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Application of matrix solid phase dispersion to the determination of imidacloprid, carbaryl, aldicarb, and their main metabolites in honeybees by liquid chromatography–mass spectrometry detection

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Abstract

A method based on matrix solid phase dispersion (MSPD) using C18 as dispersant and dichloromethane–methanol as eluent and liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) has been developed for the simultaneous determination of imidacloprid, 6-chloronicotinic acid, carbaryl, aldicarb, aldicarb sulfoxide, and aldicarb sulfone in honeybees.

The proposed method was compared with liquid–liquid extraction (LLE) combined with LC–APCI–MS analysis. Spiked blank samples were used as standards to counteract the matrix effect observed in the chromatographic determination. Recovery studies were performed at different fortification levels. Average recoveries by MSPD varied from 61% of 6-chloronicotinic acid to 99% of aldicarb sulfoxide and relative standard deviations were equal or lower than 14%. Limit of detections ranged from 0.004 mg kg^{-1} for imidacloprid to 0.09 mg kg^{-1} for 6-chloronicotinic acid. Results obtained by both methods were compared, MSPD showed higher recoveries and sensitivity than LLE for most pesticides, except for carbaryl. As MSPD is easier to perform, faster, consumes less sample and organic solvents than LLE, its application for pesticide analysis in honeybees is suggested.

Keywords: Honeybees; Pesticides; Metabolites; Liquid chromatography; Mass spectrometry

1. Introduction

Honeybees (*Apis mellifera*) are excellent bioindicators because of their morphological and ethiological characteristics as the intense foraging activity and their ability to retain and bioaccumulate in their bodies those substances which they are in close contact with during pollination. Because of their high sensitivity to phytosanitarian treatments, bees represent a valuable tool to evaluate the degree of pesticide contamination by two forms: firstly, as direct indicator of the pesticides that cause a high mortality to the colony or, in second term, as indirect indicator of those pesticides that are not particularly dangerous to the insect but are retained by them [1–3].

Although aldicarb is not toxic to bees, even when applied directly, carbaryl, and imidacloprid exhibit a high oral toxicity to bees with reported oral LD50 values of 0.18 μg/bee [4], and 40–60 ng/bee [5], respectively. Imidacloprid, which is extensively used in agricultural areas, represent one of the relatively new introduced molecules in the market. Beekeepers suspect that this insecticide is responsible for the death of numerous bees and the drastic decline of honey production in France [6] and Italy [7].

After its application, aldicarb is rapidly transformed to aldicarb sulfoxide and more slowly to aldicarb sulfone [8] and imidacloprid is degraded to the primary metabolite, 6-chloronicotinic acid [7].

Since carbaryl, imidacloprid, aldicarb and their metabolites are somewhat polar or thermally labile for the traditional gas chromatographic methods, liquid chromatography (LC) is the analytical technique of choice. Conventional UV

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or diode array detection has been replaced by mass spectrometry (MS) detection as the increased number of publications on liquid chromatography–mass spectrometry (LC–MS). Several researchers have examined the suitability of LC–MS for the analysis of the *N*-methyl carbamates and their metabolites in vegetables and fruits [8,9] but the simultaneous analysis by LC–MS of imidacloprid and its metabolite 6-chloronicotinic has not been reported yet.

Sample preparation represents one of the most critical parts of pesticide analysis in bees due to the large amount of beewax, proteins and other substances, which are readily extracted by solvents typically used in residue analysis. Most of the published methods involve liquid–liquid extraction (LLE) followed by a clean up step [10–12]. However, this technique is time-consuming, laborious, and requires large volumes of both, sample and organic solvent. Alternative procedures for the analysis of pesticides in honeybees include gel permeation chromatography (GPC) [13], supercritical fluid extraction (SFE) [14], and solid-phase microextraction (SPME) [15].

One of the most promising technique to reduce matrix interferences is matrix solid-phase dispersion (MSPD), which involves the dispersion of the sample over a solid support, and subsequent elution of the analytes with a relatively small volume of solvent [16,17]. Moreover, extraction and clean-up are performed in the same step, saving analysis time and organic solvent employed. MSPD has been successfully applied in other fat-content matrix such as liver, muscle tissue, milk, and fat [18,19]. Morzycka [20] developed a method for determining low and medium polarity pesticides in honeybees by combination of MPSD (silica and florisil as dispersing phase and dichloromethane as eluent) with gas chromatography (GC) and nitrogen–phosphorus detector. In addition, our laboratory has previously reported the analysis of 22 organophosphorous in honeybees by MSPD followed by LC–APCI–MS [21].

The aim of this paper was to analyze carbaryl, imidacloprid and its metabolite 6-chloronicotinic acid, and aldicarb and its metabolites aldicarb sulfoxide and aldicarb sulfone. Only a few reports have been published about the analysis these pesticides and their metabolites, although these products are of special interest due to their toxicity and simultaneous presence in the environment. Finally, the performance of the proposed method, MSPD using C18 as dispersant phase was compared with a procedure based on LLE with dichloromethane and acetone, which is frequently used in monitoring programs [2].

2. Experimental

2.1. Reagents and chemicals

Pesticides standards (imidacloprid, 6-chloronicotinic acid, carbaryl, aldicarb, aldicarb sulfoxide, aldicarb sulfone) were all purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Methanol (HPLC grade), dichloromethane (organic trace analysis), acetone (pesticide analysis) and petroleum ether (HPLC grade) were supplied by Merck (Darmstadt, Germany). Deionized water (<4 cm MΩ resistivity) was obtained from Milli-Q SP Reagent Water System of Millipore (Bedford, MA, USA).

Celite 545 (diatomaceous earth) was supplied by Sigma–Aldrich (Madrid, Spain), and anhydrous sodium sulfate, sodium chloride, ammonium chloride and orthophosphoric acid 85%, by Panreac (Barcelona, Spain). Trifluoroacetic acid, ammonium formiate and Florisil (60–100 mesh) were purchased from Sigma–Aldrich. C18, C8 and phenyl, with particle diameter of approximately 50 mm and a pore diameter of 60 Å from Analysis Vínicos (Tomelloso, Spain), and graphitized nonporous carbon material, ENVI-Carb (120–400 mesh), 40–100 mm particle size, 100 $m^2 g^{-1}$ surface area, from Supelco (Madrid, España). Sample lyophilization was carried out with a Drywinner Heto 1.0–60/CT 60 Cooling Trap (Allerod, Denmark).

2.2. Standard preparation

A stock standard solution of each pesticide was prepared by dissolving 10 mg of each compound in 10 ml of methanol and stored at 4 °C. Appropriate aliquots of individual stock solutions were diluted with methanol to prepare a mixed solution containing 100 μg ml^{-1} of imidacloprid, 200 μg ml^{-1} of aldicarb, aldicarb sulfoxide, aldicarb sulfone, carbaryl and 600 μg ml^{-1} of 6-chloronicotinic acid. Intermediate solutions at concentrations ranging from 10 to 60 and 1 to 6 μg ml^{-1} were prepared in methanol and used for accuracy, precision, and sensitivity test. To prevent matrix effects that might result in an over estimation of recoveries, working standard solution were prepared in extracts obtained after MSPD and LLE from pesticide-free honeybee samples.

2.3. Instrumentation and chromatographic conditions

The equipment used was a Hewlett-Packard (Palo Alto, CA) HP-1100 series LC–MS detector system equipped with an autosampler, a binary solvent pump, and a MS detector consisting of a single quadrupole mass analyzer with an atmospheric pressure chemical ionization (APCI) interface in positive ionization mode (PI).

The analytical column was a Luna C18 (150 mm × 4.6 mm i.d., 5 μm particle size), protected by a security-guard cartridge C18 (4 mm × 2 mm i.d.), both from Phenomenex (Ceshire, UK). The mobile phase was a methanol: aqueous solution with trifluoroacetic acid at pH 3 and the gradient used at a flow rate of 1 ml/min was: 10–90% methanol in 15 min and back to the initial conditions in 5 min.

The operating conditions of the APCI interface were capillary voltage, 4000 V; corona current, 4 μA; vaporizer temperature, 325 °C; nebulizer gas pressure, 4 bar; and drying gas flow rate, 4 l/min with a temperature of 350 °C (both, nebulizer and drying gas were nitrogen). Full-scan LC/MS chromatograms were obtained by scanning from *m/z* 100 to 400 with a scan time of 0.75 s. Time-scheduled selected-ion monitoring (SIM) of the most abundant ions of each compound was used for quantification. The optimum fragmentor voltage were pre-set for each group of ions monitoring at the same time and automatically tuned using the instrument control utilities included in the software.

2.4. Sample preparation

Honeybee samples were obtained during winter time from environmental monitoring stations located in Bologna surroundings. Winter bees are pesticide free, as bees are not active in this period; this fact was confirmed before the validation study by the analysis of blank samples. Once in the laboratory, the samples were immediately lyophilized to eliminate the matrix putrefaction process and stored at −18 °C.

2.4.1. LLE of honeybees

Three grams of bees, thoroughly pounded and homogenized, were placed in a 250 ml-glass beaker and vigorously mixed during 5 min with 80 ml of acetone. The mixture was filtered through a Buchner funnel packed with a layer of Celite (5–10 mm). One hundred millilitres of a coagulating solution (10 g of ammonium chloride, 20 ml of ortophosphoric acid and 800 ml of water) were added to the filtrate and allowed to stand for 30 min with occasional stirring and then filtered with Celite. After filtration, the sample was diluted with 100 ml of 2% aqueous NaCl (w/v) and extracted with 100 ml of dichloromethane. The organic phase was passed through a filter containing anhydrous sodium sulfate to eliminate water residues. This last extraction was repeated with 50 ml of dichloromethane. The combined extraction fractions were collected and evaporated to dryness in a rotary evaporator at 45 °C and 250 mbar. The sample was reconstituted with 1.5 ml of methanol filtered through a 0.45 μm PTFE-acrodisk, Análisis Vínicos (Tomellosos, Spain) and 5 μL were then injected in the LC–MS system.

2.4.2. MSPD of honeybees

One gram of honeybees was placed into a glass mortar and gently blended with 1 g of C18 for 5 min using a pestle. This mixture was introduced into a 100 mm × 9 mm i.d. glass chromatography column fitted with a coarse frit (No. 3) and covered with a plug of silanized glass wood at the top. The pesticides were eluted with 10 ml of a dichloromethane:methanol solution (85:15, v/v) that was allowed to elute dropwise by applying a slight vacuum. The eluent was collected and evaporated to dryness under a stream of nitrogen at 45 °C. The extract was reconstituted with 0.5 ml of methanol. Then, the sample was filtered through a 0.45 μm PTFE-acrodisk and 5 μl were injected in the LC–MS system.

3. Results and discussion

3.1. LC–MS determination

As imidacloprid metabolite, 6-chloronicotinic acid was not well resolved by methanol–water gradient, different modifiers were selected to obtain an overall optimum response. Addition of modified with acid characteristics or changes on ionic strength of the aqueous solution allowed to improve MS detectability and chromatographic behavior [22]. Ten and 5 mM ammonium formate buffer were assayed without positive results. Nevertheless, the addition of trifluoroacetic acid in the mobile phase (pH 3) allowed satisfactory chromatographic behavior of the analytes and the separation of the five compounds was accomplished within 15 min.

APCI is the most applied interface for this type of compounds as provides few fragment ions, reproducible spectra and makes possible to monitor precisely and simultaneously various pesticides and their metabolites [8,23,24]. The conditions of the LC–MS were optimized and the appropriate ions selected by flow injection analysis (FIA) with the syringe pump of the individual solutions of the pesticides (10 μg ml^{-1}). The production of positive or negative ions depends considerably on the acidity of the pesticide and the compounds studied showed maximum sensitivity in positive ion APCI analysis. Table 1 summarizes the chemical structures, molecular weighs, base peak and the most abundant ions (with the relative abundance) of the mass spectra with different fragmentor voltages in PI mode. Other parameters of MS as vaporizer, drying gas temperature, and capillary voltage were assayed but did not drastically improve sensitivity nor even influence fragmentation. The protonated molecule was the most abundant ion for all the compounds, except aldicarb, for which the most abundant ion was the fragment $[M–OCONHCH_3]^+$. Imidacloprid and carbaryl quantification was achieved choosing the protonated molecule and their corresponding fragment ions. 6-Chloronicotinic acid, aldicarb, and metabolites were only quantified with the protonated molecule because of their poor fragmentation.

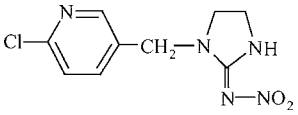
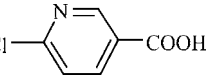
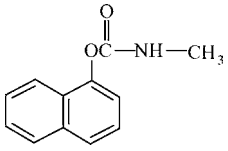
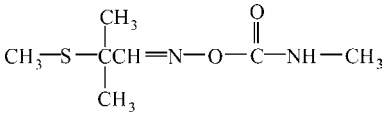
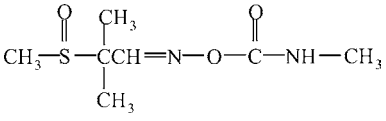
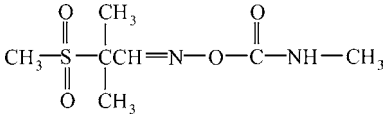
Aldicarb sulfoxide fragment ion (*m/z* 132) was not used for quantification because of the matrix interferences observed at the same retention time. Time scheduled selected ion monitoring (SIM) of the most abundant ion of each compound used for quantification is shown in Table 2.

The detector response was linear over the range of injected amounts, from 0.25 to 50 μg ml^{-1}, with excellent correlation for all compounds ($r > 0.992$). For repeatability and intermediate precision studies of the LC–MS procedure, five replicate determinations on the same day and on five different days of a standard solution were carried out. Relative standard deviations (R.S.D.s) ranged from 5 to 12% for run-to-run precision and from 8 to 16% for the day-to-day precision.

3.2. MSPD extraction

Different parameters that affect MSPD extraction as dispersant agent, clean-up and eluent solvent were studied. Nonpolar solid phase C18, C8, and carbon were tested for matrix dispersion as shown in Table 3. Although recoveries were similar in all cases, C18 provided cleaner extract as lipids showed greater affinity for this phase. Elution of these pesticides was carried out with different methanol proportions to establish the best elution procedure. Elution with 100% of methanol provided high recoveries ($R > 100\%$) but the extracts obtained were dirty because of waxes and pigments. In contrast, elution with 100% of dichloromethane gave low mean recovery ($R < 50\%$). Different proportion of methanol and dichloromethane were assayed but the best results were obtained with the mixture dichloromethane:methanol (85:15, v/v) as reduced the amount of co-extractives with satisfac-

Table 1
Molecular and fragment ions and their relative abundance obtained by LC–APCI–MS at different fragmentation voltages

Pesticide (Mw)	*m/z* and tentative ion	Abundance (%)				
		20 V	40 V	60 V	100 V	140 V
Imidacloprid (255) 	256 $[M+H]^+$	50	75	100	35	–
	209 $[M–NO_2]^+$	–	–	–	35	40
	175 $[M–NO_2–Cl]^+$	–	–	–	35	65
6-Chloronicotinic acid (157) 	158 $[M+H]^+$	35	40	60	100	13
Carbaryl (201) 	202 $[M+H]^+$	50	60	15	–	–
	145 $[M–OCNHCH_3]^+$	–	20	85	85	15
Aldicarb (190) 	191 $[M+H]^+$	–	–	–	–	–
	116 $[M–OCONHCH_3]^+$	40	85	100	10	-
	89 $[M–CH{=}NOCONHCH_3]^+$	–	–	20	25	-
Aldicarb sulfoxide (206) 	207 $[M+H]^+$	75	85	30	–	–
	132 $[M–OCONHCH_3]^+$	5	30	100	5	–
Aldicarb sulfone (222) 	223 $[M+H]^+$	50	100	65	2	–
	166 $[M–CONHCH_3]^+$	–	–	20	3	–
	148 $[M–OCONHCH_3]^+$	–		20	8	–

tory recoveries. In order to wash out matrix interferences, two purification steps were assayed. Firstly, petroleum ether was used to eliminate polar compounds previous to pesticide elution and in second term, 0.5 g of Florisil was placed at the bottom of the MSPD column to remove interference from the matrice. In both cases interferences were eliminated but recoveries also decreased, so, purification steps were no longer used. Finally, the method proposed was C18 as solid phase and a mixture of dichloromethane:methanol as eluent (85:15).

3.3. MSPD versus LLE

The MSPD method proposed in this paper was compared to the established LLE procedure used in a monitoring program for the analysis of organophosphorus and some carbamates in honeybees [2]. Matrix interference studies were carried out for both procedures comparing the calibration curves of standard solutions prepared in methanol with standard solution prepared in honeybee matrix. The intensity of the suppression and enhancement effect depends on the interface used, the sample

Table 2
SIM conditions for determining pesticides by LC–APCI–MS

Pesticide	Retention time (min)	Time (min)	Monitor ion (*m/z*)	Fragmentation (V)
Aldicarb sulfoxide	5.97	0.00–6.50	207	40
Aldicarb sulfone	6.63	6.50–8.00	223	40
Imidacloprid	9.25	8.00–10.00	256	100
			209	
			175	
6-Chloronicotinic acid	10.97	10.00–11.50	158	100
Aldicarb	11.71	11.50–13.00	116	60
Carbaryl	13.41	13.00–15.00	202	60
			145	

Table 3
Recovery (%) and R.S.D.s of the studied pesticides in honeybee samples spiked at 0.3 mg kg^{-1} using different sorbent material

Pesticide	C18	C8	Carbon
Aldicarb sulfoxide	98 ± 7	95 ± 10	94 ± 15
Aldicarb sulfone	70 ± 10	74 ± 12	73 ± 10
Imidacloprid	71 ± 13	68 ± 14	71 ± 15
6-Chloronicotinic acid	63 ± 11	60 ± 9	58 ± 10
Aldicarb	73 ± 15	71 ± 14	68 ± 11
Carbaryl	72 ± 10	70 ± 12	74 ± 13

pre-treatment procedure, the matrix nature and the pesticide considered. Both methods showed a slight enhancement and ion suppression of the response (ranging from 0 to 15 dependent of the compound studied).

Limits of detection (LODs) and limits of quantifications (LOQs) are shown in Table 4. The LODs defined as the concentration that yields a signal-to-noise of at least 3:1, was ranged from 0.003 to 0.08 mg kg^{-1} by LLE and from 0.004 to 0.09 mg kg^{-1} by MSPD. LOQs estimated as those concentrations of analyte which yield a signal-to-noise of at least 10 ranged from 0.01 to 0.2 mg kg^{-1} by LLE and from 0.02 to 0.3 mg kg^{-1} by MSPD.

Recoveries and precision studies of MSPD and LLE were performed on three honeybee samples spiked at two different levels of each compound as show in Table 5. Average recoveries by LLE ranged from 29% for 6-chloronicotinic acid and 84% for carbaryl when samples were spiked at 0.6 and 0.3 mg kg^{-1}, respectively. MSPD recoveries ranged from 61% for 6-chloronicotinic acid and 99% for aldicarb sulfoxide spiked at 0.6 and 0.2 mg kg^{-1}, respectively. The relative standard deviation (R.S.D.) ranged from 3 to 16% by LLE and from 6 to 14% by MSPD. LLE recoveries were not acceptable for aldicarb metabolites and 6-chloronicotinic acid but MSPD recoveries were satisfactory for all compounds, although recovery of carbaryl was higher by LLE. The relatively low recoveries of aldicarb could be due to some uncontrollable loss of the pesticide occurred during the solvent removal step. These results are in agreement with other works that report the poor stability of aldicarb at high temperatures [17,25].

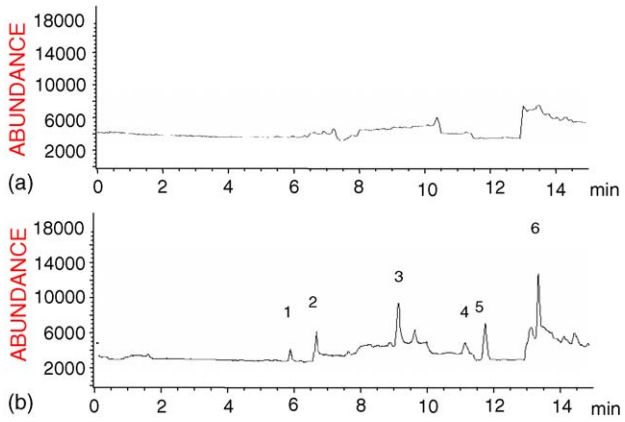


Fig. 1. LLE–LC–MS chromatogram of (a) blank honeybee sample and (b) spiked honeybee sample at 0.2 mg kg^{-1} of aldicarb sulfoxide, aldicarb sulfone, aldicarb and carbaryl, 0.1 mg kg^{-1} of imidacloprid, and 0.6 mg kg^{-1} of 6-chloronicotinic acid. Peak number: 1. aldicarb sulfoxide, 2. aldicarb sulfone, 3. imidacloprid, 4. 6-chloronicotinic acid, 5. aldicarb and 6. carbaryl.

Chromatograms obtained of a blank honeybee sample and of a spiked honeybee sample after LLE and MSPD are illustrated in Figs. 1 and 2, respectively. The absence of any ion mass signal at the same retention time as target pesticides suggest that there were not matrix compounds that might give a false positive signal.

From this data, it can be seen that the best extraction efficiency was obtained by MSPD. In general, MSPD with C18

Table 4
LODs and LOQs of honeybees by LLE and MSPD

Pesticide	LLE		MSPD	
	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Aldicarb sulfoxide	0.08	0.2	0.02	0.06
Aldicarb sulfone	0.02	0.04	0.008	0.02
Imidacloprid	0.006	0.02	0.004	0.02
6-Chloronicotinic acid	0.04	0.2	0.09	0.3
Aldicarb	0.01	0.03	0.01	0.04
Carbaryl	0.003	0.01	0.005	0.02

Table 5
LLE and MSPD recoveries and relative standard deviations (R.S.D.s) for honeybee samples at two spiking levels ($n = 3$)

Pesticide	Added standard (mg kg^{-1})	Recovery (%) ± R.S.D.%		Added standard (mg kg^{-1})	Recovery (%) ± R.S.D.%	
		LLE	MSPD		LLE	MSPD
Aldicarb sulfoxide	0.2	37 ± 10	99 ± 12	2	38 ± 3	91 ± 12
Aldicarb sulfone	0.2	39 ± 12	72 ± 14	2	49 ± 16	72 ± 11
Imidacloprid	0.1	67 ± 9	70 ± 14	1	65 ± 7	71 ± 6
6-Chloronicotinic acid	0.6	29 ± 10	61 ± 11	6	21 ± 4	67 ± 9
Aldicarb	0.2	67 ± 7	70 ± 13	2	66 ± 5	69 ± 11
Carbaryl	0.2	84 ± 14	74 ± 8	2	85 ± 10	76 ± 8

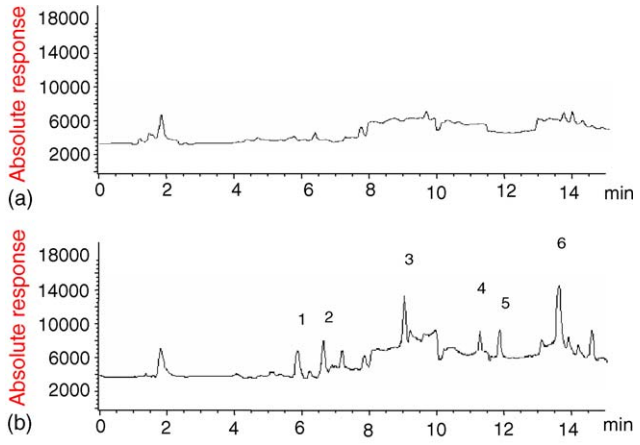


Fig. 2. MSPD-LC–MS chromatogram of (a) blank honeybee sample and (b) spiked honeybee sample at 0.2 mg kg^{-1} of aldicarb sulfoxide, aldicarb sulfone, aldicarb, and carbaryl, 0.1 mg kg^{-1} of imidacloprid, and 0.6 mg kg^{-1} of 6-chloronicotinic acid. Peaks identification as Fig. 1.

offers simplicity and a significant reduction of the required volume of organic solvents compared with LLE method.

4. Conclusion

Imidacloprid, 6-chloronicotinic acid, carbaryl, aldicarb, aldicarb sulfoxide, and aldicarb sulfone were efficiently extracted by MSPD with C18 and then determined and identified by LC–MS. Honeybees are complex because of the relatively large and variable amounts of wax residues. However, the MSPD procedure did not require additional clean-up steps because sample extraction and clean-up were carried out in the same step. MSPD takes advantage over LLE of simplicity and rapidity (30 min versus 2 h) and also requires small samples (1 g versus 3 g) and volumes of solvent (10 ml versus 230 ml). In addition, using the MSPD combined with LC–MS in SIM mode for multi-residues screening allows an unequivocal identification of the pesticides studied and problems with co-eluting pesticides are solved.

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