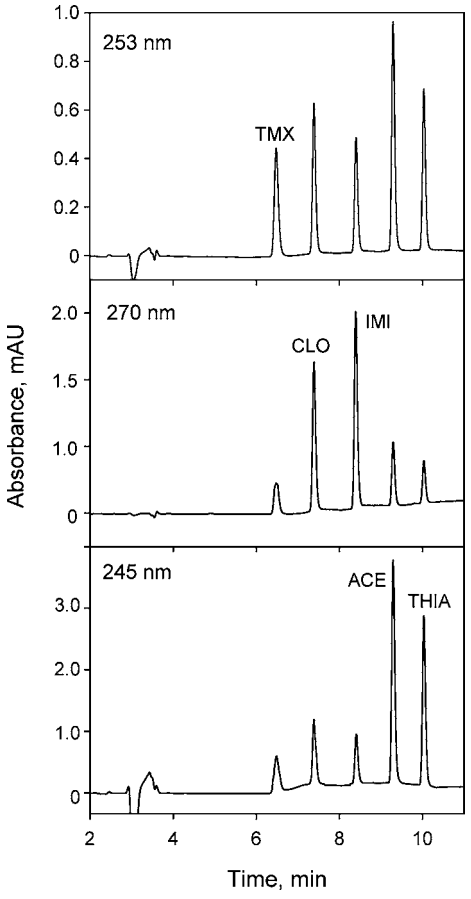

Figure 1. Elution profiles obtained from a spiked honey sample using the SPE-DLLME-LC-DAD procedure monitored at 253, 270, and 245 nm. Concentrations for the spiked honey: 6 ng/g for TMX and CLO and 2.5 ng/g for IMI, ACE, and THIA.

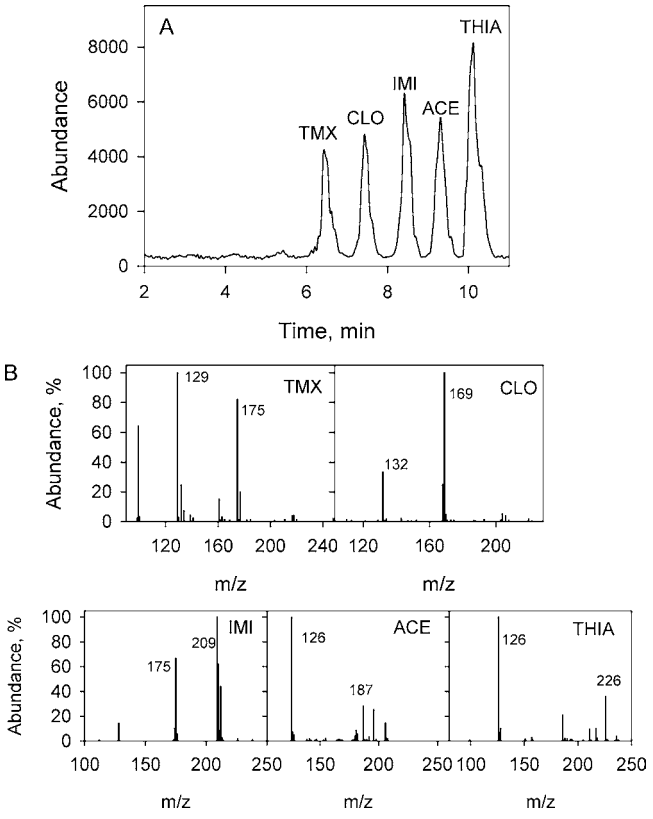

Figure 2. (A) Total ion chromatogram obtained by using SPE-DLLME combined with LC-APCI-IT-MS/MS for a spiked honey sample at a concentration level of about 5-fold the corresponding quantification limits. (B) Mass spectra of the compounds under the selected conditions.

and 2 g of honey previously dissolved in 10 mL of water was loaded. ACN was used for elution of the analytes, different volumes (0.5, 0.75, 1.5, and 3 mL) being assayed at flow- rates in the 1−5 mL/min range. Volumes of 0.5 and 0.75 mL were insufficient to elute the retained compounds. Total recoveries were attained with 1.5 mL of ACN. No significant differences were obtained for elution flow rates in the studied range, and so 5 mL/min was adopted.

Preliminary experiments were carried out to optimize the DLLME procedure using 1.5 mL of ACN containing the neonicotinoid insecticides at a 250 ng/mL concentration level. When extraction solvents of higher density than water were assayed, they accumulated rapidly at the bottom of the conical bottom tube and were easy to collect. CCl_4, $CHCl_3$, CH_2Cl_2, and $C_2H_2Cl_4$ were considered as extractants. When 100 μL of extractant and 1.5 mL of ACN as disperser solvent were used, well-defined settled volumes were recovered with all of the organic solvents, except with dichloromethane, which provided a drop volume lower than 20 μL. Best recoveries were obtained with $CHCl_3$. To study the effect of the extraction solvent volume, 1.5 mL of ACN containing different volumes of $CHCl_3$ (50, 100, 150, and 200 μL) was submitted to the DLLME procedure. Peak area increased up to 100 μL and then remained constant or a slight decrease was attained, probably due to a dilution effect. Consequently, 100 μL of the organic extractant was used.

The influence of ionic strength was evaluated at 0−30% (w/v) NaCl concentrations in the aqueous phase (10 mL). Concentration levels higher than 20% (w/v) could not be used because the organic phase did not settle. Whereas a slight increase of sensitivity was observed for THIA, CLO, and IMI when NaCl concentration increased up to 20% (w/v), the contrary effect was attained for ACE and THIA. Therefore, 10% (w/v) was selected as a compromise value.

To evaluate the influence of the pH in the aqueous phase, aliquots of ultrapure water were adjusted to pH values ranging between 3 and 8 with a 0.01 M phosphate buffer solution, in the presence of the optimized NaCl concentration. The volume of the sedimented organic solvent did not vary, and no significant differences in the analytical signal were appreciated for the target analytes. Consequently, samples were analyzed without pH adjustment in the aqueous phase.

Mixtures of extractant, disperser, and the aqueous phase were manually shaken for different times between a few seconds and 5 min before the mixture was submitted to centrigufation for 5 min at 4000 rpm. Similar peak areas were obtained in all cases, indicating that the DLLME procedure was practically time-independent, this being one of the most important advantages of the technique. Consequently, only a few seconds was needed to extract the analytes. Neither the volume of the sedimented phase nor the sensitivity for the analytes changed when samples were centrifuged for different times in the interval 1−10 min at 4000 rpm or at different centrifugation speed ranging from 2000 to 4000 rpm for 2 min. The centrifugation conditions finally adopted were 4000 rpm and 2 min. The volume of

sedimented phase was 80 μL approximately. The results prove that the sample cleanup by SPE allows an efficient preconcentration of the analytes using DLLME, an analytical methodology that can be characterized as rapid, easy to operate, and environmentally friendly.

Analytical Characteristics of the Methods. The standard additions method was used to investigate the possibility of matrix interference. Table 2 shows the slopes for different honey samples using the optimized experimental conditions, as well as those obtained for aqueous calibration, both using DAD and MS/MS. The peak area was used as analytical parameter. The standard additions method was applied by analyzing two aliquots of the unfortified sample and fortified at three different concentration levels. A statistical study was carried out to compare the slope values using the one-sample *t* test, which operated by comparing different slopes obtained from honeys with the aqueous slope for each analyte. When using DAD, slopes of the standard additions calibration graphs, with fortification levels in the 5−500 ng/g depending on the compound, were similar to those of aqueous standards (*p* values in the range of 0.22−0.83) in all cases, confirming that the matrix did not interfere and quantification can be carried out against aqueous standards. Nevertheless, when using MS/MS detection, coeluting matrix compounds suppressed the ionization of the target analytes, with the exception of ACE and THIA in some honey matrices, as can be observed in Table 2. Matrix-matched calibration was used for the quantification of the samples using a blank honey, because the comparison of the slopes of the standard additions calibration graphs, with fortification levels in the 0.5−100 ng/g range depending on the compound, provided no significant differences between them (*p* values between 0.49 and 0.75). The linear concentration ranges were between 1 and 7500 ng/g when using DAD and between 0.1 and 1000 ng/g when using MS/MS. Correlation coefficients were better than 0.996 in all cases. The detection and quantification limits were calculated on the basis of 3 and 10 times the standard deviation of the intercept of the calibration graphs, external aqueous calibration DAD, and matrix-matched calibration using a heather honey (Table 3). The repeatability was calculated using the relative standard deviation of the peak areas for 10 successive fortifications of a honey sample at 2 concentration levels (Table 3).

The optimized SPE-DLLME-LC-MS/MS procedure provided an important enhancement of sensitivity, dependent on the compound, and a lower sample consumption with respect to previous works in which dispersive solid-phase extraction[30] (increase of sensitivity in the 5−30 range, depending on the compound) or direct SPE without a DLLME stage was used (sensitivity enhacement as high as about 3000-fold in the case of IMI).[23,29]

Analysis of the Samples and Recovery Studies. The SPE-DLLME-LC-MS/MS method was applied to the analysis of 13 different honey samples, and no insecticides were detected above their detection limits. Consequently, the honeys analyzed met the EU regulations related to neonicotinoid insecticides.[7] The reliability of both optimized procedures was checked by recovery studies for three different honey samples spiked at two concentration levels. The recoveries of the insecticides from spiked honey samples varied between 90 and 104%, with an average recovery ± SD (*n* = 90) of 96 ± 4, as can be seen in Table 4, for the LC-MS/MS method. No significant differences were observed in the RSD values obtained for the recoveries of the different insecticides in each honey sample analyzed, the values ranging between 1.5 and 10% for eucalyptus honey and heather honey, respectively. When recovery studies were carried out for the same honey samples fortified at concentrations of 10 and 100 ng/g for TMX and CLO and of 5 and 50 ng/g for IMI, ACE, and THIA, using the SPE-DLLME-LC-DAD method, an overall average recovery value ± SD (*n* = 90) of 94 ± 8 was obtained.

Figure 1 depicts the chromatograms obtained using SPE-DLLME-LC-DAD for a spiked honey sample at concentration levels in the 5.0−15.0 ng/g range, depending on the compound. When using DAD, peaks were identified continuously measuring the spectrum while the solute passed through the flow cell, thus confirming the identity and the purity of the peaks. Good agreement was found between the spectra of the different peaks obtained for the standards and the spiked samples. Figure 2 shows the total ion chromatogram obtained for the same spiked honey sample, using the optimized SPE-DLLME-LC-MS/MS, at concentration levels roughly corresponding to 5-fold the quantification limit of each compound. The identity of the analytes was confirmed by comparing the retention time and the signal intensity ratios of the two MS/MS transitions with those obtained using aqueous standards. The chromatograms obtained for unspiked aliquots of different honey samples showed the absence of interfering peaks at the analyte elution times, using both DAD and MS/MS.

AUTHOR INFORMATION

Corresponding Author

*Phone: +34 86 88 87 406. Fax: +34 86 88 87 682. E-mail: hcordoba@um.es.

Funding

We acknowledge Comunidad Autónoma de la Región de Murcia (CARM, Fundación Séneca, Project 15217/PI/10) and the Spanish MEC (CTQ2012-34722) for financial support. G. Férez acknowledges a fellowship financed by CARM.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACE, acetamiprid; APCI, atmospheric pressure chemical ionization; CLO, clothianidin; DAD, diode array detection; DLLME, dispersive liquid−liquid microextraction; GC, gas chromatography; IMI, imidacloprid; IT, ion trap; LC, liquid chromatography; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; SPE, solid-phase extraction; THIA, thiacloprid; TMX, thiamethoxam

REFERENCES

(1) Maienfisch, P.; Huerlimann, H.; Rindlisbacher, A.; Gsell, L.; Dettwiler, H.; Haettenschwiler, J.; Sieger, E.; Walti, M. The discovery of thiamethoxam: a second-generation neonicotinoid. *Pest Manag. Sci.* **2001**, *57*, 165−176.

(2) Xu, T.; Jacobsen, C. M.; Cho, I. K.; Hara, A. H.; Li, Q. X. Application of an enzyme-linked immunosorbent assay for the analysis of imidacloprid in wiliwili tree, *erythrina sandwicensis* O. Deg, for control of the wasp *Quadrastichus erythrinae*. *J. Agric. Food Chem.* **2006**, *54*, 8444−8449.

(3) Green, T.; Toghill, A.; Lee, R.; Waechter, F.; Weber, E.; Peffer, R.; Noakes, J.; Robinson, M. Thiametoxam induced mouse liver tumors and their relevance to humans. Part 2: Species differences in responses. *Toxicol. Sci.* **2005**, *86*, 48−55.

(4) Duzguner, V.; Erdogan, S. Accute oxidant and inflammatory effects of imidacloprid in the mammalian central nervous system and liver in rats. *Pestic. Biochem. Physiol.* **2010**, *97*, 13−18.
(5) Nauen, R.; Ebbinghaus-Kintscher, U.; Schmuck, R. Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: apidae). *Pest Manag. Sci.* **2001**, *57*, 577−586.
(6) Willians, I. Insect pollination and crop production: a European perspective. In *Pollination Bees − The Conservation Link between Agriculture and Nature*; Kevan, P., Imperatriz Fonseca, V. L., Eds.; Ministry of Environment: Brazil, 2002; pp 56−65.
(7) EU Pesticides database, Pesticide EU-MRLs Regulation (EC); http://www.ec.europa.eu/sanco_pesticides (accessed Jan 2013).
(8) Obana, H.; Okihashi, M.; Akutsu, K.; Kitagawa, Y.; Hori, S. Determination of acetamiprid, imidacloprid, and nitenpyram residues in vegetables and fruits by high-performance liquid chromatography with diode-array detection. *J. Agric. Food Chem.* **2002**, *50*, 4464−4467.
(9) Singh, S. B.; Foster, G. D.; Khan, S. U. Microwave-assisted extraction for the simultaneous determination of thiametoxam, imidacloprid, and carbendazim residues in fresh and cooked vegetable samples. *J. Agric. Food Chem.* **2004**, *52*, 105−109.
(10) Rossi, S.; Sabatini, A. G.; Cenciarini, R.; Ghini, S.; Girotti, S. Use of high-performance liquid chromatography-UV and gas chromatography-mass spectrometry for determination of the imidacloprid content of honeybees, pollen, paper filters, grass, and flowers. *Chromatographia* **2005**, *61*, 189−195.
(11) Zhou, Q.; Ding, Y.; Xiao, J. Sensitive determination of thiamethoxam, imidacloprid and acetamiprid in environmental water samples with solid-phase extraction packed with multiwalled carbon nanotubes prior to high-performance liquid chromatography. *Anal. Bioanal. Chem.* **2006**, *385*, 1520−1525.
(12) Watanabe, E.; Baba, K.; Eun, H. Simultaneous determination of neonicotinoid insecticides in agricultural samples by solid-phase extraction cleanup and liquid chromatography equipped with diode-array detection. *J. Agric. Food Chem.* **2007**, *55*, 3798−3804.
(13) Seccia, S.; Fidente, P.; Montesano, D.; Morrica, P. Determination of neonicotinoid insecticides residues in bovine milk samples by solid-phase extraction clean-up and liquid chromatography with diode-array detection. *J. Chromatogr., A* **2008**, *1214*, 115−120.
(14) Ford, K. A.; Casida, J. E. Comparative metabolism and pharmacokinetics of seven neonicotinoid insecticides in spinach. *J. Agric. Food Chem.* **2008**, *56*, 10168−10175.
(15) Gupta, M.; Shanker, A. Persistence of acetamiprid in tea and its transfer from made tea to infusion. *Food Chem.* **2008**, *111*, 805−810.
(16) Chin-Chen, M. L.; Esteve-Romero, J.; Carda-Broch, S. Determination of the insecticide imidacloprid in fruit juices using micellar high-performance liquid chromatography. *J. AOAC Int.* **2009**, *92*, 1551−1556.
(17) Tapparo, A.; Giorio, C.; Marzaro, M.; Marton, D.; Soldà, L.; Girolami, V. Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedling obtained from coated seeds. *J. Environ. Monitor.* **2011**, *13*, 1564−1568.
(18) Wu, Q.; Li, Z.; Wang, C.; Wu, C.; Wang, W.; Wang, Z. Dispersive solid-phase extraction clean-up combined with dispersive liquid-liquid microextraction for the determination of neonicotinoid insecticides in vegetable samples by high-performance liquid chromatography. *Food Anal. Methods* **2011**, *4*, 559−566.
(19) Blasco, C.; Fernández, M.; Picó, Y.; Font, G.; Mañes, J. Simultaneous determination of imidacloprid, carbendazim, methiocarb and hexythiazox in peaches and nectarines by liquid chromatography-mass spectrometry. *Anal. Chim. Acta* **2002**, *461*, 109−116.
(20) Obana, H.; Okihashi, M.; Akutsu, K.; Kitagawa, Y.; Hori, S. Determination of neonicotinoid pesticide residues in vegetables and fruits with solid phase extraction and liquid chromatography mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 2501−2505.
(21) Bonmatin, J. M.; Moineau, I.; Charvet, R.; Fleche, C.; Colin, M. E.; Bengsch, E. R. A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants, and in pollens. *Anal. Chem.* **2003**, *75*, 2027−2033.
(22) Marín, A.; Martínez Vidal, J. L.; Egea Gonzalez, F. J.; Garrido Frenich, A.; Glass, C. R.; Sykes, M. Assessment of potential (inhalation and dermal) and actual exposure to acetamiprid by greenhouse applicators using liquid chromatography-tandem mass spectrometry. *J. Chromatogr., B* **2004**, *804*, 269−275.
(23) Fidente, P.; Seccia, S.; Vanni, F.; Morrica, P. Analysis of nicotinoid insecticides residues in honey by solid matrix partition clean-up and liquid chromatography-electrospray mass spectrometry. *J. Chromatogr., A* **2005**, *1094*, 175−178.
(24) Seccia, S.; Fidente, P.; Attard Barbini, D.; Morrica, P. Multiresidue determination of nicotinoid insecticide residue in drinking water by liquid chromatography with electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2005**, *553*, 21−26.
(25) Ferrer, I.; Thurman, E. M.; Fernández-Alba, A. R. Quantitation and accurate mass analysis of pesticides in vegetables by LC/TOF-MS. *Anal. Chem.* **2005**, *77*, 2818−2825.
(26) Di Muccio, A.; Fidente, P.; Attard Barbini, D.; Dommarco, R.; Seccia, S.; Morrica, P. Application of solid-phase extraction and liquid chromatograpy-mass spectrometry to the determination of neonicotinoid pesticide residues in fruits and vegetables. *J. Chromatogr., A* **2006**, *1108*, 1−6.
(27) Pirard, C.; Widart, J.; Nguyen, B. K.; Deleuze, C.; Heudt, L.; Haubruge, E.; De Pauw, E.; Focant, J. F. Development and validation of a multi-residue method for pesticide determination in honey using on-column liquid-liquid extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2007**, *1152*, 116−123.
(28) Mezcua, M.; Ferrer, C.; García-Reyes, J. F.; Martínez-Bueno, M. J.; Albarracín, M.; Claret, M.; Fernández-Alba, A. R. Determination of selected non-authorized insecticides in peppers by liquid chromatography time-of-flight mass spectrometry and tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1384−1392.
(29) Kamel, A. Refined methodology for the determination of neonicotinoid pesticides and their metabolites in honey bees and bee products by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *J. Agric. Food Chem.* **2010**, *58*, 5926−5931.
(30) Tanner, G.; Czerwenka, C. LC-MS/MS analysis of neonicotinoid insecticides in honey: methodology and residue findings in Austrian honeys. *J. Agric. Food Chem.* **2011**, *59*, 12271−12277.
(31) Xiao, Z.; Li, X.; Wang, X.; Shen, J.; Ding, S. Determination of neonicotinoid insecticides residues in bovine tissues by pressurized solvent extraction and liquid chromatography-tandem mass spectrometry. *J. Chromatogr., B* **2011**, *879*, 117−122.
(32) Xie, W.; Han, C.; Qian, Y.; Ding, H.; Chen, X.; Xi, J. Determination of neonicotinoid pesticides residues in agricultural samples by solid-phase extraction combined with liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2011**, *1218*, 4426−4433.
(33) Martel, A.; Lair, C. Validation of a highly sensitive method for the determination of neonicotinoid insecticides residues in honeybees by liquid chromatography with electrospray tandem mass spectrometry. *Int. J. Environ. Anal. Chem.* **2011**, *91*, 978−988.
(34) Navalón, A.; González-Casado, A.; El-Khattabi, R.; Vilchez, J. L.; Fernández-Alba, A. R. Determination of imidacloprid in vegetable samples by gas chromatography-mass spectrometry. *Analyst* **1997**, *122*, 579−581.
(35) Lodevico, R. G.; Li, Q. X. Determination of total imidacloprid residues in coffee by gas chromatography-mass spectrometry. *Anal. Lett.* **2002**, *35*, 315−326.
(36) Mateu-Sánchez, M.; Moreno, M.; Arrebola, F. J.; Martínez Vidal, J. L. Analysis of acetamiprid in vegetables using gas chromatography-tandem mass spectrometry. *Anal. Sci.* **2003**, *19*, 701−704.
(37) Ono, Y.; Yamagami, T.; Nishina, T.; Tobino, T. Pesticide multiresidue analysis of 303 compounds using supercritical fluid extraction. *Anal. Sci.* **2006**, *22*, 1473−1476.
(38) Liu, L.; Hashi, Y.; Qin, Y.; Zhou, H.; Lin, J. Development of automated online gel permeation chromatography-gas chromatograph mass spectrometry for measuring multiresidual pesticides in agricultural products. *J. Chromatogr., B* **2007**, *845*, 61−68.

(39) Zhang, B.; Pan, X.; Venne, L.; Dunnum, S.; McMurry, S. T.; Cobb, G. P.; Anderson, T. A. Development of a method for the determination of 9 currently used cotton pesticides by gas chromatography with electron capture detection. *Talanta* **2008**, *75*, 1055−1060.
(40) Watanabe, E.; Eun, H.; Baba, K.; Arao, T.; Ishii, Y.; Endo, S.; Ueji, M. Rapid and simple screening analysis for residual imidacloprid in agricultural products with commercially available ELISA. *Anal. Chim. Acta* **2004**, *521*, 45−51.
(41) Kim, H.; Shelver, W. L.; Li, Q. X. Monoclonal antibody-based enzyme-linked immunosorbent assay for the insecticide imidacloprid. *Anal. Chim. Acta* **2004**, *509*, 111−118.
(42) Girotti, S.; Maiolini, E.; Ghini, S.; Eremin, S.; Mañes, J. Quantification of imidacloprid in honeybees: development of a chemiluminescent ELISA. *Anal. Lett.* **2010**, *43*, 466−475.
(43) Wang, R.; Wang, Z.; Yang, H.; Wang, Y.; Deng, A. Highly sensitive and specific detection of neonicotinoid insecticide imidacloprid in environmental and food samples by a polyclonal antibody-based enzyme-linked immunosorbent assay. *J. Sci. Food Agric.* **2012**, *92*, 1253−1260.
(44) Watanabe, E.; Miyake, S.; Baba, K.; Eun, H.; Endo, S. Immunoassay for acetamiprid detection: application to residue analysis and comparison with liquid chromatography. *Anal. Bioanal. Chem.* **2006**, *386*, 1441−1448.
(45) Kim, H.; Liu, S.; Keum, Y.; Li, Q. X. Development of an enzyme-linked immunosorbent assay for the insecticide thiamethoxam. *J. Agric. Food Chem.* **2003**, *51*, 1823−1830.
(46) Rezaee, M.; Yamini, Y.; Mohammad, F. Evolution of dispersive liquid-liquid microextraction method. *J. Chromatogr., A* **2010**, *1217*, 2342−2357.
(47) Zgoła-Grześkowiak, A.; Grześkowiak, T. Dispersive liquid-liquid microextraction. *Trends Anal. Chem.* **2011**, *30*, 1382−1399.
(48) Asensio-Ramos, L. M.; Ravelo-Pérez, L. M.; González-Curbelo, M. A.; Hernández-Borges, J. Liquid phase microextraction applications in food analysis. *J. Chromatogr., A* **2011**, *1218*, 7415−7437.