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Matrix solid-phase dispersion associated to gas chromatography for the assessment in honey bee of a group of pesticides of concern in the apicultural field

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ABSTRACT

A method based on matrix solid-phase dispersion (MSPD) associated to gas chromatography-flame photometric detection (GC-FPD), GC-electron capture detection (GC-ECD) and GC-mass spectrometry (GC–MS) for confirmation purposes, was developed for the determination of a representative group of twelve pesticides in honeybee with particular concern in the apicultural field (fipronil, thiamethoxam, acetamiprid, acrinathrin, metamidophos, dimetoathe, diazinon, chlorpyrifos, methidathion, profenophos, azinphos methyl and coumaphos). Factors influencing the extraction efficiency of MSPD were investigated and optimized through response surface method. The use of octadecylsilyl (C18) sorbent combined with a florisil clean-up and acetonitrile-methanol (99:1) elution was the optimal condition for the extraction of the selected pesticides. Under this condition the recovery of pesticides at the limit of quantification of the method (0.007 to 0.050 $\mu g\,g^{-1}$) ranged from 68 to 102% with RSDs for within-laboratory reproducibility ≤20%. The proposed method was applied to the analysis of honeybees collected in 68 field hives from areas of great apicultural and agricultural development in central Chile. In 65% of these samples eight different pesticides were detected. Pesticides most frequently found were chlorpyrifos (34% of the samples, <0.017–0.067 $\mu g\,g^{-1}$), acrinathrin (32% of the samples, <0.020–0.026 $\mu g\,g^{-1}$) and diazinon (10% of the samples at values <0.015 $\mu g\,g^{-1}$). The incidence of these pesticides in bees can be related to their high employ in central Chile, use to combat the varroosis in hives and hydrophobicity.

1. Introduction

Honeybees (*Apis mellifera*) are the natural and economically most important group of pollinators worldwide; 35% of the world food crop production depends on pollinators [1]. The decline of pollinating species, which has grown over the last decades, may lead to a parallel decrease of plant species or vice versa. More specifically, there is a great concern about the decline of honeybee in several parts of the world. In this sense, the worldwide fact most recently observed is the acute depopulation of hives (a honeybee vanishing), which has been called Čolony Collapse Disorder (CCD)ťhat was first named in 2007 [2]. Along with the decline in honey production, the loss of pollinators has had a negative impact on the reproduction of multiple crops [3]. The possible causes of CCD include parasites, bacteria, fungi, viruses, pesticides, deficient nutrition, management practices and environmental factors. However, recent studies postulate that a combination of these factors could be responsible, principally parasites and the exposure to cocktails of agrochemical as pesticides of different class [4–8]. Although no single pesticide has been shown to cause CCD, the additive and synergistic effects of multiple pesticide exposures may contribute to declining honey bee health [9–12].

It is rare to find exposure to a single pesticide in bees; usually they are exposed to various insecticides, fungicides, and acaricides, among others. Between them, neonicotinoid, organophosphorus pesticides (OPPs) and halogenated pesticides (HPs) are included. In recent years it has been postulated that neonicotinoid pesticides could be a trigger of CCD. Some authors have done a wide overview on the effect of neonicotinoids on bees and their relationship with CCD [1,13,14]. Thiamethoxam and acetamiprid belongs to this group of pesticides where the first one is highly toxic for bees and bumble bee [15,16]; while acetamiprid is less toxic compared to the other neonicotinoids [17]. Recently it has been reported that thiamethoxam significantly reduce the reproductive capacity of male

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honeybees (drones) by decrease of sperm viability and living sperm quantity [18]. On the other hand, fipronil like the neonicotinoids is considered one of the probable causes of CCD by increasing the mortality of honeybees previously infected by *Nosema ceranae* [19,20] and inducing behaviors that reduce foraging efficiency [21]. Otherwise, residues of OPPs have been frequently detected in matrices of honeybee colonies, and their potential risk to the colonies reported [22–25]. While results of some studies suggest that OPPs would not be directly involved with the cause of colony losses [24], they may be interacting with other stressors or in combination with other pesticides. An example is the synergistic effect observed between the acaricide coumaphos and the fungicide chlorothalonil on bee larvae mortality [12]. It has also been observed that bumble bee colonies exposed to chlorothalonil produced fewer workers, lower total bee biomass, and had lighter mother queens than control colonies [26]. As a result, the use of pesticides in agriculture has indubitable repercussions on the environment and a highly probable negative impact on the sustainability of bee colonies, which has become a serious environmental concern that need a continuous monitoring through adequate analytical method.

The determination of volatile pesticides in honey bees has been performed by gas chromatography (GC) coupled to tandem mass spectrometry (GC–MS/MS) [27,28] or to single mass spectrometry (GC–MS) [29] for qualitative and quantitative purposes. Although these GC detection techniques are widely used for the analysis of pesticide residues in bees, the selective detection systems are used to evaluate the performance of new methods, including nitrogen-phosphorus (NPD) [30–33] and electron capture (ECD) [30,33]. On the other hand, although the hyphenated techniques based on mass spectrometry have high selectivity, the fatty matrices require sample extraction and purification to remove partially or totally the lipidic components co-extracted with the target compound. Thus, the determination of residues of pesticides in bees by chromatographic methods is a challenging analytical problem because they have a high content of fats, waxes, pigments and other compounds of varying polarity; which can be co-extracted with analytes and cause problems in the chromatographic detection, particularly by blocking active sites in liners and columns.

The reported process for the extraction of pesticides from honeybees involves a solid-liquid extraction with acetonitrile [27,28], diethylether [29], acetone [32], methylene chloride from sample adsorbed on diatomaceous earth [33] followed by a cleaning step with dispersive solid phase extraction (dSPE) based on the QuEChERS method [27,28,34,35], gel permeation chromatography [29,33], solid phase extraction [29] or solid phase micro-extraction [32]. All these approaches involve two separate steps (extraction followed by cleaning) which is time consuming and increases the handling of samples. On the other hand, matrix solid phase dispersion (MSPD) permits to carry out the extraction and clean-up in one step simplifying the treatment of samples. However, this method has been scarcely proposed for the extraction of pesticides from honeybees before chromatographic analysis [30,31,36,37].

In this study we have optimized a MSPD method for the extraction, determination by gas chromatography-with selective detectors (FPD and ECD) and confirmation by GC–MS; of twelve pesticides of particular relevance for honeybee and/or a wide use in crop protection; with the aim of obtaining good recoveries and decreasing as much as possible the potential matrix effect onto detection. Pesticides included were fipronil, thiamethoxam, acetamiprid, acrinathrin, metamidophos, dimetoathe, diazinon, chlorpiryphos, methidathion, profenophos, azinphos methyl and coumaphos. In this manner we proposed a common sample treatment together with different chromatographic systems, considering the different types of instruments that a laboratory can have depending on the availability of resources. To our knowledge, this is the first report on the determination of thiamethoxam and acetamiprid, together with organophosphorus and halogenated pesticides by gas chromatography in honeybee. Residues of the selected pesticides were determined in honeybees collected in field hives from central Chile characterized by its great apicultural and agricultural development.

Table 1
Log K_{ow}, solubility in water at 20 °C and vapor pressure at 25 °C of the studied pesticides [39].

Compound	log K_{ow}	Sol. water (mg/l)	Vapor pressure (mPa)
Thiamethoxam	−0.13	4,100	6.6×10^{-6}
Fipronil	3.8	3.78	0.002
Acetamiprid	0.8	2,950	1.7×10^{-4}
Acrinathrin	6.3	0.002	4.4×10^{-5}
Methamidophos	−0.8	200,000	2.3
Dimethoate	0.7	39,800	0.25
Diazinon	3.7	60	12
Chlorpyriphos	4.7	1.05	1.43
Methidathion	2.6	240	0.25
Profenophos	4.8	28	2.53
Azinphos methyl	3.0	28	5.0×10^{-4}
Coumaphos	4.1	1.5	0.013

2. Experimental

2.1. Chemicals and reagents

The pesticides used (fipronil, thiamethoxam, acetamiprid, acrinathrin, metamidophos, dimetoathe, diazinon, chlorpyrifos, methidathion, profenophos, azinphos methyl and coumaphos) had a purity of ≥98% (Sigma-Aldrich ®, St. Louis, MO, USA). Table 1 summarized some relevant properties of these compounds. All solvents used were residue analysis grade (Merck ®, Darmstadt, Germany). Triphenylphosphate (TPP) and pentachloronitrobenzene (PCNB) (Sigma-Aldrich ®, St. Louis, MO, USA) were used as internal standard for GC-FPD and GC-ECD determinations, respectively. Stock solutions were prepared in acetonitrile at 1 g L^{-1}. Working standard solutions were diluted with acetonitrilie for spiking purposes. Clean-up® unbonded silica (15 mL, 2 g); Clean-up® carbon graphitized non-porous (6 mL, 0.5 g) and Enviro-clean® florisil (15 mL, 2 g) solid phase extraction (SPE) cartridges and Selectrasorb® bulk sorbent end-capped C18 for matrix solid phase dispersion (MSPD) were provided by UCT ® (Bristol. PA, USA).

2.2. Chromatographic analysis

2.2.1. GC-FPD

A Hewlett Packard (Agilent; Little Falls, DE, USA) model 5890 Series II ® gas chromatograph equipped with split/splitless injector and FPD was employed. A BPX5 (SEG Analytical Science, Australia) capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used. Helium and nitrogen (99.995%) were selected as carrier and auxiliary gas, respectively. Pesticides were separated and determined under the following conditions: injector temperature, 250 °C; detector temperature, 280 °C; column temperature program: 70 °C, held for 2 min; increased at a rate of 25°/min up to 250 °C; increased at a rate of 50 °C/min up to 320 °C; held for 7 min A 1-μL volume of the extract was injected in the splitless mode (1.5 min purge). Carrier gas flow in the column was 1.8 mL/min. Hydrogen, 75 mL/min and air, 100 mL/min were used as combustion gases. Under these conditions, the mixture of eight pesticides and internal standard (TPP) was well resolved in a run time of 18 min. Since the matrix-induced chromatographic response enhanced effect previously reported [31,38], all the chromatographic analysis were performed using spiked extracts of free residues honeybee as matrix-matched standard.

2.2.2. GC-μECD

An Agilent (Santa Clara, CA, USA) model 7890B ® gas chromatograph equipped with automatic injector and a μECD was employed. An HP-5 capillary column (30m × 0.32 mm i.d., 0.25 μm film thickness) was used. Argon-methane (99.995%) mixture was selected as carrier gas. Halogenated pesticides were separated and determined under the following conditions: injector temperature, 250 °C; detector temperature, 320 °C; column temperature program, 120 °C held for 1 min, increased at 15° / min up to 190 °C, increased at 8°/min up to 230 °C, increased at 20°/min up to 280 °C held for 6 min A 1-μL volume of the extract was injected in the splitless mode (1 min purge). The carrier gas flow in the column was 1.3 mL min^{-1}. Under these conditions, the mixture of 5 pesticides and the internal standard PCNB was well resolved in 20 min. The chromatographic analysis was performed using spiked extracts of free residues honeybee as matrix-matched standard.

2.2.3. GC–MS

Analyses were performed with a Thermo Scientific (Austin, TX, USA) Trace 1300 ® model gas chromatograph equipped with automatic injector AL 1310 ®, couple to ISQ ® model single quadrupole system. An HP-5 capillary column (30m × 0.32 mm i.d., 0.25 μm film thickness) was used. Pesticides were separated and determined under the following conditions: injector temperature, 250 °C; column temperature program, 70 °C held for 5 min, increased at 15° / min up to 190 °C, increased at 8°/min up to 230 °C, increased at 20°/min up to 240 °C and held for 6 min A 1-μL volume of the extract was injected in the splitless mode (1 min purge). Under these conditions, the mixture of pesticides was well resolved in 23 min.The temperatures of the transfer line and ionization source were set at 250, and 200, respectively. The mass spectrometer was operated in EI generating electrons with a kinetic energy of 70 eV and SRM acquisition mode. All compounds were monitored in full scan mode in the range *m/z* 50–550, using EI mode. A minimum of two MS/MS transitions were selected for each compound. Table S1, in Supplementary data, shows the target extracted *m/z* corresponding to the ions of each compound used for identification of residues in positive samples.

2.3. Sample collection

Honeybee samples used for spiking and validation studies were obtained during autumn of south hemisphere (April 2015) from hives localized in the experimental apiary on the laboratory located at the Instituto de Ciencias, Facultad de Medicina Clínica Alemana, Universidad del Desarrollo, Santiago, Chile. On the other hand, honeybees were collected from the O'Higgins and Valparaíso region of Chile in November 2015 and November 2016 (spring of south hemisphere), respectively. In the first one nine apiaries (32 colonies) were sampled from the zone of Rengo (12 colonies from 3 apiaries); Chimbarongo (8 colonies from 2 apiaries); Codegua (4 colonies from 2 apiaries) and Peumo (8 colonies from 2 apiaries). In Valparaíso region twelve apiaries (36 colonies) were sampled from the zone of Casablanca, Catemu-Panquehue, ConCon-Quintero and Quillota (3 colonies from 3 apiaries in each one). Each apiary is seen as a flock and a single colony as an individual. The selected areas are characterized by a significant apicultural and agricultural development with a recognized use of pesticides to control pests in crops. 50 foraging bees were collected during the morning at the entrance to the hive. The insects were euthanized by immersion in a solution of ethanol 70% v/v and stored in the same solution for their transport to the laboratory carried out within 24 h. At the laboratory bees were separated from the solution, homogenized in a mini food processor and stored in portion of 1 g in Eppendorf ® tubes at −20 °C until the analysis. Before eliminate the ethanol solution this was analyzed for assess a possible extraction of pesticides; however, residues of the compounds were not detected in this solution.

Table 2
Experimental factors and overall desirability (D) of the central composite design for the optimization of MSPD.

Run	Volume eluting solvent / mL	Methanol content / % v/v	D
1	12 (−1)	2 (−1)	0.672
2	18 (1)	2 (−1)	0.447
3	12 (−1)	8 (1)	0.602
4	18 (1)	8 (1)	0.509
5	10 (−1.41)	5 (0)	0.136
6	20 (1.41)	5 (0)	0.000
7	15 (0)	0 (−1.41)	0.881
8	15 (0)	10 (1.41)	0.851
9	15 (0)	5 (0)	0.000
10	15 (0)	5 (0)	0.000

() Coded values.

2.4. MSPD-SPE of honeybees

A sample of 1.0 *g* of bees previously homogenized was placed into a mortar and gently blended with 2 *g* of bulk sorbent C18 for 5 min using a pestle to obtain a semi-dry homogeneous mixture. During this procedure the sample was sprayed with 0.200 ml of spiking solution in acetonitrile. This mixture was introduced into the 2 *g* florisil (co-sorbent) cartridge and covered with a plug of silanized glass wood at the top. The prepared column was prewashed with 10 ml of hexane and the eluate was discarded. The analyte was eluted dropwise with 12 ml of acetonitrile-methanol (99:1) by applying a slight vacuum. The eluent was collected and evaporated to dryness using a vacuum rotary evaporator equipped with a 50 °C water bath; the residue was reconstituted with 1.0 ml of acetone with the assistance of an ultrasonic bath and centrifuged (20,000 rcf, 20 °C) for 5 min before the GC analysis.

2.5. Optimization of MSPD

The co-sorbent phase (silica, carbon graphitized and florisil) used for clean-up in MSPD was evaluated using different mixtures of solvent for elution. Subsequently a central composite design was set up to optimise the MSPD procedure through the evaluation of response surface. Honeybee samples spiked with pesticides at 0.08 and 0.1 μg g^{-1} were used depending on the proportion of the area obtained in the chromatogram for each compound. The effect of two factors on extraction efficiency and clean-up was evaluated: Volume of eluting solvent (10–20 mL) and % methanol in this solvent (0–10% v/v in acetonitrile). The central experiment was replicated three times to calculate experimental error (Table 2, total 10 experiments). Due to the variability of pesticide properties the desirability function was used to simplify the OPPs response matrices. With this previous analysis, each individual response *"i"* is associated with its own partial desirability function (d_i). This varies from 0 to 1 according to the closeness of the response to its target value. The *n* individual desirability values are then combined as geometric means to obtain the overall desirability ($D = (d_1\ d_2\ d_3\ \ldots\ dn)^{1/n}$) for each experimental condition, whose values can be utilised for optimization purposes into the domain. Statistical software (Statgraphics Centurion XV for Windows, Rockville, MD) was used to build the experimental design and to analyze data from experimental values.

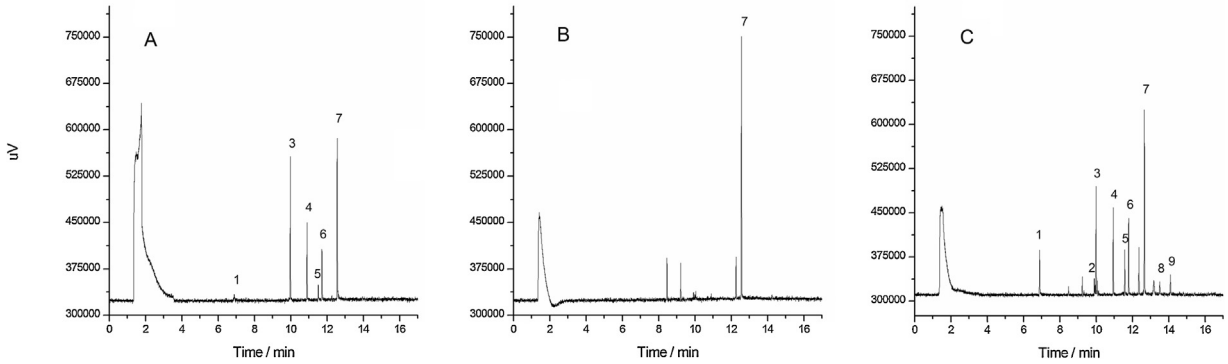

Fig. 1. GC–FPD chromatogram obtained for (a) a standard in acetonitrile, (b) a honeybee blank extracts and (c) a spiked honeybee blank extracts. Pesticides: 1 = methamidophos (0.015 μg g^{-1}); 2 = diamethoate (0.045 μg g^{-1}); 3 = diazinon (0.045 μg g^{-1}); 4 = chlorpyriphos (0.045 μg g^{-1}); 5 = methidathion (0.045 μg g^{-1}); 6 = profenophos (0.045 μg g^{-1}); 7 = TPP (internal standard 0.1 μg g^{-1}); 8 = azinphos methyl (0.150 μg g^{-1}) and 9 = coumaphos (0.075 μg g^{-1}).

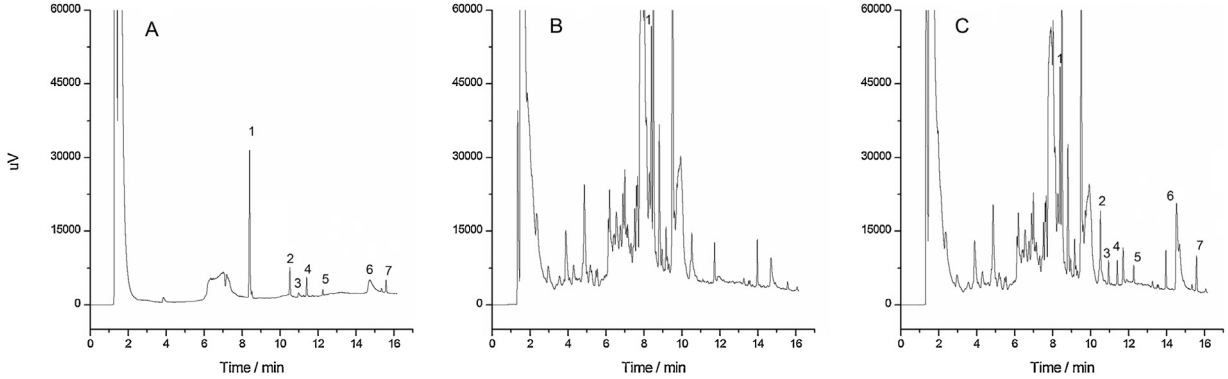

Fig. 2. GC–ECD chromatogram obtained for (a) a standard in acetonitrile, (b) a honeybee blank extracts and (c) a spiked honeybee blank extracts. Pesticides: 1 = PCNB (internal standard 0.1 μg g^{-1}); 2 = chlorpyriphos (0.045 μg g^{-1}); 3 = thiamethoxam (0.030 μg g^{-1}); 4 = fipronil (0.030 μg g^{-1}); 5 = profenophos (0.045 μg g^{-1}); 6 = acetamiprid (0.120 μg g^{-1}) and 7 = acrinathrin (0.060 μg g^{-1}).

3. Results and discussion

3.1. Gas chromatography determination

Besides their relevance due to the negative effects on honeybee the selected pesticides present a wide variety in hydrophobicity and volatility (see Table 1) which influences their gas chromatographic response and determination at residue levels. Thus, the chromatographic response of standard of pesticide prepared in acetonitrile and diluted in honeybee blank extracts were obtained and compared. Fig. 1a and c; Fig. 2a and c shows FPD and ECD chromatogram of the pesticides in acetonitrile and bee matrix, respectively. Pesticides in the bee matrix showed higher response that those in acetonitrile. Methamidophos in GC-FPD; thiamethoxam, acetamiprid and profenophos in GC-ECD showed the higher values. For some compounds even no chromatographic signal was observed in solvent (Fig. 1a; dimetoathe, azinphos methyl and coumaphos in GG-FPD). On the other hand, thiamethoxam and acetamiprid showed minimal peaks and/or peaks with tail in GC-ECD (Fig. 2a). This is probably due to interactions with the injection system, the column or partial thermal decomposition. However, for all these compounds well-defined chromatographic peaks were obtained in the presence of the matrix. This "matrix-induced chromatographic response enhanced" effect [38] is caused when matrix components are present to fill active sites in the inlet system, thus reducing analyte interactions and increasing their transference to the chromatographic column. The compounds more susceptible to this effect are polar and/or strong hydrogen-bond acids and/or bases exemplified by the presence of phosphate, hydroxyl, amino, imidazole, benzimidazole, carbamate and urea functional groups [38]. Thus, matrix-matched calibration solutions were used for calibration purposes in order to avoid quantitative errors. The chromatographic programs used allow a good resolution of the pesticides mixture in 18 and 20 min for GC-FPD and GC-μECD, respectively. To assess the selectivity of the method, extracts from blank bee sample were injected and compared with those of bee samples spiked with the compounds. No chromatographic interference was observed at the elution time of each compound in the blank samples analyzed by GC-FPD (Fig. 1b). However, in GC-ECD (Fig. 2b) was observed co-elution of chlorpyrifos and partial co-elution of acetamiprid and acrinathrin with matrix interference, which were more evident due to the lower selectivity and higher sensitivity in GC-ECD than GC-FPD detection. However, the determination of chlorpyrifos was made by GC-FPD, where this problem was not present, while acetamiprid and acrinathrin had sufficient resolution with the interference of the matrix to carry out its determination.

Only a reduced number of works on the determination of thiamethoxam or acetamiprid by GC-ECD has been reported including potato tuber [40], pineapple [41] and some crops [42], with limit of quantification ranging 0.015 to 0.050 mg kg^{-1}. To our knowledge, they have never been simultaneously analyzed alone or with other pesticides in honeybee or in any other matrix through GC-ECD as we proposed in this work.

3.2. Optimization of MSPD

First silica, florisil and graphitized carbon were evaluated as co-sorbent phase for clean-up in MSPD. Initially, 15 mL of solvent with increasing polarity were tested for pesticide elution

Table 3
Analytical characteristics of the MSPD and GC method for the pesticides studied.

Detection	Pesticide	Linear range (μg g^{-1})	R	(S_b / b)×100[a]	$S_{y/x}$ / b[b] (μg g^{-1})	rLOQ[c] (μg g^{-1})	mLOQ[d] (μg g^{-1})
GC-μECD	Thiamethoxam	0.010–0.3	0.990	6.5	0.001	0.010	0.010
	Fipronil	0.010–0.2	0.988	6.9	0.001	0.010	0.020
	Acetamiprid	0.040–0.2	0.983	8.5	0.005	0.040	0.047
	Acrinathrin	0.020–0.3	0.987	8.2	0.003	0.020	0.020
GC-FPD	Methamidophos	0.005–0.2	0.972	10.8	0.001	0.005	0.007
	Dimethoate	0.008–0.2	0.989	6.7	0.001	0.008	0.016
	Diazinon	0.015–0.2	0.975	10.3	0.002	0.015	0.015
	Chlorpyriphos	0.015–0.2	0.989	6.7	0.002	0.015	0.017
	Methidathion	0.015–0.2	0.985	7.8	0.002	0.015	0.028
	Profenophos	0.015–0.2	0.986	7.5	0.002	0.015	0.015
	Azinphos methyl	0.050–0.5	0.990	7.3	0.007	0.050	0.050
	Coumaphos	0.025–0.5	0.989	6.7	0.004	0.025	0.030

[a] Linearity as % of slope variability.
[b] Analytical sensitivity.
[c] Limit of quantification from regression model = $10(S_{y/x} / b)[(n-2)/(n-1)]^{1/2}$.
[d] Limit of quantification from the method = 10 SD (n = 5) at the lower level of the lineal range.

(ethyl acetate, ethyl acetate-methanol and acetonitrile). For C18-GCB combination the higher recoveries were obtained with ethyl acetate and ethyl acetate-methanol. However, even with these solvents some compounds not exceeded 50% recovery. Colorless extracts and a residue that was insoluble in acetone were obtained with the three elution solvents evaluated. In the case of C18-SiO and C18-florisil combinations the recovery increased as the polarity of solvent increased, reaching 60–80% for the pesticides with acetonitrile. In both cases, light yellow extract were obtained due to pigments not adsorbed by silica or florisil and a small yellow residue insoluble in acetone, which was removed by ultracentrifugation before the chromatographic analysis. However, from the three evaluated sorbent/co-sorbent the pair C18-florisil produced the chromatograms with lower matrix interferences. Thus, MSPD with C18 as sorbent and florisil as co-sorbent was selected for further optimization.

In a second stage, the volume of eluting solvent (10–20 mL) and % methanol in this solvent (0–10% v/v in acetonitrile) were optimized through a central composite design. The pesticides affected by these factors in the studied experimental range were fipronil, acritatrin, methamidophos, and azinphos-methyl; while the other compounds had recovery higher or equal than 90% for the entire experimental range. Thus, only the pesticides affected by the experimental factors were included in the multiple response optimization, where the partial desirability for each pesticide was obtained by maximization of their recovery (unilateral, weight = 1, impact = 3). The overall desirability for each experiment of the design is shown in Table 2. The highest recoveries were obtained for a low volume of solvent and minimum content of MeOH. The optimal condition finally selected was 12 mL of acetonitrile with 1% of methanol, which allowed recoveries higher or equal than 70% and chromatograms with less interferences. The model as fitted for the optimization represents the data adequately since the regression was significant (F = 14.6; p-value 0.012) and the coefficient of determination was 0.950.

3.3. Validation and analytical performance of the method

The developed method was validated following principally the SANTE guide of the European Commission (11813/2017) [43]. To obtain low limit of quantification in the bee matrix split-less injection was selected. The analytical characteristics of the method for the determination of pesticides by GC-ECD or GC-FPD are summarized in Table 3. Linearity was studied in different ranges depending on the chromatographic response of each compound; measuring a matrix-matched standard calibration curve prepared in extracts of blank matrix. Linear calibration graphs were constructed by least-square regression of peak area ratio versus concentration (analyte/IS) of the calibration standards. The responses of all compounds were linear in the range under study, with r values of 0.972–0.990. Moreover, the parameter $(Sb / b) \times 100$, where S_b is the standard deviation of the slope and b is the slope, which gives a better representation of linearity (slope variability) was lower or equal than 10.8%. Analytical sensitivity ($S_{y/x}$ / b; where $S_{y/x}$ is the standard error of the estimation), which indicates the minimal difference in concentration detected by the method, was between 0.001 and 0.007 μg g^{-1}. The limit of quantification from the regression model (rLOQ) was obtained, as 10 ($S_{y/x}$ / b)[(n-2)/(n-1)]$^{1/2}$; where n is the number of pairs of points (in this case the four of lower concentration), and ranged from 0.005 for methamidophos to 0.050 μg g^{-1} for azinphos-methyl. The limit of quantification of the method (mLOQ) was obtained by spiking free residue honeybee at the lower level of the lineal range and subjecting it to the sample preparation method (ten times the standard error of the signal obtained for five extracts). The mLOQ obtained ranged from 0.007 to 0.050 μg g^{-1} and were similar to rLOQ values. Additionally, the LOQ of the GC–MS method used for confirmation purposes was determined, obtaining values similar to those observed for GC-ECD and CG-FPD (0.003–0.021 ug g^{-1}).

Accuracy and precision experiments were assessed by repeated analysis of blank samples spiked with three concentrations of the pesticides (LOQ, 3LOQ and 5LOQ; n = 5). Results are shown in Table 4. The mean recovery at the limit of quantification ranged from 68 to 102%; while for the intermediary concentration ranged from 68 to 108%. Precision was assessed as within-laboratory reproducibility (RSD_{wR}; n = 5) with values ≤20%. The lower recoveries were obtained for compounds with intermediary hydrophobicity

Table 4
Percent recoveries (%) ± within-laboratory reproducibility (RSD_{wR} %) of the pesticides spiked to bees at LOQ, 3 × LOQ and 5 × LOQ.

	Recovery ± RSD_{wR}% (n = 5)		
Pesticide	LOQ	3 × LOQ	5× LOQ
Thiamethoxam	80 ± 13	80 ± 15	84 ± 12
Fipronil	83 ± 7	75 ± 9	85 ± 19
Acetamiprid	91 ± 13	108 ± 20	72 ± 18
Acrinathrin	76 ± 8	68 ± 8	71 ± 20
Methamidophos	90 ± 13	81 ± 13	84 ± 12
Dimethoate	74 ± 20	85 ± 12	78 ± 10
Diazinon	72 ± 10	73 ± 12	70 ± 6
Chlorpyriphos	77 ± 8	77 ± 13	74 ± 7
Methidathion	68 ± 8	75 ± 7	75 ± 9
Profenophos	75 ± 7	77 ± 16	70 ± 9
Azinphos methyl	102 ± 6	101 ± 9	78 ± 8
Coumaphos	86 ± 20	77 ± 10	81 ± 10

Table 5
Comparison with other reported methods that include the determination of the target pesticides in honey bees.

Pesticide	Sample treatment / Detection	Recovery range / % (lower fortification value)	LOQ	Ref
200 multiclass pesticides	QuEChERS / LC–MS/MS, GC–MS/MS	95–107% (0.010 ug g^{-1}) RSD 3–10%	0.001–0.005 μg g^{-1}	[27]
150 Multiclass pesticides	S-L extraction, low temperature precipitation of fatty and dSPE / GC–MS/MS	82–106% (0.010–0.500 μg g^{-1}) RSD 6–33%	Not informed	[28]
150 Multiclass pesticides	MSPD / GC-NPD, GC-ECD	60–107% (0.010–0.050 μg g^{-1}) RSD 1–4%	0.008–0.020 μg g^{-1}	[30]
12 Multiclass insecticides	MSPD / GC-NPD	96–112% (0.020–0.160 μg g^{-1}) RSD 3–7%	0.030–0.120 μg g^{-1}	[31]
18 organophosphorus insecticides	S-L extraction, SPME / GC-NPD	69–250% (0.010 μg g^{-1}) RSD 2–30%	0.001–0.004 μg g^{-1}	[32]
29 organophosphorus and carbamates insecticides	S-L supported on diatomaceous earth, GPC / GC-NPD, GC-ECD	80–96% (0.330–0.440 μg g^{-1}) RSD 6–20%	0.001–0.043 μg g^{-1}	[33]
115 Multiclass insecticides	QuEChERS / LC-ESI-MS/MS	80–106% (0.05 μg g^{-1}) RSD 4–14%	0.001–0.013 μg g^{-1}	[34]
80 Multiclass pesticides	QuEChERS / GC-ToF and LC–MS/MS	60–125% (0.010 μg g^{-1}) RSD 11–27%	0.003–0.027 μg g^{-1}	[35]
22 organophosphorus insecticides	MSPD / LC–MS	76–98% (0.010 μg g^{-1}) RSD 9–12%	0.100–0.260 μg g^{-1}	[37]
12 multiclass concern pesticides	MSPD / GC-FPD, GC-μECD	60–101% (0.007–0.050 μg g^{-1}) RSD 6–20%	0.007–0.050 μg g^{-1}	This work

Table 6
Pesticide residues detected in honeybee from Valparaíso (V) and O'Higgins (VI) regions of Chile.

		Positive samples and concentration range () / μg g^{-1}							
Region / zone	Samples	Thiamethoxam	Fipronil	Acetamiprid	Acrinathrin	Methamidophos	Diazinon	Chlorpyriphos	Coumaphos
V/Casablanca	9	-[a]	2 (<0.020)	1 (0.057)	4 (<0.020–0.026)	–	–	–	–
V/Catemu-Panquehue	9	–	–	2 (<0.047–0.100)	5 (<0.020)	–	1 (<0.015)	–	–
V/ConCon-Quintero	9	–	1 (<0.020)	1 (<0.047)	5 (<0.020)	1 (<0.007)	–	1 (<0.017)	–
V/Quillota	9	–	–	–	1 (<0.020)	3 (<0.007)	–	–	–
VI/Chimbarongo	8	1 (<0.010)[b]	–	–	5 (<0.020)	–	4 (<0.015)	7 (<0.017–0.067)	–
VI/Codegua	4	–	–	–	1 (<0.020)	–	–	4 (<0.017)	–
VI/Peumo	8	–	–	–	–	–	2 (<0.015)	4 (<0.017)	–
VI/Rengo	12	–	–	–	1 (<0.020)	–	–	7 (<0.017)	1 (<0.030)
Overall	68	1 (<0.010)	3 (<0.020)	4 (<0.047–0.100)	22 (<0.020–0.026)	4 (<0.007)	7 (<0.015)	23 (<0.017–0.067)	1 (<0.030)

[a] Residue was lower than the LOD.
[b] Lower that the mLOQ.

(dimethoate, diazinon, methidathion) at the lower concentration spiked. With only some particular exception, these values are in accordance to the SANTE guide of the European Commission (11813/2017) that stipulates recovery values in the range 70%–120% with $RSD_{wR} \leq 20\%$ [43]. Moreover, according to this guide, recoveries outside this range may be accepted, specifically if is low but consistent (i.e. demonstrating good precision as in our results) and if the basis for this is well established (in this case due to the distribution of the analyte in the MSPD procedure). Finally, these results compared favourably with those reported in previous works on the liquid and gas chromatographic determination of the target pesticides in honeybee with different extraction method. This comparison is summarized in Table 5. For a comparable fortification level, the recoveries and RSD for the proposed method are similar to those reported in literature. Similar values of LOQ are also observed. Although the FPD detector is not widely used, the limits of quantification obtained with it were similar to those obtained with other detectors, being therefore an alternative in the determination of the pesticides evaluated in bee. Moreover, the present work includes the determination of acrinathrin in bees, an acaricide of widespread use in beehives against varroosis. Only one of the reported works has also included this pyrethroid [28].

3.4. Real sample analysis

The compounds tested in this work have been selected based on their potential negative impact in the apicultural field and on the sales of pesticides that are widely used in the agriculture of the central region of Chile [44]. Results of the analysis of real samples collected in central Chile are summarised in Table 6. Overall, for 44 samples (65%), eight of the twelve tested pesticides were detected. Dimethoate, methidathion, profenophos, and azinphos methyl were not detected in any sample. The number of different pesticides in an individual sample ranged from 0 to 3, and the mean, median and mode values were equal to one pesticide per sample. A total of 25 samples had only one pesticide at detectable levels, 14 samples had two pesticides and 5 samples had three pesticides. Besides, in Chimbarongo and Codegua was observed the highest occurrence and number of residues per sample (median and mode of two compounds). On the other hand, the highest detection rate was observed for the residues of chlorpyriphos; twenty three samples (34%) contained this pesticide in the range of <0.017–0.067 μg g^{-1}. Twenty two samples (32%) contained achrinatrin residues ranging from <0.020 to 0.026 μg g^{-1}. Diazinon residues were detected in seven samples (10%) at values <0.015 μg g^{-1}. Chlorpiryphos and diazinon were among the pes-

Table 7
Comparison with results obtained on the determination of the target pesticides in bees in other countries.

Country [Ref]	Samples	Positive samples and concentration range () / μg g^{-1}					
		Thiamethoxam	Fipronil	Dimethoate	Diazinon	Chlorpyriphos	Coumaphos
Chile [This work]	68[a]	1 (<0.010)	3 (<0.020)	–	7 (<0.015)	23 (<0.017–0.067)	1 (<0.030)
Uruguay [6]	31[b]	–	2 (0.150–0.170)	–	–	–	–
France [23]	307[a]	–	7 (<0.0003–0.0007)	–	–	–	14 (<0.037–25)
Egypt [24]	16[a]	–	–	–	9 (0.0002–0.0005)	1 (0.032)	–
Poland [27]	73[b]	2 (0.044–0.275)	3 (0.034–0.271)	30 (0.0014–1.6)	–	38 (0.002–3.3)	–
Poland [28]	25[b]	–	10 (0.010–0.064)	9 (0.238–4.9)	–	5 (0.010–0.056)	–
Poland [30]	33[b]	–	3 (0.008–0.017)	3 (0.258–7.3)	–	9 (0.010–576)	–
Greece [34]	27[b]	–	1 (0.082)	–	–	–	2 (0.013–0.020)
France [35]	145[a]	–	–	–	1 (<0.027)	4 (0.003–0.180)	28 (0.004–0.047)

[a] Workers bees.
[b] Poisoned dead bees.

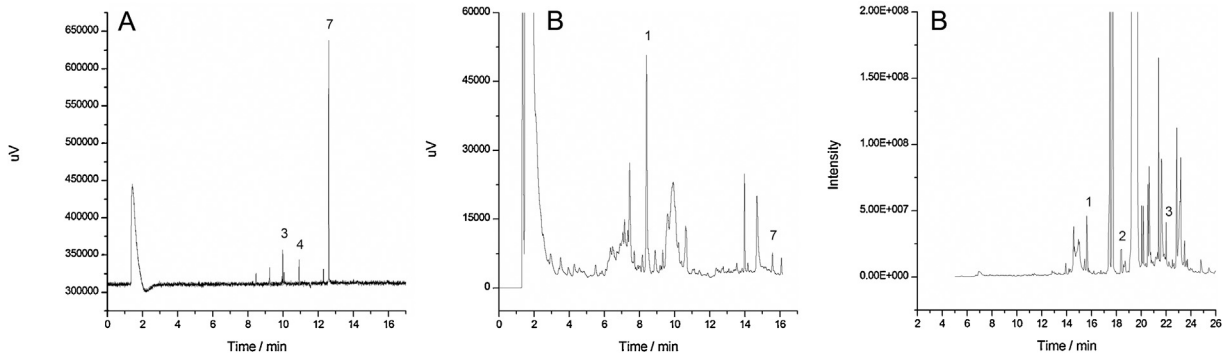

Fig. 3. GC–FPD (a), GC-ECD (b) and GC–MS (c) chromatograms obtained for the honeybee sample from Chimbarongo zone. Pesticides in GC–FPD and GC-ECD as indicated in Figs. 1 and 2. Pesticides in GC–MS: 1 = Diazinon; 2 = chlorpyriphos; 3 = acrinathrin.

ticides with the highest sales in O'Higgins and Valparaíso region of Chile in the 2012 [44]. Even more, the sales of chlorpyrifos in O'Higgins region is three times higher than in Valparaiso region, which could be one of the causes of its higher incidence in that region. The use of achrinatrin by beekeepers to combat the varroosis explains its high frequency of detection in the samples. The frequency of occurrence of this compound was higher in the samples of the Valparaíso region. On the other hand, chlorpyriphos and acrinathrin are highly lipophilic compounds (see Table 1). Thus, bees can accumulate lipo-soluble and/or widespread used pesticides in crop protection and be considered as bio-indicators of the presence of these compounds in the environment. In Table 7 are compared the residue levels of the target pesticides found in bees from central Chile and other previously published data from different countries. Overall, fipronil and organophosphorus pesticides dominates the residues detected in bees; where chlorpyrifos showed one of the highest frequencies of appearance in Chile and Poland [27,28,30]; whereas in France the highest frequency has been obtained for coumaphos related to its use to combat varroa [23,35]. As shows the cases of colony mortality are accompanied by high concentration of residues in dead bee samples. Pesticide presence in bees is expected when colonies perish due to pesticide exposure. However, sub ug g $^{-1}$ levels are detected in the insects when honey bees forage in any conventional agricultural or urban setting, such as those collected in this study.

Along with the quantification of pesticide residues in the positive samples by GC-FPD/μECD, their identity was confirmed through GC–MS analyses, under the chromatographic conditions detailed in Section 2.2.3. The GC-FPD, GC-μECD and GC–MS chromatograms of one of the honeybee sample from Chimbarongo zone with three detected pesticides (diazinon, chlorpyriphos and achrinatrin) are shown in Fig. 3.

4. Conclusions

The optimized MSPD and GC–FPD / GC-μECD method is simple and sensitive to determine a group of pesticides with potential risk for bees, varied hydrophobicity and volatility, at sub μg g^{-1} level in those insects; including the neonicotinoids thiamethoxam and acetamiprid. Due to the "matrix- induced chromatographic response enhancement effect" caused by co-extracted compounds from samples, matrix-matched calibration method should be used to obtain accurate results. The study and optimization approach of MSPD through experimental design has permitted to assess the variability of the pesticides with different physic-chemical properties; and to count with a common sample treatment for different chromatographic systems. By applying the method to field samples from the central region in Chile, residues of the most used pesticide in hive fumigation against varroosis, crop protection and with a lipophilic character (acrinathrin, chlorpyriphos and diazi-

non) were the most frequently found in these samples at sub ug g^{-1} levels. These facts demonstrate that bees can be considered as bio-indicators of the presence of these compounds in the environment and that be vulnerable to his exposure.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.06.062.

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