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Multi-residue analysis of 80 environmental contaminants in honeys, honeybees and pollens by one extraction procedure followed by liquid and gas chromatography coupled with mass spectrometric detection

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ABSTRACT

One of the factors that may explain nowadays honeybees' colonies losses is the increasing presence of chemicals in the environment. The aim of this study is to obtain a global view of the presence of environmental contaminants in beehives and, develop a fast, cheap and sensitive tool to analyze environmental contaminants in apiarian matrices. A multi residue analysis was developed to quantify 80 environmental contaminants, pesticides and veterinary drugs, belonging to different chemical classes, in honeys, honeybees and pollens. It consists in a single extraction, based on a modified "QuEChERS method", followed by gas chromatography coupled with Time of Flight mass spectrometry (GC-ToF) and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The "QuEChERS method" combines salting-out liquid-liquid extraction with acetonitrile and a dispersive-SPE clean up. It was adjusted to honey and especially to honeybee and pollen, by adding a small fraction of hexane in acetonitrile to eliminate lipids that interfere with mass spectrometry analysis. This method, combined with accurate and sensitive detection, allowed quantification and confirmation at levels as low as 10 ng/g, with recoveries between 60 and 120%. Application to more than 100 samples of each matrix was achieved for a global view of pesticide presence in the honeybee environment. Relatively high percentages of honeys, honeybees and pollens were found to be contaminated by pesticides used to combat varroa but also by fungicides like carbendazim and ubiquitous contaminants.

1. Introduction

Nowadays, it is well-known that bee mortality has never been so high all over the world. One of the hypotheses to explain this mortality is the increasing use of pesticides. To check this hypothesis, a global view of beehives contamination is needed and ecotoxicological studies on honeybees have to be conducted, which requires reliable and sensitive analytical methods. Furthermore, honeybees are subject to a large range of molecules, including pesticides and also antibacterial substances that can be used by farmers [1]. Consequently, to survey beehives contamination, fast and cheap multi-residue analytical methods have to be developed.

The most universal extraction method to analyze a wide range of pesticides is the "QuEChERS method". This method consists in two steps, liquid-liquid extraction, and purification by dispersive Solid Phase Extraction (dSPE). QuEChERS stands for quick, easy, cheap, efficient, rugged and safe. This method was first introduced by Anastassiades et al. [2] in 2003. The original method was developed in order to extract pesticides from fruits and vegetables. Initially, it consisted in salting-out liquid–liquid extraction, using acetonitrile, $MgSO_4$ and NaCl salts and a dispersive SPE step based on primary and secondary amine bonded silica (PSA). One of the QuEChERS method advantages is to be a simple method, easily adjustable. Consequently, since 2003, the two steps of this method have been optimized and adjusted several times. In 2005, Lehotay et al. [3] added acetate salts in order to buffer the liquid–liquid extraction and avoid the degradation of base-sensitive pesticides. In 2008, Przybylski et al. [4] published a QuEChERS method adjusted for high fat matrices, adding a small fraction of hexane in acetonitrile, to remove lipids from the extract. Regarding the second step, in 2006, Leandro et al. [5] used PSA and octadecyl bonded silica (PSA/C18) instead of PSA bonded silica to eliminate apolar interferences of the matrix. In 2010, Mullin et al. [6] adapted successfully this method to wax, pollen, bees and beebread, coupled with analysis

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Table 1
Compounds studied in this work and their chemical class; OH, organohalogens; OPP, organophosphorous; Pyr, pyrethroid; IGR, insect growth regulator; Syn, synergist.

Compound	Class	Compound	Class	Compound	Class
Aldrin	*OH*	Cadusaphos	*OPP*	Bitertanol	*Triazole*
Bromopropylate	*OH*	Chlorpyriphos	*OPP*	Cyproconazole	*Triazole*
Chlorothalonil	*OH*	Chlorpyriphos-methyl	*OPP*	Flusilazole	*Triazole*
DDD o,p′	*OH*	Coumaphos	*OPP*	Myclobutanil	*Triazole*
DDT p,p′	*OH*	Diazinon	*OPP*	Propiconazole	*Triazole*
Dicloran	*OH*	Dichlorvos	*OPP*	Penconazole	*Triazole*
Dicofol	*OH*	Dimethoate	*OPP*	Tebuconazole	*Triazole*
Dieldrin	*OH*	Ethoprofos	*OPP*	Triadimenol	*Triazole*
Endosulfan alpha	*OH*	Fenitrothion	*OPP*	Paclobutrazide	*Triazole*
Endosulfan beta	*OH*	Malathion	*OPP*	Clothianidin	*Nicotinoid*
Endosulfan sulphate	*OH*	Methamidophos	*OPP*	Imidacloprid	*Nicotinoid*
Lindane	*OH*	Parathion	*OPP*	Thiamethoxam	*Nicotinoid*
Hexachlorobenzene	*OH*	Phenthoate	*OPP*	Abamectin	*Avermectin*
Methoxychlor	*OH*	Phosalone	*OPP*	Eprinomectin	*Avermectin*
Tetradifon	*OH*	Phosmet	*OPP*	Ivermectin	*Avermectin*
Benalaxyl	*Amide*	Phoxim	*OPP*	Moxidectin	*Avermectin*
Prochloraz	*Amide*	Tolclofos-methyl	*OPP*	Bupirimate	*Pyrimidine*
Bifenthrin	*Pyr*	Triphenylphosphate	*OPP*	Fenarimol	*Pyrimidine*
Cyfluthrin	*Pyr*	Carbaryl	*Carbamates*	Procymidone	*Dicarboximide*
Cypermethrin	*Pyr*	Carbendazim	*Carbamates*	Vinclozolin	*Dicarboximide*
Deltamethrin	*Pyr*	Carbofuran	*Carbamates*	Iprodione	*Dicarboximide*
Esfenvalerate	*Pyr*	Diethofencarb	*Carbamates*	Imazalil	*Imidazole*
Permethrin	*Pyr*	Fenoxycarb	*Carbamates*	Amitraz	*Formamidine*
Tau-fluvalinate	*Pyr*	Methiocarb	*Carbamates*	Clofentezine	*Tetrazine*
λ cyhalothrin	*Pyr*	Methomyl	*Carbamates*	Hexythiazox	*Thiazolidine*
Piperonyl Butoxide	*Syn*	Thiophanate-methyl	*Carbamates*	Pyriproxifen	*IGR*
				Buprofezin	*IGR*

by LC–MS/MS and GC–MS/MS: the usual 15 g sample was reduced to 3 g and for GC analysis the final extract was further purified by SPE, using a dual layer cartridge which contains PSA and graphitized carbon black (GCB). Finally, Barakat et al. [7] published an analytical method of honey which contained a supplementary concentration step in order to reduce method limits of detection. Wilkowska et al. recently published a review about the numerous applications of the QuEChERS method on food matrices [8].

Regarding analytical techniques, GC–EI–MS and LC–ESI–MS/MS are the most used techniques for multi-residue analysis of pesticides in food [9,10] and bee products [1,11] but they are both subject to strong matrix effects which can significantly reduce or enhance the analyte response and lead to wrong quantification. Consequently, solutions have to be found to reduce them and to take them into account for quantification. Most of them have been reviewed in 2003 by Hajslová et al. [12]. Regarding matrix effects in pesticide analysis by GC, an innovative solution was published by Anastassiades et al. [13] which consist in adding compounds called "analyte protectants" (AP) that interact more than pesticides with active sites present in the chromatographic system. There is no equivalent considering LC analysis but, according to Gros et al. [14], the dilution of sample extracts is an efficient strategy to reduce ion suppression.

In light of these concerns, the aim of this study was to develop a simple, fast, sensitive and reliable analytical method for trace analysis of a large number of environmental contaminants in beehives matrices. A list of 80 compounds (Table 1) covering more than 14 families of contaminants were chosen on geographical, economical and scientific (persistence in the environment, bioaccumulation) criteria [15]. This list contains a majority of pesticides but also veterinary drugs and a synergist. Physicochemical properties of these compounds are so different that two separation techniques were necessary: gas and liquid chromatography [16]. Consequently, the extraction method chosen had to be compatible with GC and LC, which is one of the characteristics of the QuEChERS method. Regarding honey, the protocol applied in this study was based on the one published by Barakat et al. [7], who added a concentration step in order to reach limits of detection in the range of environmental concentration. The main difference was that the buffer used in our work was citrate instead of acetate, according to the Standard Method EN 15662 [17]. However, the same method could not be applied to honeybees and pollens which contain high amounts of lipids. The addition of hexane at the first step of QuEChERS, as suggested by Przybylski et al. [4] for high fat matrices extraction, was successfully applied. Analytical techniques used in this work are among the most efficient technique available: LC–MS/MS and GC-ToF. LC–MS/MS is well-known for its great sensitivity and as a reliable tool for quantification. Unlike LC–MS/MS, few studies used GC-ToF as a tool for quantification [18–22]. High acquisition rate and greater selectivity compare to simple quadrupole make GC-ToF a powerful instrument for analysis [23]. Furthermore, thanks to new technology like dynamic range enhancement [20], the dynamic range of recent GC-ToF is comparable to GC–MS, which allows accurate quantification, even in very complex matrices such as pollen. Finally, in this work, special attention was brought to lower matrix effect. Matrix matched calibration was used for quantification and two strategies were tested: dilution of the extract and the use of AP.

In conclusion, this paper presents an original analytical approach which consists in one simple extraction method for each matrix coupled with GC and LC analysis and a comprehensive validation of the whole method. The method developed in this work is expected to be applied as a fast and reliable tool for routine analysis of a large range of compounds at trace level that is in the range of 10–50 ng/g. Application to a large number of samples was made in order to check the robustness of the method but also to obtain a global view of environmental contaminants presence in beehives and to compare the contamination of honeys, honeybees and pollens.

2. Materials and method

2.1. Materials

All compounds were obtained from Sigma–Aldrich with purity higher or equal than 97% (St. Quentin Fallavier, France), except tau-fluvalinate 93.8%, cypermethrine 95.1%, malathion 96.1%, fenitrothion 95.4%, ethoprophos 93.1%, tefluthrin 96.8%, and mox-

idectin 92.3%. Methiocarb (95%) were obtained from Chemservice, and piperonyl butoxide (92.5%), Cyhalothrin-lambda, hexythiazox, eprinomectin (95.0%), cadusafos, and cypermethrine (92.0%) were obtained from Cluzeau (Ste Foy la Grande, France). Isotopically labeled compounds were carbaryl-d_7 (98.8%), malathion-d_7 (99%) and carbendazime-d_4 (99.2%) chlorpyriphos-methyl-d_6 (98%) obtained from Cluzeau. Stock standard solutions of each compound at 1000 mg/L were prepared in acetonitrile (ACN), acetone or methanol, except carbendazim in dimethylformamide and stored at −18 °C. A mixture of these standards at 10 mg/L, prepared in ACN, stored at −18 °C was stable for at least 6 months.

Three compounds were used as analyte protectants (AP) for GC analysis: 3-ethoxy 1-2 propanediol (98%, Aldrich), D-(+)-gluconic acid δ-lactone (99%, Sigma), shikimic acid (99%, Aldrich). Standard solutions of each compound at 50 mg/mL were prepared in 30/70 water/ACN and stored at 4 °C. A mixture of these standards, called "AP mix", was prepared in ACN, stored at 4 °C, leading to respective concentrations of 30, 10 and 5 mg/mL for 3-ethoxy 1-2 propanediol, D-(+)-gluconic acid δ-lactone, shikimic acid.

LC–MS ACN and methanol, hexane, ammonium formate, formic acid were obtained from Fluka (Sigma–Aldrich). The water used was purified by a Milli-Q water system (Millipore, France). "Citrate QuEChERS kits" were obtained from Agilent Technologies: salts are packaged separately and consist in 4 g of anhydrous $MgSO_4$, 1 g of sodium chloride, 1 g of sodium citrate dihydrate and 500 mg of disodium citrate sesquihydrate. Fifteen mL centrifuge tubes of PSA and PSA/Cl8 dispersive SPE were purchased from Carlo-Erba: PSA tubes contain 900 mg of anhydrous $MgSO_4$, 150 mg of PSA bonded silica and PSA/C18 tubes, 900 mg of anhydrous $MgSO_4$, 150 mg of PSA bonded silica and 150 mg of C18 bonded silica.

2.2. Sample collection

Blank matrix samples were constituted by samples collected in Ouessant Island during the summer 2008 (1 kg of honeybees, 1 kg of trap pollen, 1 kg of honey) and were checked for no contamination.

Other samples were collected during the beekeeping seasons 2008 and 2009 (4 samplings per year: April/May, June/July, July/August, September/October). They concerned 16 apiaries of the "Région des Pays de la Loire" (Western France) located in four types of landscapes (bocage, large-scale farming, gardening/orchards, urban area) and two control apiaries (less inhabited landscapes) located in Atlantic islands (Island of Yeu and Island of Ouessant) [15]. For each period, samples were collected in several colonies of every apiary (honey, foraging bees and trap pollen) and repackaged to obtain one pool per apiary. All samples were stored at −20 °C until analysis.

2.3. Sample preparation

The overall sample preparation strategy is represented in Fig. 1.

2.3.1. Honey

First, 5 g of honey are weighed in a 50 mL centrifuge tube, 10 mL of water are added. The tube is then shaken to dissolve honey. When the mixture is homogeneous, 10 mL of acetonitrile (ACN), "citrate QuEChERS" salts (described in Section 2.1) and 200 μL of an isotopically labeled compounds working solution (carbaryl-d_7, malathion-d_7 and carbendazime-d_4) at 1 mg/L are added. For calibration point, a suitable volume of a working standard solution at 1 mg/L is also added. Next, the tube is immediately shaken by hand, vortexed one minute and then centrifuged for 2 min at 5000 × *g*. Afterwards, 6 mL aliquot of supernatant are added in a pre-prepared 15 mL PSA tube (described in Section 2.1). Then, this tube is immediately shaken by hand, vortexed 10 s and centrifuged for 2 min at 5000 × *g*. Finally, 4 mL of the extract, sampled in a 10 mL glass cone-ended centrifuge tube, are evaporated until 50 μL are left, and the remaining extract is kept at −18 °C until analysis.

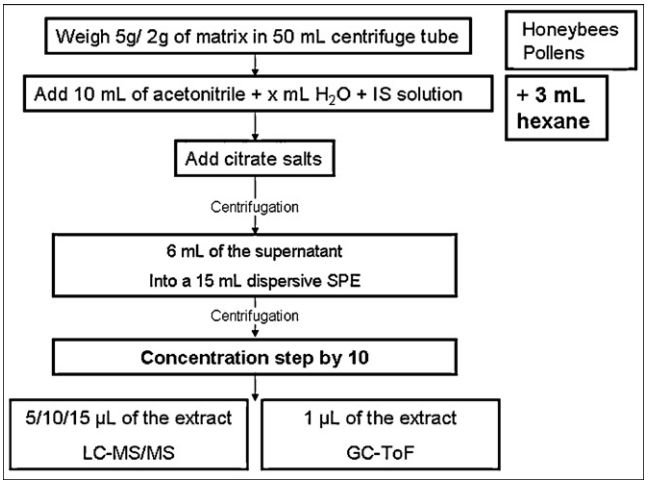

Fig. 1. Sample preparation strategy based on QuEChERS method.

2.3.2. Honeybees

First, about 10 g of honeybees is sampled and ground with a Microtron MB 550 (Kinematica, Switzerland). After that, 5 g of honeybees ground are weighed in a 50 mL centrifuge tube, in which 10 mL of ACN, 3 mL of water, 3 mL of hexane and "citrate QuEChERS" salts and 200 μL of an isotopically labeled compounds working solution (carbaryl-d_7, malathion-d_7 and carbendazime-d_4) at 1 mg/L are then added. For calibration point, a suitable volume of a working standard solution at 1 mg/L is also added. Next, the tube is immediately shaken by hand, vortexed 1 min and then centrifuged for 2 min at 5000 × *g*. Afterwards, 6 mL of the acetonitrile fraction (below the hexane fraction) is added in a pre-prepared 15 mL PSA/C18 tube (described in Section 2.1). Then, this tube is immediately shaken by hand, vortexed 10 s and centrifuged for 2 min at 5000 × *g*. Finally, 4 mL of the extract, sampled in a 10 mL glass cone-ended centrifuge tube, are evaporated until 50 μL are left, and the remaining extract is kept at −18 °C until analysis.

2.3.3. Pollens

2 g of pollens are weighed in a 50 mL centrifuge tube, in which 10 mL of ACN, 8 mL of water, 3 mL of hexane and "citrate QuEChERS" salts are then added. Next, the sample preparation is the same as for honeybees.

2.3.4. Reconstitution of the extract

Just before analysis, 80 μL of a chlorpyriphos-methyl-d_6 working solution at 1 mg/L and 270 μL of ACN are added to obtain a final volume of 400 μL. An aliquot of 100 μL is diluted by 10 in 90/10 mobile phase/ACN for honey and honeybees and in 90/10 mobile phase/ACN and 100% ACN (see Section 3.1.2) for pollens, regarding LC–MS/MS analysis. An aliquot of 90 μL is taken and mixed with 10 μL of AP mix (see Section 2.1) for GC-ToF analysis.

2.4. LC–MS/MS

The system used was a Waters 2695 series Alliance HPLC (Waters, Milford, MA) coupled to a triple quadrupole mass spectrometer Quattro from Micromass (Manchester, UK) equipped with a Z-spray electrospray interface (ESI). Data were processed with MassLynx 4.1.

The chromatographic separation was performed on a Nucleodur Sphinx RP-C18 (50 x 2 mm, 1.8 μm) column from Macherey-Nagel

with in-line filter "krudkatcher" 0.5 μm porosity (Phenomenex). The column oven temperature was set to 40 °C; the flow rate was 300 μL/min. Samples were analyzed with the mobile phase (A) water with ammonium formate 0.3 mM and 0.05% formic acid and (B) methanol. Samples were analyzed in positive mode with the following elution program: from 0 to 5 min linear gradient from 98 to 68% (A), from 5 to 8 min, step at 68% (A), from 8 to 13 min, linear gradient to 100% (B), from 13 to 21 min, step at 100% (B) and 21.1 to 31 min step at 100% (A). Injection volume and solvent depend on the matrix: 15 and 10 μL in 90/10 mobile phase (A)/ACN for honey and honeybees respectively; 5 μL in 90/10 mobile phase (A)/ACN and 100% ACN (see Section 3) for pollens.

Electrospray ionization was performed in the positive mode. The electrospray source parameters were capillary voltage 3.2 kV, the temperatures of the source and desolvatation 120 and 350 °C, respectively, the nitrogen flow was adjusted to 80 L/h for the cone gas and 550 L/h for the desolvatation gas. For each compound, cone voltage and collision energies of two MRM transitions were optimised from a continuous flow of a standard injection (10 mg/l solution in 50/50 (A) and (B) at 10 μL/min) to obtain the maximum intensities. Parent ions and fragment ions selected for confirmation are listed in Table 2. MRM 1 is used for quantification and MRM 2 for confirmation. The analytical run is divided into 10 periods. Interscan and interchannel delays were optimized to 0.03 s.

2.5. GC-ToF

GC-ToF analysis was carried out with a 6890 Agilent gas chromatograph (Agilent Technologies, Avondale, USA) coupled to a Time of Flight (ToF) mass spectrometer GCT Premier from Waters. Data were processed with MassLynx 4.1.

Chromatographic separation was performed on a 30m × 0.25 mm I.D., 0.25 μm film thickness DB-XLB capillary column. Helium (purity 99.999%) was used as a carrier gas at a constant flow of 1 mL/min. Initial oven temperature was set at 80 °C for 1 min, followed by a linear ramp to 220 °C at a rate of 25 °C/min. Subsequently, the temperature was raised to 290 °C at a rate of 10 °C/min, hold 6.4 min, followed by a ramp to 300 at a rate of 30 °C/min and a hold time of 9.7 min, leading to a total run time of 30 min. A split-spitless injector set at 280 °C was always used and injections were performed in the splitless mode. Injection volume and solvent was 1 μL in 90/10 ACN/AP mix (see Section 2.1). A solvent delay of 4 min was applied. Transfer line temperature was set at 250 °C and the source temperature at 200 °C.

The mass spectrometer was operated in the electron impact mode (EI, 70 eV). Multichannel plate voltage was set at 2800 V, acquisition rate at 10 spectra/s (i.e. 5 spectra/s with "Dynamic Range Enhancement" mode on) and pusher interval at 40 μs. Acquisition was performed in the full scan mode with a scan range of m/z 50–550. Calibration was done using the calibration wizard, with heptacosa as the reference. The mass resolution was around 5000 FMWH for m/z 218.9856. During acquisitions, an internal standard, pentafluorochloro benzene, was introduced continuously into the EI source, from a reference reservoir at 50 °C and through a reference inlet at 120 °C. The mass m/z 201.9609 was used as lock mass.

2.6. Method validation

2.6.1. Method validation plan

The developed method was validated following mainly the International Conference on Harmonisation (ICH) [24]. The validation strategy is represented in Fig. 2. It was performed on 3 days with concentration ranges between 4 and 60 ng/g regarding honeys and honeybees and between 10 and 150 ng/g considering pollens.

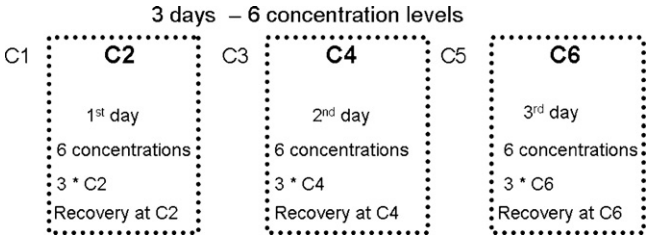

Fig. 2. Validation plan on three days; C1, C2, C3, C4, C5 and C6 corresponds to 4, 10, 20, 30, 40 and 60 ng/g for honeys and honeybees and to 10, 25, 50, 75, 100 and 150 ng/g for pollens.

More precisely, each day, 6 samples of blank matrix spiked with 6 levels of concentration (C1, C2, C3, C4, C5 and C6) were extracted to determine linearity and Intermediate precision; two more samples of blank matrix spiked with one level (first day C2, second day C4, third day C6) were extracted to determine repeatability ($n = 3$); Three samples of blank matrix were extracted and spiked just before analysis, at the same level as for repeatability, to determine recoveries. To resume, this validation strategy consists in 11 experiments per day, during 3 days.

2.6.2. Method validation parameters

For LC/GC analysis, the method limit of detection (LOD) was determined as the analyte concentration that produced a peak signal of three times the background noise from the chromatogram, regarding MRM 2/confirmation m/z. The method limit of quantification (LOQ) was determined as the analyte concentration that produced a peak signal of ten times the background noise from the chromatogram regarding MRM 1/quantification m/z, and at which the MRM/ion ratio is consistent with the MRM/ion ratio of a standard (Relative Standard Deviation (RSD) < 20%), respectively.

Other validation parameters were determined on the peak areas of the compound in the blank matrix sample spiked before extraction ($A_{\text{extraction}}$) and in the blank matrix sample spiked just before analysis (A_{analysis}). Recovery was expressed as the ratio $A_{\text{extraction}}/A_{\text{analysis}}$ in percentage. Repeatability was expressed as the RSD of $A_{\text{extraction}}$ of samples extracted the same day, at the same concentration. Intermediate precision was expressed as the RSD of $A_{\text{extraction}}$ of samples extracted in different days, at the same concentration. Regarding LC–MS/MS analysis, due to matrix effect, areas were corrected with the area of the isotopically labeled compound carbaryl-d_7 for honeys and honeybees and carbaryl-d_7 and malathion-d_7 for pollens (A_{IsoLab}). Intermediate precision was expressed as the RSD of the ratio $A_{\text{extraction}}/A_{\text{IsoLab}}$.

2.6.3. Quantification

Matrix-matched calibration was used for quantification. In each batch, 6 calibration points prepared as describe in Section 2, with concentration ranges between 4 and 60 ng/g for honeys and honeybees and between 10 and 150 ng/g for pollens, were injected.

Quantification was performed using QuanLynx 4.1. Considering LC–MS/MS analysis, each compound, except clofentezine, was characterized by its retention time, two MRM transitions and the MRM ratio which was obtained by the ratio between the MRM 1 and MRM 2 areas (Table 2). Regarding GC-ToF analysis, each compound was characterized by its retention time and two masses with mass windows between 0.02 and 0.1 Da and the ion ratio (Table 3). The ion ratio was calculated by the ratio between the quantification m/z and confirmation m/z areas. Eight analytes, in bold in Tables 2 and 3, were analyzed both by LC–MS/MS and GC-ToF.

In a batch, each sample was injected twice. Instrumental performance was checked with the signal area of chlorpyriphos-methyl-d_6 present in each sample and the injection of Quality

Table 2
LC–MS/MS acquisition method parameters.

Compound	t_R (min)	MRM 1	Cone voltage (V)	CE (eV)	MRM 2	CE (eV)	MRM ratio
Methamidophos	1.70	142 > 94	20	15	142 > 125	15	2.6
Amitraze I[a]	3.32	122 > 107	28	15	122 > 77	30	1.6
Carbendazime-d_4	4.73	196 > 164	25	15			
Carbendazime	4.83	192 > 160	18	15	192 > 132	30	4.5
Methomyl	6.47	163 > 88	10	8	163 > 106	10	1.4
Thiamethoxam	7.22	292 > 211	20	15	292 > 181	20	1.6
Clothianidine	7.97	250 > 169	15	13	250 > 132	15	1.4
Imidacloprid	8.40	256 > 175	20	20	256 > 209	15	1.1
Amitraze II[a]	10.90	150 > 107	20	20	150 > 132	15	2.3
Imazalil	12.40	297 > 159	35	20	297 > 201	20	3.7
Carbofuran	13.04	222 > 165	18	15	222 > 123	20	1.2
Thiophanate-methyl	13.09	343 > 151	18	20	343 > 311	10	5.9
Carbaryl	13.10	202 > 145	12	10	202 > 117	20	6.4
Carbaryl-d_7	13.10	209 > 152	20	20			
Methiocarbe	14.40	226 > 121	15	20	226 > 169	10	1.5
Diethofencarbe	14.40	268 > 226	10	10	268 > 180	15	2.2
Cyproconazole	14.60	292 > 70	15	15	292 > 125	25	4.4
Triadimenol	14.84	296 > 70	12	15	296 > 99	15	9.3
Malathion-d_7	14.85	338 > 128	15	15			
Fenoxycarbe	15.20	302 > 88	20	20	302 > 116	10	1.3
Iprodione	15.27	330 > 245	25	15	330 > 288	10	7.3
Prochloraz	15.40	376 > 308	12	10	376 > 70	20	2.3
Clofentezine	15.60	303 > 138	18	15			
Phoxim	15.57	299 > 129	14	8	299 > 153	8	1.8
Coumaphos	15.56	363 > 227	22	25	363 > 307	20	3.2
Chlorpyriphos-methyl	15.70	322 > 125	22	20	322 > 290	15	1.5
Chlorpyriphos-methyl-d_6	15.70	328 > 131	15	15			
Piperonyl butoxide	16.00	356 > 119	17	30	356 > 177	15	2.7
Pyriproxyfen	16.03	322 > 96	20	20	322 > 227	15	5.2
Hexythiazox	16.17	353 > 168	22	25	353 > 228	15	1.3
Eprinomectin	16.49	915 > 186	20	20	915 > 330	15	5.6
Abamectin	16.47	891 > 305	10	20	891 > 567	15	0.7
Moxidectin	16.80	640 > 528	15	10	640 > 498	15	4.5
Ivermectin	16.86	893 > 569	20	15	893 > 307	25	1

[a] Amitraze I, 2,4-dimethylphenylamine; Amitraze II, N-(2,4 dimethylphenyl) formamide.

Control samples. Extraction performance was checked following the signal areas of carbaryl-d_7, malathion-d_7 and carbendazime-d_4.

3. Results and discussion

3.1. Method optimization

3.1.1. Sample extraction

Regarding the extraction methodology, our main criterion was to find one that gives acceptable recoveries for all analytes with only one protocol. In preliminary studies, based on previous work and literature, three methodologies were tested: solid phase extraction (SPE) for honeys [25], matrix solid phase extraction (MSPD) for pollens and honeybees [26], and the QuEChERS method [7,27], based on the Standard Method EN 15662 [17]. Regarding SPE, depending on the type of phase used, poor recoveries were obtained either for the most polar compounds such as methamidophos, or the most apolar ones such as hexachlorobenzene. The use of MSPD led to poor recoveries regarding the most polar compounds. Finally, the QuEChERS method was the methodology that gave the best recoveries for all analytes with one protocol, so our extraction was based on this method with a few optimizations. First, regarding dSPE of honeybees and pollens, PSA/C18 was preferred to PSA/GCB. Indeed, GCB retained apolar analytes such as hexachlorobenzene and aldrin leading to poor recoveries.

One QuEChERS disadvantage is that it leads to lower concentration of the sample compare to other sample preparation method [19]. Indeed, instrumental limits of detection of GC-ToF led to method limits of detection between 0.03 and 0.1 μg/g which are between 3 and 10 times higher than the value of the maximum residue limits required in the European directives [28]. Consequently, a concentration step by evaporation was added which was satisfactory for honey extraction. Nevertheless, regarding honeybees and pollens, further optimization was needed because this concentration step led to strong ion suppression in LC-MS/MS and saturation of the detector in GC-ToF.

GC-ToF analysis of honeybees and pollens extracts revealed high amounts of fatty acids and fatty acid esters. Indeed, these molecules are the main constituents of beeswax [29]. The addition of a very apolar solvent such as hexane in the extraction step has already been proved to be an efficient way to remove this kind of compounds in baby food [4] and was successfully applied to honeybees and pollens. Different volumes of hexane between 1 and 5 mL were tested. Volumes smaller than 3 mL did not remove enough apolar interferences and volumes higher than 3 mL led to low recoveries of apolar pesticides such as hexachlorobenzene and aldrin. Consequently, a final volume of 3 mL of hexane was chosen.

3.1.2. LC–MS/MS

Optimization of chromatographic conditions of multi-residue analysis is always challenging because of the diversity of physicochemical properties of target analytes. In this work, the most difficult part was to find optimum conditions for the class of avermectins. Indeed, these last compounds are macrocyclic lactones. They were rarely included in multi-residue analysis [30] and to the best of our knowledge had never been analyzed in honeybees although their potential toxicity towards insects is known [31].

The most suitable type of LC column for avermectins is C_8 column [32,33]. But a C_8 column did not retain polar compounds, such as methamidophos and carbendazim. A C_{18} column was also tested but led to poor resolution regarding avermectins. Then, a special type of C_{18} column, namely "Nucleodur Sphinx RP" from Macherey-Nagel, was tested. Its phase present a balanced ratio of

Table 3
GC–ToF acquisition method parameters.

Compound	t_R (min)	Quantification ion m/z	Mass window (Da)	Confirmation ion m/z	Mass window (Da)	Ion ratio
Dichlorvos	4.89	109.007	0.02	184.974	0.03	2.4
Ethoprophos	7.07	157.963	0.02	96.952	0.03	1.7
Cadusafos	7.32	158.972	0.02	157.963	0.03	1.3
Hexachlorobenzene	7.76	283.807	0.02	285.808	0.03	1.2
Dimethoate	7.77	87.016	0.03	93.007	0.03	1.6
Dichloran	7.78	175.993	0.03	177.993	0.05	2.2
Diazinon	7.80	152.096	0.02	304.105	0.03	0.7
Lindane	8.06	218.913	0.03	180.936	0.03	1.0
Vinclozoline	8.57	284.996	0.03	212.003	0.03	2.1
Chlorpyrifos-methyl	8.62	285.926	0.04	287.925	0.03	1.4
***Chlorpyriphos-methyl-d*$_6$**	8.62	291.963	0.04	293.960	0.03	1.4
Tolclofos-methyl	8.72	264.989	0.02	266.986	0.03	2.6
Chlorothalonil	8.86	265.878	0.03	263.887	0.03	1.4
Malathion-d$_7$	8.89	174.085	0.02	131.018	0.05	0.7
Malathion	8.93	173.079	0.03	127.037	0.03	1.8
Fenitrothion	9.02	277.017	0.03	260.017	0.03	1.9
Chlorpyrifos	9.16	198.913	0.02	196.920	0.03	0.9
Aldrin	9.25	262.862	0.02	260.855	0.03	1.6
Parathion	9.33	291.042	0.05	109.012	0.03	2.3
4,4′-dichlorobenzophenone	9.60	138.999	0.03	250.018	0.05	43.2
Phenthoate	9.75	273.985	0.06	124.980	0.03	3.6
Penconazole	9.86	248.098	0.02	158.978	0.05	1.3
Procymidone	9.96	283.017	0.03	285.011	0.03	1.7
Triadimenol	9.97	168.115	0.03	112.051	0.03	1.0
Paclobutrazol	10.34	236.059	0.03	125.015	0.03	3.1
Endosulfan I	10.49	236.842	0.03	240.893	0.04	1.1
Bupirimate	10.60	273.097	0.03	193.145	0.05	6.7
Buprofezine	10.65	105.056	0.02	106.066	0.03	2.4
o,p-DDD	10.85	235.007	0.02	237.005	0.03	1.4
Flusilazole	10.88	233.060	0.02	206.053	0.04	3.3
Dieldrin	10.88	262.859	0.03	264.861	0.03	1.7
Myclobutanil	10.99	179.032	0.04	150.010	0.03	2.7
Cyproconazole	11.29	222.043	0.03	138.999	0.05	2.4
Endosulfan II	11.69	236.842	0.03	240.906	0.05	0.7
Benalaxyl	11.70	148.112	0.02	206.117	0.03	4.4
Propiconazole	12.03	259.033	0.02	172.954	0.03	1.0
Propargite	12.04	135.081	0.04	173.094	0.05	1.7
p,p-DDT	12.05	235.008	0.05	237.001	0.03	1.7
Piperonyl Butoxide	12.09	176.083	0.03	177.087	0.05	3.2
Triphenylphosphate	12.14	326.070	0.03	325.057	0.05	1.2
Endosulfan sulphate	12.37	271.822	0.04	273.808	0.03	1.2
Tebuconazole	12.39	125.013	0.05	250.077	0.1	0.5
Bifenthrin	12.47	181.104	0.02	166.076	0.03	9.1
Bromopropylate	12.73	340.902	0.03	338.905	0.03	2.0
Methoxychlor	12.85	227.106	0.02	228.112	0.03	3.7
Phosmet	13.02	160.041	0.03	161.041	0.05	12.3
Pyriproxyfen	13.42	136.076	0.03	226.098	0.03	9.7
Cyhalothrin-lambda	13.49	181.066	0.02	197.036	0.05	1.6
Tetradifon	13.50	158.970	0.03	228.889	0.03	1.2
Phosalone	13.67	182.004	0.05	184.000	0.05	4.1
Fenarimol	14.11	138.992	0.02	219.037	0.03	1.0
Bitertanol	14.53	170.073	0.02	168.113	0.05	8.3
Permethrin	14.50	183.079	0.05	184.089	0.05	7.1
Coumaphos	15.05	362.019	0.05	225.986	0.03	2.4
Cyfluthrin	15.23	206.055	0.05	227.076	0.03	1.5
Cypermethrin	15.54	163.008	0.07	165.008	0.03	2.0
tau-Fluvalinate	17.19	250.060	0.05	252.060	0.03	10.9
Esfenvalerate	17.54	125.013	0.05	225.079	0.1	1.7
Deltamethrin	18.66	252.917	0.05	181.067	0.05	0.9

C_{18} and propylphenyl ligands which allows π–π interactions with aromatic compounds such as avermectins. A better resolution was obtained with this column and two particle size 1.8 and 3 μm were tested in order to increase it as much as possible. As the HPLC system used in this study does not bear high pressure, the flow was limited to 300 μL/min. But even if it is not the optimum flow with a 1.8 μm column, a gain of sensitivity was observed with the 1.8 μm column compare to the 3 μm, as seen in Fig. 3a. Finally, the composition of the mobile phase has also to be optimized. Fig. 3b presents the signal to noise of several compounds, with various composition of aqueous phase. For most of the compounds, the best sensitivity is obtained with formic acid. But to analyze avermectins, the presence of ammonium is necessary, especially for abamectin and ivermectin which are not ionized into $[M+H]^+$ but in $[M+NH_4]^+$. A compromise was found with 0,05% formic acid and ammonium formate at 0.3 mM.

Finally the injection solvent was optimized. For the most polar compounds that are firstly eluted, with an injection solvent composed of only acetonitrile or with a high percentage of acetonitrile, a lack of resolution is observed. Indeed, as the gradient of mobile phase started at 90% of water, it is necessary to have a minimum percentage of water of 90% to obtain a good peak shape. This injection solvent was kept for honey and honeybees. But in pollens extract, for the latest eluted compounds which are more apolar, the

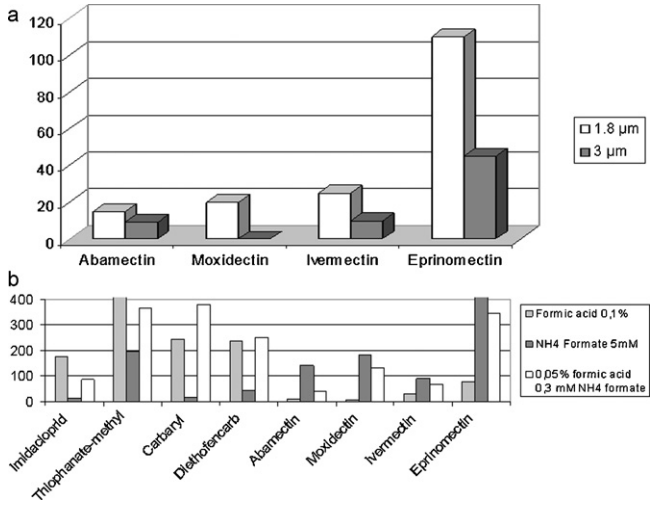

Fig. 3. Optimization of liquid chromatography for avermectins analysis: influence of column granulometry and buffer aqueous phase on signal to noise.

peak area was divided by a factor higher than 10 with an injection solvent which contains 90% of water. This difference may be due to interactions between apolar interferences of the matrix and apolar pesticides, when the percentage of water is too high. Consequently, to analyze pollens extracts, two injections were necessary, one at 90% of water for the earliest eluted compounds (i.e. $t_R < 15$ min (Table 2)) and one at 100% of acetonitrile.

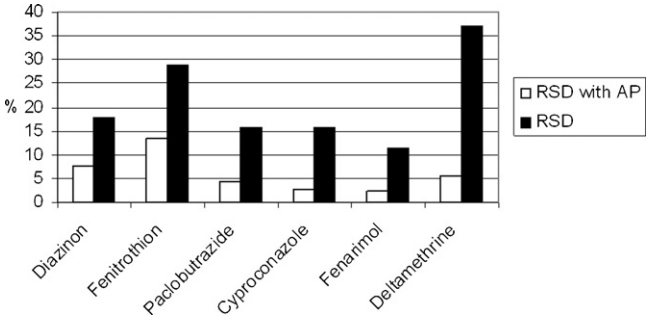

Fig. 5. Influence of the use of analyte protectants on the analysis of honeys of different floral origins by GC-ToF.

3.1.3. GC-ToF

To improve sensitivity, the noise has to be as low as possible which is not easy in GC-ToF since a reference standard has to be continuously injected in the source to insure good mass accuracy. Consequently, to calibrate the mass spectrometer, it is important to choose a compound which has a mass spectrum with abundant ions in a large mass range such as heptacosa. But during acquisitions, the use of heptacosa as a reference standard led to a high level of noise. To reduce this level, pentafluorochloro benzene was preferred as reference standard during acquisitions.

Another critical parameter regarding the noise is the mass window [20]. As an example, the impact of different values of mass window from 0.02 to 0.1 Da is shown in Fig. 4. This figure represents the extracted ion chromatogram of propiconazole (m/z 259.041) of

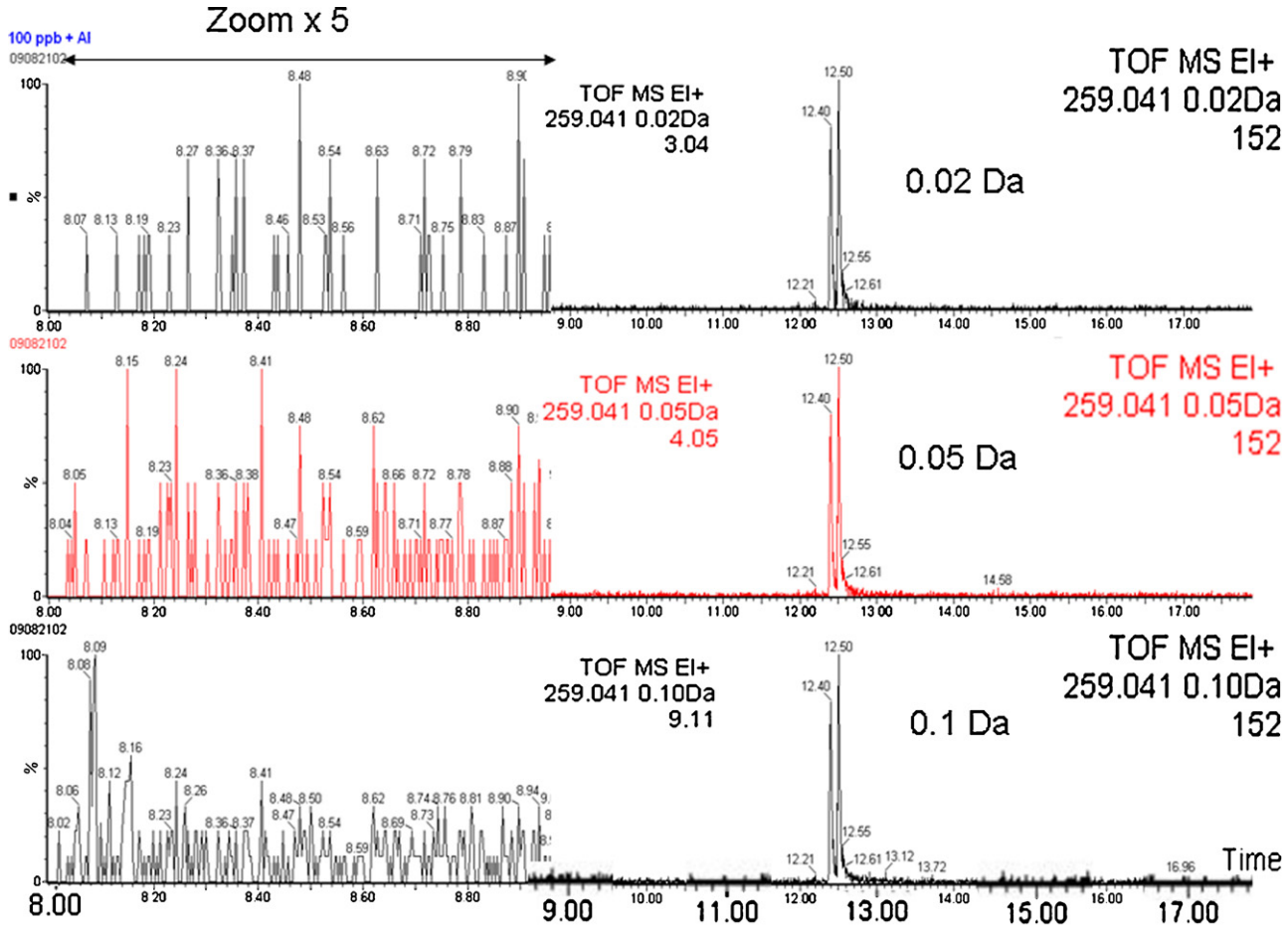

Fig. 4. Influence of mass window width in GC-ToF analysis.

Table 4
Method performance and validation: Limits of Detection (LOD) and Quantification (LOQ), recoveries (%), repeatability (RSD%) and Inter-day (Inter-d) precision (RSD%) obtained in honeys.

Compound	LC/GC	MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery ± RSD%			Inter-d precision		
					n = 3			RSD% *n* = 3		
					10 ng/g	30 ng/g	60 ng/g	C2	C4	C6
Metamidophos	LC	–	10.0	40.1	70 ± 8	75 ± 13	73 ± 2	9	24	25
Amitraze I	LC	–	10.0	37.0	82 ± 19	76 ± 20	75 ± 17	11	25	24
Carbendazime-d$_4$	LC	–			91 ± 5	85 ± 5	86 ± 3	11	23	23
Carbendazime	LC	> 100	0.5	4.0	103 ± 13	99 ± 7	95 ± 7	10	24	24
Methomyl	LC	20	0.1	10.5	98 ± 6	92 ± 1	94 ± 2	10	24	24
Thiamethoxam	LC	10	0.3	4.0	104 ± 5	94 ± 3	94 ± 2	10	24	24
Clothianidine	LC	10	0.3	4.3	88 ± 1	92 ± 5	89 ± 1	10	24	25
Imidacloprid	LC	50	0.2	3.9	103 ± 4	95 ± 8	93 ± 4	10	24	24
Amitraze II	LC	–	0.3	4.3	102 ± 6	94 ± 5	97 ± 3	11	24	23
Imazalil	LC	50	0.7	4.1	100 ± 6	94 ± 9	95 ± 3	10	24	23
Carbofuran	LC	–	0.03	3.8	103 ± 5	92 ± 5	95 ± 2	10	24	24
Thiophanate-methyl	LC	> 100	0.3	10.3	92 ± 34	60 ± 27	74 ± 28	10	24	23
Carbaryl	LC	–	0.1	3.8	99 ± 4	96 ± 1	96 ± 3	9	24	24
Carbaryl-d$_7$	LC	–			118 ± 4	86 ± 3	96 ± 3	–	–	–
Methiocarbe	LC	50	0.01	4.1	101 ± 2	92 ± 5	96 ± 2	10	23	25
Diethofencarbe	LC	–	0.04	3.8	100 ± 2	91 ± 6	95 ± 1	10	24	24
Cyproconazole	LC	50	0.2	3.5	100 ± 11	88 ± 5	96 ± 4	10	24	22
Triadimenol	LC	–	1.0	6.4	106 ± 4	89 ± 10	97 ± 4	9	21	22
***Malathion-d*$_7$**	LC	–			100 ± 1	94 ± 3	101 ± 4	10	24	25
Fenoxycarbe	LC	–	0.1	4.1	105 ± 3	90 ± 7	103 ± 7	11	24	21
Iprodione	LC	–	9.7	19.5	91 ± 11	104 ± 17	98 ± 5	12	24	28
Prochloraz	LC	–	0.2	11.4	103 ± 5	89 ± 5	94 ± 1	10	23	26
Clofentezine	LC	–	1.0	3.9	99 ± 12	98 ± 1	104 ± 20	12	22	11
Phoxim	LC	20	0.1	7.3	87 ± 6	91 ± 19	111 ± 13	10	23	14
Coumaphos	LC	–	0.3	3.0	102 ± 11	97 ± 12	108 ± 12	10	24	16
Chlorpyriphos-methyl	LC	–	0.4	5.2	89 ± 12	85 ± 15	115 ± 17	10	24	13
Piperonyl butoxide	LC	–	0.2	9.0	99 ± 4	93 ± 9	101 ± 7	10	24	20
Pyriproxyfen	LC	50	1.5	4.3	70 ± 11	85 ± 28	119 ± 20	11	22	12
Hexythiazox	LC	–	0.1	4.0	76 ± 3	83 ± 29	121 ± 20	10	24	14
Eprinomectin	LC	–	9.7	29.1	64 ± 19	68 ± 10	107 ± 7	11	22	21
Abamectin	LC	–	10.2	30.6	94 ± 34	81 ± 41	112 ± 17	10	24	27
Moxidectin	LC	–	18.7	nq	nd	±	6 ± 35	nd	12	21
Ivermectin	LC	–	23.5	70.4	nd	95 ± 15	120 ± 17	nd	24	11
Dichlorvos	GC	–	5.8	14.6	136 ± 55	96 ± 9	93 ± 21	36	16	19
Ethoprofos	GC	–	1.3	6.4	91 ± 4	102 ± 6	98 ± 8	15	8	10
Cadusaphos	GC	–	3.6	8.9	89 ± 2	98 ± 9	102 ± 6	12	7	14
Hexachlorobenzene	GC	–	0.2	3.9	81 ± 5	83 ± 5	94 ± 8	18	13	14
Dimethoate	GC	–	13.6	18.2	nd	136 ± 27	63 ± 30	nd	43	39
Dichloran	GC	–	19.0	57.0	nd	93 ± 23	119 ± 28	nd	20	26
Diazinon	GC	–	7.4	10.5	98 ± 10	100 ± 4	101 ± 5	13	6	7
Lindane	GC	10	1.2	3.4	104 ± 15	91 ± 9	98 ± 4	11	13	6
Chlorothalonil	GC	–	22.2	33.3	nd	23 ± 38	23 ± 40	nd	37	40
Chlorpyriphos-methyl	GC	–	0.1	5.2	84 ± 5	87 ± 5	102 ± 6	19	8	13
Tolclofos-methyl	GC	50	0.1	3.0	88 ± 3	87 ± 2	101 ± 6	22	8	12
Vinclozoline	GC	–	4.0	10.1	78 ± 7	96 ± 1	106 ± 9	25	16	23
Fenitrothion	GC	10	6.2	15.5	96 ± 3	108 ± 12	102 ± 5	18	14	8
***Malathion-d*$_7$**	GC	–			100 ± 8	92 ± 2	99 ± 4	12	1	5
Malathion	GC	20	5.5	11.7	119 ± 15	89 ± 7	100 ± 2	9	5	3
Chlorpyrifos	GC	–	3.2	8.0	93 ± 6	93 ± 3	103 ± 7	14	5	10
Parathion	GC	–	4.6	11.4	95 ± 8	106 ± 10	100 ± 5	19	16	12
Aldrin	GC	10	0.2	4.5	84 ± 6	86 ± 7	95 ± 7	22	2	12
4,4′-dichlorobenzophenone	GC	–	3.6	17.9	72 ± 7	123 ± 18	121 ± 4	47	40	41
Penconazole	GC	–	5.4	13.5	100 ± 2	98 ± 10	105 ± 7	10	8	13
Phenthoate	GC	–	0.3	14.4	103 ± 6	96 ± 5	101 ± 5	16	12	14
Triadimenol	GC	–	11.2	16.0	93 ± 14	115 ± 15	102 ± 4	18	4	8
Procymidone	GC	–	1.3	3.7	116 ± 23	96 ± 3	100 ± 5	24	9	11
Paclobutrazol	GC	–	7.5	16.2	104 ± 11	115 ± 19	98 ± 7	8	6	10
Endosulfan I	GC	10	5.1	12.7	77 ± 21	83 ± 7	104 ± 10	31	18	18
Bupirimate	GC	–	5.7	14.2	87 ± 7	103 ± 6	105 ± 6	17	9	14
Flusilazole	GC	50	4.1	10.3	105 ± 8	121 ± 18	97 ± 5	17	11	5
Myclobutanil	GC	–	10.7	32.2	67 ± 22	106 ± 12	105 ± 19	26	11	27
Buprofezine	GC	50	23.9	35.9	nd	104 ± 14	94 ± 5	nd	17	9
o,p-DDD	GC	50	0.3	3.7	90 ± 1	89 ± 3	101 ± 6	21	8	13
Dieldrin	GC	10	3.9	29.5	nd	90 ± 1	98 ± 13	nd	14	14
Cyproconazole	GC	50	4.0	10.1	94 ± 4	110 ± 12	104 ± 8	14	8	17
Endosulfan II	GC	10	10.3	30.9	87 ± 20	106 ± 6	104 ± 5	30	11	31
Benalaxyl	GC	–	5.7	14.2	84 ± 6	90 ± 3	102 ± 5	27	10	14
Propiconazole	GC	–	11.1	42.5	114 ± 9	116 ± 10	104 ± 4	18	5	6
Endosulfan sulphate	GC	10	1.2	3.4	95 ± 5	89 ± 9	104 ± 7	22	6	9
p,p-DDT	GC	50	21.9	65.8	nd	nd	91 ± 25	nd	nd	33
Propargite	GC	–	17.1	25.6	nd	115 ± 16	103 ± 5	nd	15	12

Table 4 (*Continued*)

Compound	LC/GC	MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery ± RSD%			Inter-d precision		
					n = 3			RSD% *n* = 3		
					10 ng/g	30 ng/g	60 ng/g	C2	C4	C6
Tebuconazole	GC	50	12.8	25.6	112 ± 35	118 ± 17	102 ± 3	44	23	4
Triphenylphosphate	GC	–	0.7	9.3	124 ± 32	96 ± 4	103 ± 4	26	9	12
Piperonyl Butoxide	GC	–	3.6	9.0	91 ± 4	122 ± 14	105 ± 6	5	4	12
Bifenthrine	GC	–	3.3	12.9	85 ± 2	96 ± 4	104 ± 5	22	7	13
Phosmet	GC	50	3.9	9.8	113 ± 14	116 ± 12	99 ± 10	26	36	25
Bromopropylate	GC	100	0.3	3.9	100 ± 5	105 ± 8	105 ± 7	7	5	10
Methoxychlor	GC	–	3.9	9.8	122 ± 15	126 ± 15	105 ± 14	40	40	39
Tetradifon	GC	–	3.3	5.7	75 ± 7	92 ± 2	101 ± 6	26	10	17
Phosalone	GC	50	4.1	10.2	111 ± 7	103 ± 9	101 ± 4	8	13	7
Pyriproxyfen	GC	50	7.5	10.7	84 ± 22	100 ± 6	102 ± 7	28	12	14
Cyhalothrin-lambda	GC	20	6.7	9.6	94 ± 23	100 ± 7	102 ± 6	4	3	8
Fenarimol	GC	–	8.1	16.3	nd	109 ± 1	97 ± 4	nd	10	13
Bitertanol	GC	–	11.0	16.5	110 ± 16	116 ± 26	105 ± 5	nd	5	11
Coumaphos	GC	–	3.7	9.2	128 ± 20	109 ± 14	100 ± 7	26	16	21
Permethrin	GC	–	4.3	10.7	49 ± 19	98 ± 5	89 ± 20	30	28	26
Cyfluthrin	GC	–	12.3	30.8	85 ± 10	110 ± 17	100 ± 7	25	13	9
Cypermethrin	GC	50	4.5	37.6	55 ± 27	102 ± 6	100 ± 11	37	35	39
tau-Fluvalinate	GC	10	3.7	9.1	87 ± 9	115 ± 7	104 ± 7	12	10	11
Esfenvalerate	GC	–	10.1	30.2	nd	102 ± 18	101 ± 7.3	nd	34	15
Deltamethrin	GC	–	6.9	17.3	104 ± 19	104 ± 11	106 ± 10	18	10	17

nd, non detected.

a standard at 100 μg/L with a zoom on the noise from 8 to 9 min. Thanks to the GC-ToF mass accuracy, it is possible to decrease the mass window, to extract procymidone ion, to 0.02 Da, without decreasing the signal. At the same time, reducing the mass window to 0.02 Da induces a decrease of the noise by a factor of 3. As seen in Table 2, for most of the compounds, good results are obtained with a mass window of 0.02 or 0.03 Da, but for some compounds it is too narrow leading to a decrease of the signal. Consequently it is very important to adjust the mass window for each mass.

3.1.4. Calibration and matrix effect

LC-ESI–MS/MS and GC-EI–MS are both subject to strong matrix effects (ME) which can significantly reduce or enhance the analyte response and lead to wrong quantification. No doubt that applying external calibration to complex matrices such as honey, honeybees and pollens would lead to high errors in quantification. Therefore three calibration strategies could be used in this study: internal standard, standard addition and matrix-matched calibrations. Internal standard calibration is the most efficient method to correct ME but it requires the use of isotope labeled standards of the target analytes [34]. Standard addition is also an efficient method but very time-consuming especially when a large number of samples have to be analyzed. Finally matrix-matched calibration is an efficient method to correct ME, only if a blank matrix is available and if the ion suppression/enhancement effects due to the matrix used for calibration are the same as the effects of the samples analyzed. Indeed, this last condition does not concerned honeybees but honeys and pollens samples can be very different between each other. Previous work on honeys [25] showed different intensities of ME depending on the floral origin of honey, chestnut honey being the type of honey that led to the strongest ME. Regarding pollen samples, their composition depends on the period of sampling, i.e. available flowers for foraging.

In this work, internal standard calibration could not be used because a lot of isotope-labeled standards corresponding to the target analytes were not commercially available or very expensive. One of the objectives of this study was to apply the analytical method to a large number of samples to get an overview of environmental contamination so standard addition calibration could not be used. Finally as blank matrix (see Section 2.2) was available, matrix-matched calibration was used. Even with the use of matrix-matched calibration, our experience on honeys suggests us to look for strategies to reduce ME. Dilution of the extract in LC–MS/MS was chosen. Regarding GC-ToF, the addition of AP based on the work published by Payá et al. [35] was tested. To check the efficiency of this strategy, three honeys of different floral origins were spiked at 60 ng/g, extracted and analyzed with and without AP. Fig. 5 presents the relative standard deviations obtained in these experiments for some compounds. It shows that the addition of AP decreased significantly matrix effects and allowed accurate quantification as discussed in the following section.

3.2. Method validation and performance

The purpose of method validation is to ensure that an analytical methodology is accurate, specific, reproducible and robust over the specified range that a compound will be analyzed [36]. Numerous guidelines have been published regarding method validation but some validation parameters are common. The method validation plan used in this study and calculation of different parameters are described in Section 2.6. Parameters determined were linearity, recovery, repeatability and intermediate precision, over a range of concentration between 4 and 60 ng/g for honeys and honeybees and between 10 and 150 ng/g for pollens due to lower sensitivity in this matrix and limit of quantification (LOQ) and limit of detection (LOD). Criteria of validation were as follow: regression coefficient higher than 0.99 for linearity, recoveries between 60 and 120%, RSD lower than 20% for repeatability and lower than 25% for intermediate precision. Results are presented in Tables 4–6 for honeys, honeybees and pollens respectively.

Over the range of concentrations chosen, all the targeted analytes were detected in honeys but 2 compounds (chlorothalonil and procymidone) in honeybees and 5 (dichloran and 4 avermectins) in pollens were not detected. Good linearity was observed for all compounds in the three matrices, even in GC-ToF analysis. Indeed, thanks to the Dynamic Range Enhancement, regression coefficients higher than 0.99 were obtained. Thanks to the extraction, no saturation of the detector was observed except in honeybees and pollens at the same retention time as procymidone and triadimenol which explain their no detection. Nevertheless, triadimenol signal was only interfered in honeybees in which it was still possible to analyze it by LC–MS/MS.

Table 5
Method performance and validation: limits of detection (LOD) and quantification (LOQ), recoveries (%), repeatability (RSD%) and inter-day (Inter-d) precision (RSD%) obtained in honeybees.

Compound	LC/GC	LOD (ng/g)	LOQ (ng/g)	Recovery ± RSD%			Inter-d precision		
				$n = 3$			RSD% $n = 3$		
				10 ng/g	30 ng/g	60 ng/g	C2	C4	C6
Metamidophos	LC	0.8	10.0	60 ± 22	67 ± 2	62 ± 1	9	19	19
Amitraze I	LC	18.5	27.8	nd	58 ± 20	57 ± 24	nd	17	20
Carbendazime-d_4	LC			69 ± 8	72 ± 3	74 ± 1	4	17	11
Carbendazime	LC	0.6	4.0	76 ± 19	74 ± 2	80 ± 4	10	16	12
Methomyl	LC	0.3	10.5	85 ± 10	84 ± 7	86 ± 3	12	16	10
Thiamethoxam	LC	0.6	4.0	75 ± 11	83 ± 3	71 ± 3	13	21	5
Clothianidine	LC	0.9	10.6	80 ± 15	75 ± 2	91 ± 2	16	16	11
Imidacloprid	LC	0.4	9.6	74 ± 27	77 ± 4	83 ± 2	12	11	3
Amitraze II	LC	4.3	10.8	80 ± 3	75 ± 4	81 ± 5	10	19	6
Imazalil	LC	1.4	10.2	80 ± 8	72 ± 6	78 ± 3	19	20	4
Carbofuran	LC	0.1	3.8	85 ± 5	79 ± 1	79 ± 4	13	10	6
Thiophanate-methyl	LC	4.1	10.3	64 ± 27	88 ± 5	63 ± 21	42	28	11
Carbaryl	LC	0.4	3.8	83 ± 16	82 ± 5	89 ± 2	2	12	5
Carbaryl-d_7	LC			78 ± 17	85 ± 7	71 ± 4	–	–	–
Methiocarbe	LC	0.4	10.3	80 ± 17	82 ± 8	80 ± 4	15	16	6
Diethofencarbe	LC	0.2	3.8	82 ± 11	84 ± 3	86 ± 4	12	4	5
Cyproconazole	LC	2.0	10.1	79 ± 7	78 ± 5	95 ± 3	7	12	9
Triadimenol	LC	9.6	16.0	83 ± 8	81 ± 3	90 ± 1	17	22	12
Malathion-d_7	LC			79 ± 4	85 ± 13	85 ± 5	11	4	10
Fenoxycarbe	LC	0.6	4.1	81 ± 18	106 ± 14	83 ± 19	15	16	19
Iprodione	LC	9.7	19.5	nd	85 ± 11	117 ± 6	24	17	28
Prochloraz	LC	0.7	4.6	85 ± 6	83 ± 11	99 ± 1	16	18	4
Clofentezine	LC	1.0	3.9	75 ± 20	104 ± 11	79 ± 20	27	29	20
Phoxim	LC	1.8	7.3	86 ± 17	100 ± 1	86 ± 19	1	25	23
Coumaphos	LC	0.4	3.7	90 ± 14	107 ± 13	93 ± 15	8	24	17
Chlorpyriphos-methyl	LC	5.2	13.0	79 ± 12	108 ± 12	95 ± 17	12	19	13
Piperonyl Butoxide	LC	0.1	3.6	90 ± 15	94 ± 11	85 ± 20	3	24	17
Pyriproxyfen	LC	2.1	4.3	85 ± 11	99 ± 11	87 ± 27	2	26	26
Hexythiazox	LC	0.8	3.9	77 ± 22	104 ± 12	88 ± 18	12	24	13
Eprinomectin	LC	3.9	9.7	66 ± 7	88 ± 8	87 ± 24	33	41	41
Abamectin	LC	10.2	20.4	nd	94 ± 4	84 ± 9	nd	23	44
Moxidectin	LC	3.7	9.4	81 ± 22	96 ± 13	79 ± 16	17	37	3
Ivermectin	LC	11.7	23.5	nd	114 ± 11	71 ± 22	nd	29	46
Dichlorvos	GC	5.8	14.6	46 ± 22	90 ± 11	101 ± 8	44	10	9
Ethoprofos	GC	0.6	3.6	88 ± 10	85 ± 5	91 ± 11	9	5	9
Cadusaphos	GC	1.0	8.9	85 ± 7	83 ± 7	92 ± 9	6	6	7
Hexachlorobenzene	GC	0.8	3.9	36 ± 12	41 ± 8	43 ± 9	17	6	9
Dimethoate	GC	3.6	27.3	125 ± 27	92 ± 8	80 ± 6	25	11	8
Dichloran	GC	38.0	nd	nd	85 ± 17	98 ± 18	nd	14	13
Diazinon	GC	6.3	14.7	83 ± 22	88 ± 4	86 ± 6	19	5	8
Lindane	GC	1.0	5.2	89 ± 8	89 ± 7	89 ± 8	10	10	8
Chlorothalonil	GC	nd	nd	nd	nd	nd	nd	nd	nd
Chlorpyriphos-methyl	GC	0.3	5.2	88 ± 1	86 ± 8	87 ± 8	8	8	7
Tolclofos-methyl	GC	0.3	3.0	92 ± 3	86 ± 7	89 ± 8	8	6	7
Vinclozoline	GC	4.0	10.1	61 ± 5	81 ± 16	94 ± 8	19	13	6
Fenitrothion	GC	1.1	6.2	94 ± 6	98 ± 4	88 ± 6	10	10	7
Malathion-d_7	GC			79 ± 8	92 ± 6	86 ± 1	16	9	6
Malathion	GC	7.8	15.6	78 ± 44	87 ± 8	94 ± 10	38	16	8
Chlorpyrifos	GC	0.8	3.2	85 ± 11	84 ± 7	85 ± 11	8	5	8
Parathion	GC	1.6	8.0	90 ± 6	91 ± 6	85 ± 9	5	9	8
Aldrin	GC	4.5	22.3	34 ± 40	46 ± 9	50 ± 9	30	7	7
4,4′-dichlorobenzophenone	GC	3.6	9.0	94 ± 10	82 ± 5	89 ± 6	15	4	8
Penconazole	GC	1.9	13.5	84 ± 12	88 ± 18	85 ± 14	11	17	10
Phenthoate	GC	0.6	14.4	92 ± 6	91 ± 6	93 ± 9	10	7	8
Triadimenol	GC	nd	nd	nd	nd	nd	nd	nd	nd
Procymidone	GC	nd	nd	nd	nd	nd	nd	nd	nd
Paclobutrazol	GC	4.3	10.8	94 ± 14	102 ± 16	80 ± 15	30	31	29
Endosulfan I	GC	5.1	38.0	122 ± 10	116 ± 20	62 ± 17	28	46	34
Bupirimate	GC	5.7	14.2	108 ± 11	99 ± 12	89 ± 12	22	18	20
Flusilazole	GC	2.1	10.3	98 ± 16	91 ± 14	90 ± 10	15	11	9
Myclobutanil	GC	10.7	21.4	nd ± nd	95 ± 13	82 ± 16	nd	26	20
Buprofezine	GC	23.9	71.8	nd	54 ± 91	58 ± 34	nd	62	40
o,p-DDD	GC	3.7	9.2	98 ± 15	91 ± 11	85 ± 13	13	14	14
Dieldrin	GC	3.9	9.8	102 ± 30	86 ± 18	77 ± 12	28	15	11
Cyproconazole	GC	1.2	4.0	93 ± 13	93 ± 8	92 ± 11	10	9	9
Endosulfan II	GC	10.3	30.9	68 ± 24	80 ± 13	63 ± 39	27	25	33
Benalaxyl	GC	5.7	28.4	nd	81 ± 9	88 ± 5	nd	12	5
Propiconazole	GC	2.6	17.0	95 ± 5	88 ± 7	88 ± 8	4	6	7
Endosulfan sulphate	GC	5.1	8.4	114 ± 22	100 ± 15	82 ± 14	17	20	13
p,p-DDT	GC	1.3	4.4	84 ± 3	82 ± 7	75 ± 11	17	10	12
Propargite	GC	11.9	34.1	nd	126 ± 16	68 ± 10	nd	22	26

Table 5 (*Continued*)

Compound	LC/GC	LOD (ng/g)	LOQ (ng/g)	Recovery ± RSD%			Inter-d precision		
				n = 3			RSD% *n* = 3		
				10 ng/g	30 ng/g	60 ng/g	C2	C4	C6
Tebuconazole	GC	5.1	17.9	90 ± 16	100 ± 15	90 ± 7	22	20	10
Triphenylphosphate	GC	0.4	9.3	91 ± 5	92 ± 3	93 ± 8	11	5	7
Piperonyl Butoxide	GC	1.1	3.6	88 ± 5	83 ± 8	87 ± 9	3	7	7
Bifenthrine	GC	1.3	5.1	78 ± 2	77 ± 4	76 ± 10	8	5	8
Phosmet	GC	9.8	19.7	nd	109 ± 11	56 ± 24	nd	38	31
Bromopropylate	GC	0.2	3.9	88 ± 7	89 ± 5	90 ± 8	7	5	7
Methoxychlor	GC	1.2	3.9	102 ± 1	91 ± 4	85 ± 8	9	7	9
Tetradifon	GC	3.3	8.2	102 ± 9	87 ± 11	86 ± 9	16	8	7
Phosalone	GC	4.1	10.2	92 ± 35	93 ± 6	75 ± 3	28	9	8
Pyriproxyfen	GC	4.3	10.7	76 ± 15	83 ± 4	86 ± 7	17	3	5
Cyhalothrin-lambda	GC	3.8	9.6	73 ± 6	94 ± 8	86 ± 9	11	11	6
Fenarimol	GC	3.3	8.1	89 ± 5	87 ± 10	88 ± 9	9	9	6
Bitertanol	GC	1.1	4.4	95 ± 6	87 ± 7	90 ± 8	6	8	6
Coumaphos	GC	3.7	9.2	90 ± 14	100 ± 3	81 ± 7	19	17	17
Permethrin	GC	4.3	10.7	89 ± 6	80 ± 9	79 ± 14	9	9	10
Cyfluthrin	GC	12.3	61.5	85 ± 4	89 ± 12	87 ± 4	7	9	3
Cypermethrin	GC	4.5	27.1	97 ± 18	94 ± 8	95 ± 4	32	28	36
tau-Fluvalinate	GC	3.7	9.1	93 ± 4	88 ± 5	92 ± 13	4	6	9
Esfenvalerate	GC	10.1	30.2	105 ± 3	95 ± 9	87 ± 12	23	17	15
Deltamethrin	GC	4.6	16.2	81 ± 6	98 ± 14	81 ± 15	26	20	15

nd, non detected.

Most RSD of repeatability respect the validation criteria for the three matrices: 18, 18 and 9 compounds present RSD higher than 20% respectively for honey, honeybee, and pollen. Most RSD of intermediate precision respect the validation criteria for honey and honeybees: 16 and 18 compounds present RSD higher than 25% respectively for honey and honeybee. But, regarding pollens, in this complex matrix, 23 compounds present RSD of intermediate precision superior to 30%. The developed extraction method led to good recoveries. Six compounds presented recoveries lower than 60%: Amitraz I, hexachlorobenzene, aldrin in honeybees and pollens and dichlorvos, imazalil, thiophanate-methyl only in pollens. Hexachlorobenzene and aldrin are very apolar compounds (Log Koc > 4, 5 [37]) and a decrease in recovery was observed as soon as hexane was added in the extract. The recovery of thiophanate-methyl can be explained by a relative basicity of pollen matrix to which this compound is sensitive. Concerning Amitraz I, dichlorvos and imazalil, recoveries are higher than 40%. Despite theses recoveries, good RSD of repeatability was obtained and accurate quantification of these compounds was still possible.

Seven compounds were troublesome to analyze in the 3 matrices: avermectins by LC–MS/MS and dichloran, dimethoate, chlorothalonil, by GC-ToF. Avermectins' analysis in a multi-residue method is difficult for two reasons. First, as described in Section 3.1.2, the best sensitivity for these compounds is obtained with the presence of ammonium contrary to the other compounds. Secondly, due to their hydrophobic property, they present an affinity to apolar interferences of the matrix that's why none of these compounds were detected in pollens. Nevertheless, acceptable results were obtained in honeybees and in honeys except for moxidectin. Regarding GC-ToF sensitivity of dichloran was quite poor: hence it was possible to analyze it at concentrations higher than 30 ng/g in honeys but in more complex matrices such as honeybees and pollens, it was not possible. Dimethoate and chlorothalonil are well-known [21] for their difficulty to be analyzed because of poor sensitivity and degradation. Therefore it would be interesting to analyze also their degradation products.

Regarding the method performance, eight analytes, in bold in Tables 4–6, were analyzed by LC–MS/MS and GC-ToF. Except for chlorpyrifos-methyl and cyproconazole in LC–MS/MS, LOD obtained with LC–MS/MS are generally lower than with GC-ToF. Besides LOD and LOQ were compared to actual MRL [28] required by the EU directives in honeys and pollens. MRL available are also listed in Tables 4–6. Regarding honeys, LOQ are all lower than MRL except LOQ of fenitrothion, endosulfan I, dieldrin, endosulfan II, and p,p-DDT. Considering pollens, LOQ obtained with LC–MS/MS technique are lower than MRL except for clothianidine, and with GC-ToF, 11 LOQ are higher than MRL. Consequently, the method performance is in concordance with the EU directives concerning the analysis by LC–MS/MS but the sensitivity of GC-ToF is not good enough for some compounds. Concerning honeybees, there is no EU directive, but LOD are lower than 7 ng/g for most compounds, with a highest LOD at 38 ng/g. The method performance is so compatible with analysis of environmental contaminants. In conclusion, this validation study shows the difficulties encountered to analyze compounds with very different physicochemical properties; the developed method allows a global view of the three matrices contamination at low concentration but accurate quantification is not possible for all the compounds.

3.3. Application to real samples

A large sampling of honeybees, trap pollen and honey was performed in 2008 and 2009 but their analysis was done in 2009 and 2010. Consequently it was important to check that no degradation could occur between sampling and analysis, so the stability of contaminants in the three matrices at −18 °C was studied on 2 years: firstly, 14 samples of each matrix were spiked at 1 and 5 μg/g. Six month later 6 of these samples (2 of each matrix) were extracted and analyzed and the others stored at −18 °C. Then the same experiment was repeated every 3 month for 2 years. Finally, no degradation was observed in these conditions of storing during 2 years.

As detailed in Section 2.6.3, instrumental and extraction performances were checked with the use of isotope labeled standards and Quality Control samples. For compounds that can be analyzed by LC–MS/MS and GC-ToF, results obtained with the two techniques were compared and a good agreement was observed.

Table 7 presents compounds detected in 142 samples of honeys, 145 samples of honeybees and 130 samples of pollens. In total, 36 compounds were detected but only 10 compounds were detected in all the matrices: metabolites of amitraz, carbendazim, thiophanate-methyl, coumaphos, flusilazole, triphenylphosphate, phosmet and

Table 6
Method performance and validation: limits of detection (LOD) and quantification (LOQ), recoveries (%), repeatability (RSD%) and inter-day (Inter-d) precision (RSD%) obtained in pollens.

Compound	LC/GC	MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery ± RSD%			Inter-d precision		
					$n = 3$			RSD% $n = 3$		
					25 ng/g	75 ng/g	150 ng/g	C2	C4	C6
Metamidophos	LC	–	2.2	25.1	62 ± 10	66 ± 8	63 ± 2	38	24	36
Amitraze I	LC	–	46.3	69.4	nd	40 ± 9	39 ± 4	nd	45	23
Carbendazime-d_4	LC	–			70 ± 2	68 ± 2	63 ± 4	36	21	37
Carbendazime	LC	> 100	0.1	1.0	81 ± 3	76 ± 4	68 ± 1	40	27	42
Methomyl	LC	20	0.8	3.2	91 ± 8	84 ± 5	86 ± 1	28	18	32
Thiamethoxam	LC	10	2.0	8.5	85 ± 10	91 ± 19	74 ± 5	9	13	24
Clothianidine	LC	10	1.4	17.0	93 ± 4	94 ± 14	81 ± 7	37	9	36
Imidacloprid	LC	50	2.6	12.0	85 ± 11	89 ± 3	75 ± 7	21	12	21
Amitraze II	LC	–	8.1	17.3	89 ± 7	87 ± 6	84 ± 2	26	14	24
Imazalil	LC	50	6.9	25.5	56 ± 16	58 ± 4	51 ± 8	31	31	39
Carbofuran	LC	–	0.4	1.0	88 ± 8	91 ± 12	85 ± 1	18	11	14
Thiophanate-methyl	LC	> 100	16.5	51.5	26 ± 10	14 ± 16	22 ± 12	nd	34	25
Carbaryl	LC	–	0.7	1.2	92 ± 2	90 ± 7	87 ± 6	16	17	18
Carbaryl-d_7	LC	–			88 ± 8	84 ± 4	86 ± 3	–	–	–
Methiocarbe	LC	50	0.2	0.5	108 ± 10	98 ± 13	82 ± 6	5	13	12
Diethofencarbe	LC	–	0.6	1.9	90 ± 8	88 ± 7	86 ± 2	27	27	29
Cyproconazole	LC	50	3.0	10.1	80 ± 20	93 ± 20	80 ± 4	13	21	22
Triadimenol	LC	–	5.6	19.2	70 ± 18	92 ± 14	82 ± 5	43	25	29
Malathion-d_7	LC	–			104 ± 8	85 ± 5	80 ± 6	–	–	–
Fenoxycarbe	LC	–	1.0	3.3	80 ± 8	80 ± 5	89 ± 2	10	2	1
Iprodione	LC	–	15.6	48.7	61 ± 6	86 ± 20	88 ± 15	15	32	27
Prochloraz	LC	–	4.9	14.8	91 ± 5	70 ± 11	63 ± 5	10	8	7
Clofentezine	LC	–	9.7	48.6	69 ± 13	64 ± 2	60 ± 7	7	26	16
Phoxim	LC	20	2.7	15.5	106 ± 5	84 ± 3	83 ± 4	4	7	10
Coumaphos	LC	–	1.8	6.0	93 ± 9	89 ± 6	84 ± 3	6	10	10
Chlorpyriphos-methyl	LC	–	15.6	52.0	8325	78 ± 17	78 ± 3	20	8	2
Piperonyl Butoxide	LC	–	6.8	22.6	106 ± 9	96 ± 6	80 ± 12	9	3	14
Pyriproxyfen	LC	50	2.1	8.6	92 ± 7	80 ± 9	82 ± 5	10	4	14
Hexythiazox	LC	–	4.8	10.2	94 ± 15	80 ± 11	75 ± 11	12	8	5
Eprinomectin	LC	–	nd	nd	nd	nd	nd	nd	nd	nd
Abamectin	LC	–	nd	nd	nd	nd	nd	nd	nd	nd
Moxidectin	LC	–	nd	nd	nd	nd	nd	nd	nd	nd
Ivermectin	LC	–	nd	nd	nd	nd	nd	nd	nd	nd
Dichlorvos	GC	–	14.6	21.9	54 ± 14	49 ± 2	61 ± 17	17	12	9
Ethoprofos	GC	–	3.2	13.7	72 ± 8	77 ± 1	79 ± 4	6	10	2
Cadusaphos	GC	–	8.9	22.3	70 ± 13	72 ± 2	72 ± 4	5	12	6
Hexachlorobenzene	GC	–	9.7	24.3	25 ± 67	21 ± 18	23 ± 15	18	17	26
Dimethoate	GC	–	9.1	45.4	nd	78 ± 18	99 ± 5	nd	35	21
Dichloran	GC	–	47.5	nd	nd	86 ± 12	126 ± 7	nd	38	15
Diazinon	GC	–	10.5	26.3	99 ± 21	85 ± 5	103 ± 10	32	25	26
Lindane	GC	10	8.6	17.2	84 ± 12	84 ± 12	85 ± 4	25	15	23
Chlorothalonil	GC	–	11.1	22.2	74 ± 7	76 ± 24	78 ± 11	50	55	50
Chlorpyriphos-methyl	GC	–	1.3	19.5	79 ± 1	74 ± 3	78 ± 4	7	8	3
Tolclofos-methyl	GC	50	1.1	11.4	122 ± 54	63 ± 35	77 ± 3	40	44	30
Vinclozoline	GC	–	1.5	12.6	57 ± 9	94 ± 7	87 ± 2	26	9	11
Fenitrothion	GC	10	3.9	19.4	82 ± 7	79 ± 5	83 ± 4	19	25	26
Malathion-d_7	GC	–			119 ± 18	75 ± 13	84 ± 1	nd	25	12
Malathion	GC	20	39.1	58.6	nd	96 ± 15	85 ± 3	nd	40	11
Chlorpyrifos	GC	–	8.0	20.0	55 ± 16	78 ± 4	74 ± 2	28	10	3
Parathion	GC	–	11.4	17.1	86 ± 2	84 ± 3	82 ± 3	12	11	12
Aldrin	GC	10	11.1	13.9	43 ± 10	42 ± 9	43 ± 2	9	10	5
4,4′-dichlorobenzophenone	GC	–	3.1	11.2	78 ± 13	85 ± 6	76 ± 3	16	17	10
Penconazole	GC	–	6.7	16.9	76 ± 4	83 ± 6	81 ± 3	19	27	18
Phenthoate	GC	–	1.4	14.4	81 ± 3	87 ± 3	93 ± 4	9	12	8
Triadimenol	GC	–	16.0	32.0	117 ± 7	89 ± 8	82 ± 7	42	11	36
Procymidone	GC	–	nd	nd	nd	nd	nd	nd	nd	nd
Paclobutrazol	GC	–	3.8	10.8	79 ± 5	92 ± 1	79 ± 8	5	14	5
Endosulfan I	GC	10	12.7	31.7	62 ± 32	66 ± 15	68 ± 9	11	12	7
Bupirimate	GC	–	2.8	21.4	101 ± 12	85 ± 4	86 ± 1	23	13	10
Flusilazole	GC	50	3.6	15.5	82 ± 6	80 ± 7	81 ± 4	7	3	7
Myclobutanil	GC	–	10.7	37.5	82 ± 11	87 ± 9	84 ± 2	7	4	9
Buprofezine	GC	50	29.9	59.9	nd	56 ± 7	77 ± 2	nd	14	6
o,p-DDD	GC	50	4.6	13.9	83 ± 6	71 ± 5	79 ± 5	35	19	10
Dieldrin	GC	10	9.8	24.6	96 ± 17	77 ± 12	71 ± 3	10	16	9
Cyproconazole	GC	50	10.1	50.4	84 ± 29	89 ± 14	79 ± 4	55	23	8
Endosulfan II	GC	10	15.5	51.5	85 ± 21	79 ± 1	81 ± 3	12	5	8
Benalaxyl	GC	–	21.3	42.7	76 ± 14	91 ± 9	86 ± 3	22	12	6
Propiconazole	GC	–	4.3	85.1	78 ± 7	88 ± 7	82 ± 4	4	16	13
Endosulfan sulphate	GC	10	8.4	21.1	91 ± 18	85 ± 9	93 ± 4	15	22	15
p,p-DDT	GC	50	11.0	27.4	72 ± 4	75 ± 4	72 ± 3	10	7	6
Propargite	GC	–	42.7	128.0	nd	68 ± 24	82 ± 5	nd	25	10

Table 6 (*Continued*)

Compound	LC/GC	MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery ± RSD%			Inter-d precision		
					n = 3			RSD% *n* = 3		
					25 ng/g	75 ng/g	150 ng/g	C2	C4	C6
Tebuconazole	GC	50	12.8	38.4	76 ± 13	77 ± 10	88 ± 9	42	23	19
Triphenylphosphate	GC	–	0.5	9.3	87 ± 5	97 ± 5	91 ± 3	23	14	13
Piperonyl butoxide	GC	–	9.0	45.2	76 ± 4	81 ± 3	81 ± 2	18	13	11
Bifenthrine	GC	–	4.5	19.3	72 ± 2	72 ± 9	67 ± 2	19	16	8
Phosmet	GC	50	14.8	24.6	108 ± 4	65 ± 35	99 ± 16	44	48	40
Bromopropylate	GC	100	1.0	14.5	87 ± 11	85 ± 8	79 ± 4	20	18	14
Methoxychlor	GC	–	2.0	9.8	82 ± 12	81 ± 2	85 ± 2	22	4	7
Tetradifon	GC	–	8.2	20.4	67 ± 22	78 ± 6	85 ± 3	9	7	8
Phosalone	GC	50	10.2	15.4	83 ± 5	77 ± 11	89 ± 6	9	10	17
Pyriproxyfen	GC	50	10.7	21.5	71 ± 3	80 ± 3	79 ± 2	18	10	5
Cyhalothrin-lambda	GC	20	23.9	47.9	91 ± 13	73 ± 13	89 ± 0	8	22	19
Fenarimol	GC	–	20.3	28.4	105 ± 12	83 ± 14	70 ± 6	36	12	9
Bitertanol	GC	–	3.9	16.5	76 ± 8	87 ± 5	81 ± 2	15	10	9
Coumaphos	GC	–	4.6	18.4	95 ± 6	79 ± 15	95 ± 6	35	36	29
Permethrin	GC	–	5.3	32.1	72 ± 7	79 ± 10	80 ± 3	23	14	6
Cyfluthrin	GC	–	76.9	230.7	80 ± 22	62 ± 12	86 ± 1	40	28	17
Cypermethrin	GC	50	56.4	169.1	nd	74 ± 11	90 ± 8	nd	12	17
tau-Fluvalinate	GC	10	4.6	22.8	79 ± 8	87 ± 2	89 ± 1	12	7	8
Esfenvalerate	GC	–	25.1	150.9	89 ± 9	56 ± 3	90 ± 5	34	31	31
Deltamethrin	GC	–	28.9	57.8	77 ± 23	69 ± 16	88 ± 8	22	40	35

nd, non detected.

tau-fluvalinate. Amitraz, coumaphos and tau-fluvalinate are pesticides that can be used by beekeepers. Triphenylphosphate is not a pesticide but a ubiquitous pollutant that was also detected in water and air by Reemtsma et al. [38]. Considering carbendazim, flusilazole and carbaryl, concentrations found in pollens were significantly higher than in the other matrices. This can be explained by the fact that pollen gives a view of contamination in the shortest period (around 3 days) and so this matrix allows the determination of acute contaminations. Compounds detected and the range of concentrations are comparable with other studies [6,39]. In con-

Table 7
List of the 36 compounds detected in real samples: percentage of samples contaminated, and the maximum concentration quantified.

Compound	Honey		Honeybees		Pollen	
	% Samples detected	ng/g Max quantified	% Samples detected	ng/g Max quantified	% Samples detected	ng/g Max quantified
Amitraze I	4	26	5	30	2	115
Carbendazime	64	88	44	66	34	2595
Imidacloprid	2	<LOQ	nd	nd	1	<LOQ
Amitraze II	68	116	15	40	15	129
Imazalil	4	<LOQ	nd	nd	nd	nd
Carbofuran	2	<LOQ	nd	nd	2	2
Thiophanate-methyl	1	5	6	2419	2	3674
Carbaryl	6	<LOQ	2	<LOQ	8	15
Diethofencarbe	1	<LOQ	nd	nd	1	3
Cyproconazole	11	4	nd	nd	1	22
Fenoxycarbe	1	<LOQ	1	20	nd	nd
Iprodione	nd	nd	nd	nd	1	<LOQ
Prochloraz	1	<LOQ	nd	nd	nd	nd
Phoxim	2	<LOQ	nd	nd	nd	nd
Coumaphos	77	29	19	47	10	40
Chlorpyriphos-methyl	1	<LOQ	nd	nd	nd	nd
Piperonyl Butoxide	8	<LOQ	2	<LOQ	nd	nd
Pyriproxyfen	4	<LOQ	1	<LOQ	5	<LOQ
Hexythiazox	1	<LOQ	1	<LOQ	nd	nd
Dimethoate	nd	nd	nd	nd	1	<LOQ
Diazinon	2	14	1	<LOQ	nd	nd
Vinclozoline	nd	nd	nd	nd	2	70
Chlorpyrifos	nd	nd	4	180	4	140
Bupirimate	1	<LOQ	nd	nd	1	<LOQ
Flusilazole	2	<LOQ	2	<LOQ	2	52
Buprofezine	1	43	nd	nd	nd	nd
Dieldrin	nd	nd	nd	nd	1	<LOQ
Endosulfan II	1	<LOQ	nd	nd	nd	nd
Benalaxyl	nd	nd	1	<LOQ	nd	nd
Propiconazole	nd	nd	1	<LOQ	nd	nd
Tebuconazole	1	<LOQ	nd	nd	nd	nd
Triphenylphosphate	2	<LOQ	24	62	10	<LOQ
Phosmet	13	42	3	62	7	78
Phosalone	nd	nd	1	<LOQ	nd	nd
Cypermethrin	1	<LOQ	1	49	nd	nd
***tau*-Fluvalinate**	5	30	7	53	3	85

nd, non detected.

clusion, this study allows the comparison of contamination of three matrices: honey is the matrix in which compounds were detected the most frequently in the lowest concentration and pollen the matrix in which compounds were detected the least frequently in the highest concentration, honeybees being the intermediate matrix.

4. Conclusion

Combination of a simple extraction method like QuEChERS with sensitive analytical techniques LC–MS/MS and GC-ToF made possible the accurate quantification of contaminants in honey, honeybees and pollen at concentrations as low as 10 ng/g. However, our analytical method comprises certain limitations: first, it does not allow the analysis of avermectins in pollens and these compounds would need the development of a specific method. Second, this study only consider a small number of pesticides metabolites, whereas certain molecules, such as imidacloprid [40] have little chance of being found in their original state, particularly in honeybees. Consequently, there is a need for extensive studies on pesticide mechanisms of metabolism in honeybees, in order to determine the major metabolites and include them in future multiresidue analytical methods. Finally, other apiarian matrices need to be studied. Royal jelly and propolis are increasingly included in cosmetics and foods respectively, making the presence of pesticide in them a potential threat to human health. In addition, wax and beebread are also interesting matrices: since waxes are always recycled by beekeepers, they are a possible source of contamination; beebread should also be studied for two reasons: it is the only source of protein for honeybees in winter and it contains pollens brought by honeybees during the rest of the year. Therefore, it could be used as a long-term surveillance matrix.

To conclude, even though the number of pesticides included in our method is not the most comprehensive found in the literature, to our knowledge our analytical method was developed for one of the largest number of families of contaminants. An extensive application of the final method revealed the presence of a large number of chemical contaminants. Concentrations found are mostly lower than 100 ng/g but some acute contaminations higher than 1 μg/g were detected. These results confirm the potential use of honeybees as environmental bioindicator.

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