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Analysis of insecticides in honey by liquid chromatography–ion trap-mass spectrometry: Comparison of different extraction procedures

Cristina Blasco, Pablo Vazquez-Roig, Matthias Onghena, Ana Masia, Yolanda Picó*

Food and Environmental Safety Research Group, Facultat de Farmàcia, Universitat de València, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, València, Spain

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ABSTRACT

The feasibility of different extraction procedures was tested and compared for the determination of 12 organophosphorus and carbamates insecticides in honey samples. In this sense, once the samples were pre-treated – essentially dissolved in hot water by stirring – and before they could be analyzed by liquid chromatography–ion trap-second stage mass spectrometry (LC–MS^2), four different approaches were studied for the extraction step: QuEChERS, solid-phase extraction (SPE), pressurized liquid extraction (PLE) and solid-phase microextraction (SPME). The main aim of this work was to maximise the sensitivity of pesticides and to minimise the presence of interfering compounds in the extract. All pesticides were linear in the range from CC_β to 1000× CC_β for the four extraction methods (three orders of magnitude). Detection capabilities (CC_β) were 0.024–1.155 mg kg^{-1} with QuEChERS, 0.010–0.646 mg kg^{-1} with SPE, 0.007–0.595 mg kg^{-1} with PLE, and 0.001–0.060 mg kg^{-1} with SPME. All the target compounds could be recovered by any of the methods, at a CC_β fortification level ranged from 28 to 90% for the SPME. In comparison, the PLE method was the most efficient extraction method with recoveries from 82 to 104%. It was followed by the QuEChERS method with recoveries between 78 and 101% and the SPE method with recoveries between 72 and 100%. The repeatability expressed as relative standard deviation (RSDs) was below 20% for all the pesticides by any of the tested extraction methods. Results obtained applying the four extraction techniques to real honey samples are analogous.

1. Introduction

Honey is mostly a mixture of sugars and contains only trace amounts of vitamins or minerals. With respect to carbohydrates, honey is mainly fructose (about 38.5%) and glucose (about 31.0%) but also includes maltose, sucrose, and other complex carbohydrates. It also contains tiny amounts of several compounds functioning as antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin. The specific composition of any batch of honey, as well as the contaminants presents in it, are dependent on the crops surrounding the beehive [1,2]. Pesticide application in crops can contaminate soil, air, water, and the flowers from which bees collect nectar for honey production. Consequently, bees and honey may serve as indicators of environmental pollution since honeybees are greatly affected by pesticides (by increasing their mortality) and transport them to the colony as contaminated nectar which ends as a contaminated honey. Pesticide residues may also be from the treatment of beehives with acaricides in the control of *Varroa jacobsonie* and *Ascosphera apis*. The honey benefits can be suppressed by pesticides introduced to honey during its processing and arising from both agricultural and beekeeping practices [1,3]. This work has been focussed in the formers since there is less information available. About 79% of the insecticides in current use in Europe are organophosphorus (OPs) and (closely related) carbamates. Both families of chemicals primarily affect the nervous system by inhibiting acetylcholinesterase (AchE) enzyme activity [4].

Owing to the complex nature of the honey matrix, there is a consensus on that efficient sample preparation, trace-level detection and identification are important aspects of analytical methods to determine pesticides in honey [5–7]. The technique most frequently used for sample extraction or clean-up is solid-phase extraction (SPE) [8–11,11–16], or solvent extraction (SE) very often followed by clean-up on an SPE column [3,9,17–22]. It greatly reduces interferences of the matrix, enhancing sensitivity and facilitating unambiguous identification and confirmation. These aspects are of great importance, especially when determining possible presence of contaminants in honey at trace levels [23–26]. However, in some cases, extraction methods, such as SPE and SE, are still not selective enough to comply with the needs of food safety and regulations. Relatively new techniques – e.g., matrix solid phase dispersion (MSPD) [27], pressurized liquid extraction (PLE)

* Corresponding author at: Universitat de València, Food and Environmental Safety Research Group, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, València, Spain. Tel.: +34 963543092; fax: +34 963544954.
E-mail address: Yolanda.Pico@uv.es (Y. Picó).

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[28], solid-phase microextraction (SPME) [29–32], stir-bar sorptive extraction (SBSE) [29,33] and dispersive liquid–liquid microextraction (DLLME) [34] – require less organic solvent, are easy to implement, allow high sample throughputs and, in general, are used with good results.

Recently, regulation (EC) No. 396/2005 of the European Parliament and of the Council, has established values for the maximum residue levels (MRLs) of pesticides in products of plant and animal origin [35]. Since September 1st 2008, the European Commission set new MRLs, which mostly are between 10 and 50 ng g^{-1} in honey. These low MRLs are mainly identified with lower limit of analytical determination, which evolves to provide better sensitivity. The majority of studies on pesticide residues determination in honey have utilized gas chromatography (GC) and liquid chromatography (LC)-based approaches. For organophosphorus and carbamates, there are a number of reviews indicating that compared to conventional GC methods, LC–MS is very straightforward, sensitive, fast and more reliable [36,37]. However, for LC–MS and/or LC with tandem MS (LC–MS^2), it is still important to apply a good extraction and preparation method, as matrix effects can impact on detection systems, generating significant noise, or altering ionization efficiency and what is more, can impact on limits of detection and quantification. To establish what method is better than other, comparison of the results is the best way. However, there are few examples of these comparisons. Blasco et al. [29] used SBSE and SPME with a poly(dimethylsiloxane) coatings to determine six organophosphates in honey. SBSE showed higher concentration capability (large quantities of sample can be handled) and greater accuracy (between 5 and 20 times) and sensitivity (between 10 and 50 times) than SPME; thus, under equal conditions, SBSE is the recommended technique for pesticide analysis in honey. The same research team [30] combined SPME (using a more suitable PDMS/DVB coated fiber) with direct desorption of the analytes from the fiber to LC–MS. Under optimal conditions, the procedure provided excellent linearity (>0.990), quantification limits (between 0.005 and 0.5 μg kg^{-1}), and precision (<19% at the quantification limits and from 6 to 14% at ten times higher concentrations). This highlights the interest of performing the comparison of the methods in their optimal conditions.

The objectives of this work were to develop a robust analytical method for the simultaneous extraction and determination of twelve insecticides (bromophos ethyl, chlorpyrifos methyl, chlorpyrifos ethyl, diazinon, fenoxycarb, fonofos, phenthoate, phosalone, pirimiphos methyl, profenofos, pyrazophos, and temephos) in honey. These pesticides were selected considering the sales in the cooperatives of the area. Different extraction methods (SPME, PLE, SPE and SPME) were compared and finally the performance of the methods was validated in naturally contaminated samples. Such a broad spectra of methodologies were never compared for the quantification of pesticides in honey. To propose a reliable and robust method, parameters such as type and volume of solvent, spiking level and amount of sample extracted were optimized. Analysis was carried out using liquid chromatography–ion trap-second stage mass spectrometry (LC–IT-MS^2) [38]. These methods were partly optimized and the method validation was according to the EU Commission Decision 2002/657/EC guidelines [38].

2. Experimental

2.1. Reagents

Pesticide standards (bromophos ethyl, chlorpyrifos methyl, chlorpyrifos ethyl, diazinon, fenoxycarb, fonofos, phenthoate, phosalone, pirimiphos methyl, profenofos, pyrazophos, and temephos) were purchased from Sigma–Aldrich (Madrid, Spain). The individual stock solutions were prepared in methanol at a concentration of 1000 mg L^{-1} and stored at −20 °C. Standard working solutions at various concentrations were prepared daily in methanol.

Deionized water was prepared from a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade acetonitrile and methanol, residue analysis ethyl acetate and dichloromethane were purchased from Merck (Darmstadt, Germany). Analytical grade sodium chloride (NaCl), anhydrous magnesium sulphate ($MgSO_4$) and silica gel 60 (0.04–0.06 mm) were obtained from Scharlau (Madrid, Spain). Bondesil Primary-Secondary Amine (PSA, 40 μm, Bondesil) was from Varian Inc. (Palo Alto, USA). Oasis HLB 200 mg sorbent/6 mL cartridges were from Waters Corp. (Milford, MA, USA).

2.2. Liquid chromatography–ion trap-mass spectrometry

The liquid chromatography–ion trap-mass spectrometry (LC–IT-MS) system consisted of an Esquire3000 Ion Trap LC–MSn system (Bruker Daltonik GmbH, Germany) and a data acquisition/processing Daltonic Esquire Control Software system 3.0. LC separation was performed on a Luna C18 column (250 mm × 4.6 mm I.D., particle size 5 μm) protected by a Securityguard cartridge C18 (4 mm × 2 mm I.D.), both from Phenomenex (Madrid, Spain). The mobile phase was a methanol/water gradient at a flow-rate of 0.7 mL/min. The gradient was 70% methanol from 0 to 15 min, followed by a linear gradient to 90% from 15 to 20 min, then increased again linearly to 95% from 20 min to 25 min, and finally, maintained at 95% methanol from 25 to 30 min and re-equilibrates to the initial conditions in 10 min.

The mass spectrometer was equipped with an APCI source, and operated in positive and negative polarity. The conditions of the source were temperature, 450 °C; capillary voltage, 2500 V; the end plate offset was fixed at −500 V; corona current, 4000 nA; nebulizer pressure, 60 psi; and drying gas flow 4 L min^{-1} at a temperature of 350 °C. The Esquire 3000 was tuned for each compound, optimizing the voltages of the lenses in the ExpertTune mode of the Daltonic Esquire Control software while infusing a standard solution (10 μg mL^{-1}) by a syringe pump at a flow rate of 0.004 mL min^{-1}, which was mixed with the mobile phase at 0.8 mL min^{-1} by means of a T piece. The optimized tune parameters were set for each compound via time segments definition. The mass spectrometer was operated in full scan and MRM modes. The trap parameters were detected in ion charge control mode using rolling averaging set at 2. Full scan mode was performed with a target of 70,000 and maximum accumulation time of 100 ms at m/z range from 100 to 500 u. MRM was carried out setting the target at 200,000 and maximum accumulation time at 200 ms for both, MS and MS^n experiments. Ions were detected at unit resolution (scan speed 10,300 u s^{-1}). Four scans were summarized for each spectrum, resulting in a spectral rate of 0.4 Hz. Collision induced dissociation (CID) was performed on the ion of interest by collisions with the helium background gas present in the trap for 40 ms. In these experiments, the most intense precursor ion of the pesticide was subjected to CID to produce a first set of fragment ions, MS–MS or MS^2. Subsequently, one of its most intense product ions was isolated and fragmented to give the next set of fragment ions, MS^3. The fragmentation steps for each compound were optimized visualizing the changes in the intensities of fragments ions, whereas the fragmentation cut-off and the fragmentation amplitude were manually varied.

2.3. Honey samples

Twenty-five honey samples of different botanical origin were obtained from honey producers of Valencian Community (Spain) during 2007 and 2008. When the honey was ready for harvest

(caped honey) the beekeeper proceeded to open the combs for the honey extraction by centrifuging. The honey samples were packaged in glass containers and dispatched for analysis in our laboratory. All samples were kept at −10 °C until analysis to evaluate pesticide residues.

2.4. Extraction procedures

2.4.1. Solid-phase microextraction (SPME)

A SPME holder for automated sampling (Bellefonte, PA, USA) and silica fibers (Supelco) coating with 50 μm carbowax/templated resins (CW/TPR) were employed. The new fibers were conditioned in methanol by stirring for 30 min and the used ones were cleaned in methanol by stirring for 15 min before extraction. The SPME device has been described elsewhere [22–24]. Honey (5 g) was weighed into 5 mL clear vials (Análisis Vinicos, Tomelloso, Spain) and 3 mL of hot water was added. The extraction was carried out for 120 min under magnetic stirring in order to improve mass transfer to the aqueous sample into the fiber coating. The SPME interface (Supelco, Bellefonte, PA, USA) consisted of a standard six-port reodyne valve equipped with a fiber desorption chamber (total volume = 60 μL), installed between the autosampler of the LC and the analytical column. Desorption was performed in static mode placing the fiber into the desorption chamber, which was previously filled with methanol–water (70:30, v/v) for 15 min. To avoid possible memory effects, the fiber was left continuously exposed to the mobile phase flow during the analysis. The injection was recorded programming the autosampler for a blank run and using the handle of the SPME interface as external start.

2.4.2. Pressurized liquid extraction method (PLE)

Honey samples (1.5 g) were dissolved in 3 mL of hot water, then, the mixture was blended with 20 g of silica for 5 min in a mortar using a pestle. This mixture was introduced into a stainless steel extraction cell (22 mL capacity), which was positioned in the pressurized liquid extraction (PLE) system connected to a four-bottle solvent controller, both from Dionex (Sunnyvale, CA, USA). Nitrogen at pressure of 10 bar was supplied to assist the pneumatic system and to purge the extraction cells. For the extraction, ethyl acetate (100% flush volume) was used at 75 °C and 1500 psi (1 psi = 6894.76 Pa) for 7 min static time, in two cycles, preheated 2 min and purge 60 s. The total volume of extract obtained under those conditions was 22 mL showing only very little variations, less than 0.5 mL, when analyzing different samples.

Each PLE extract was concentrated to ca. 1 mL in a Büchi R200 (Labortechnik, Flawil, Switzerland) rotary evaporator set at 40 °C and 250 mbar in 50 mL round-bottomed flasks. Then, the extract was transferred to a 15 mL conical tube and the round-bottomed flask was rinsed with twice 0.5 mL of methanol and evaporated to dryness using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA) provided with a nitrogen stream and a water bath at 50 °C. After solvent evaporation, it was reconstituted in 0.5 mL of methanol.

2.4.3. Solid-phase extraction with Oasis HLB

Honey (1.5 g) was mixed with 30 mL of water and agitated by a stir bar for 10 min. Pesticides were isolated using an Oasis HLB cartridge [poly(divinylbenzene-co-N-pyrrolidone)] pre-conditioned with 5 mL of methanol and 5 mL of Milli-Q water. Samples were passed through the cartridges at a flow rate of 10 mL min^{-1}, and then, the cartridge was rinsed with 5 mL of Milli-Q water and dried under vacuum for 15 min. The retained pesticides were eluted by passing first 10 mL of methanol–dichloromethane (30:70). The eluate was evaporated to 0.5 mL, using a gentle steam of nitrogen, and transferred quantitatively with methanol into a 1-mL volumetric flask, obtaining a final extract in 100% methanol.

2.4.4. QuEChERS

The sample preparation procedure entailed the weighing of 1.5 g sample into a 50 mL polypropylene centrifuge tube; (2) add 3 mL of hot water and vortex until dissolution; (3) dispense 3 mL acetonitrile to the samples and shake the tubes vigorously by hand for 30 s; pour the samples and extracts into the appropriate tubes containing 6 g $MgSO_4$ and 1.5 g NaCl, shake the tubes vigorously by hand for 1 min (avoiding formation of oversized $MgSO_4$ agglomerates); centrifuge the tubes at 3000 rcf for 2 min; transfer 1 mL of acetonitrile extract (upper layer) to the dispersive-SPE tubes containing 150 mg anhydrous $MgSO_4$ and 50 mg PSA, vortex the d-SPE tubes for 30 s and centrifuge at 3000 rcf for 2 min; transfer 0.5 mL of the final extracts into the labeled autosampler vials.

2.5. Method validation

The validation of a quantitative method according to the Commission Decision should include: trueness (recovery), repeatability, within-laboratory reproducibility, decision limit (CC_α), detection capability (CC_β), calibration curves, ruggedness [38].

CC_α was determined by analyzing at least 20 blank materials per matrix to be able to calculate the signal to noise ratio at the time window in which the analyte is expected. Three times the signal to noise ratio can be used as decision limit. This is applicable to quantitative and qualitative assays, since only two pesticides, phosalone and profenofos have a MRL of 0.05 mg kg^{-1}. CC_β was established by analyzing at least 20 blank materials per matrix fortified with the analyte(s) at the decision limit. The value of the CC_α plus 1.64 times the standard deviation of the within-laboratory reproducibility of the measured content equals the CC_β (β = 5%). Honey samples were spiked with 10 μL of the appropriate working mixtures prepared in methanol, then the samples were left 15.0 min for equilibration at room temperature, after being mixed with a vortex mixer for 2.0 min.

The linearity of the analytical methods was proved building the calibration curves for each compound using standards prepared in methanol or in extracts of honey samples not contaminated with the selected pesticides (n = 5) spiked from the CC_β to 1000× CC_β. Each level was prepared in triplicate. For the SPME methods the linearity was evaluated for the whole extraction procedure from water samples and from pesticides spiked in blank honey samples.

The accuracy is expressed as the average recovery in the samples. The recovery experiments were carried out at three concentration levels (CC_β, 10 and 50 μg kg^{-1}) in quintuplicate. The repeatability and intra-laboratory reproducibility (both expressed as the relative standard deviation, RSD) were calculated from the analysis of 5 honey samples spiked with the selected analytes at each selected fortification level. Within laboratory precision was obtained by following the same protocol but five replicates of each sample were analyzed daily through three different days.

Ruggedness was evaluated though the optimization studies carried out by selecting factors of the sample pre-treatment, clean up and analysis, which may influence the measurement results.

3. Results and discussion

3.1. Optimization of liquid chromatography–mass spectrometry

The compounds were identified by their retention time and product ions (m/z), regarding the pesticide standards as listed in Tables 1 and 2. Table 1 outline the main m/z ions and their abundance in negative and positive ionization modes for the pesticides by MS, MS^2 and MS^3 with indication of the ion selected

Table 1
Mass spectral characteristics of the studied compounds.

Compound	Precursor ions m/z (abundance)	Product ions (MS^2) m/z (abundance)	Product ions (MS^3) m/z (abundance)
Negative ionization mode			
Bromophos ethyl	351 (100)	240 (100)	204 (90)
$C_{10}H_{12}BrCl_2O_3PS$			
Chlorpyrifos methyl	302 (100)	**208**[a] (100)	172 (10)
$C_7H_7Cl_3NO_3PS$		140 (60)	168 (100)
			126 (109
Chlorpyrifos ethyl	330 (100)	302 (20)	142 (100)
$C_9H_{11}Cl_3NO_3PS$		**178**[a] (100)	
Diazinon	275 (100)	151 (100)	135 (15)
$C_{12}H_{21}N_2O_3PS$		167(80)	123 (100)
			109 (10)
Fonofos	154 (100)	124 (100)	
$C_{10}H_{15}OPS_2$		112(5)	
Phenthoate	**319**[a] (100)	**124**[a] (100)	124 (50)
$C_{12}H_{17}O_4PS_2$		110 (20)	110 (100)
Phosalone	**338**[a] (100)	167 (100)	
$C_{12}H_{15}ClNO_4PS_2$	185 (5)	291 (40)	
	168 (5)	231 (10)	
Pirimiphos ethyl	304.1 (100)	196 (60)	168 (100)
$C_{11}H_{20}N_3O_3PS$		180 (100)	140 (10)
		152 (10)	
Profenofos	**345**[a] (100)	205 (100)	167 (100)
$C_{11}H_{15}BrClO_3PS$			
Pyrazophos	**372**[a] (100)	344 (12)	220 (85)
$C_{14}H_{20}N_3O_5PS$	220 (15)	326 (17)	192 (41)
		248 (14)	148 (100)
		220[a] (100)	
Temephos	451(100)	390 (100)	390 (100)
$C_{16}H_{20}O_6P_2S_3$			360 (40)
			278 (50)
Positive ionization mode			
Chlorpyrifos methyl	323 (100)	292 (100)	292 (50)
$C_7H_7Cl_3NO_3PS$			232 (80)
			213 (100)
			178 (30)
Chlorpyrifos ethyl	350 (100)	**322**[a] (100)	293 (100)
$C_9H_{11}Cl_3NO_3PS$		293.8 (40)	199 (35)
		198 (33)	
Diazinon	334 (18)	**277**[a] (100)	249 (100)
$C_{12}H_{21}N_2O_3PS$	305 (100)	249 (50)	169 (25)
		169 (50)	153 (12)
		153 (22)	
Fenoxycarb	350 (60)	**256**[a] (50)	238 (30)
$C_{17}H_{19}NO_4$	327 (30)	116 (100)	211 (100)
	302 (100)		183 (80)
Fonofos	247 (100)	**137**[a] (100)	127 (30)
$C_{10}H_{15}OPS_2$		127 (10)	109 (100)
		109 (25)	
Phenthoate	321 (100)	275 (100)	247 (100)
$C_{12}H_{17}O_4PS_2$	136 (15)	247 (45)	
Phosalone	368 (100)	322 (100)	294(90)
$C_{12}H_{15}ClNO_4PS_2$	282 (20)	182 (50)	182 (85)
	61 (60)		171 (100)
			153 (82)
			143 (50)
			115 (53)
Profenofos	375 (100)	347 (100)	289 (15)
$C_{11}H_{15}BrClO_3PS$		333 (25)	267 (20)
		305 (50)	223 (100)
			209 (18)
			188 (55)
			144 (35)
			128 (15)
Pyrazophos	374 (100)	**346**[a] (100)	318 (50)
$C_{14}H_{20}N_3O_5PS$		318 (20)	238 (100)
		238 (95)	222 (80)
		222 (100)	210 (30)
		210 (20)	194 (10)
		194 (30)	183 (5)
		183 (10)	

Table 1 (*Continued*)

Temephos $C_{16}H_{20}O_6P_2S_3$	467 (100)	419 (20) 405 (100) 357 (15) 281 (15)	357 (80) 312 (25) 281 (100) 251 (30) 233 (50) 203 (30)

Underlined ions correspond to the precursor ions selected in the MRM program.
[a] Selected precursor ions to further fragmentation.

Table 2
Time scheduled MRM conditions for monitoring insecticides.

Time window (min)	Pesticide	Ionization mode	t_r (min)	Isolation mass m/z	Isolation with m/z	Fragmentation	
						Cut-off	Amplitude
0.0–15.0	Fenoxycarb	Positive	13.4	302	2	100	0.8
	Phenthoate	Positive	13.9	319	2	100	0.6
15.1–17.9	Fonofos	Positive	15.8	305	2	100	1.2
	Diazinon	Positive	15.9	247	2	100	0.8
	Phosalone	Positive	16.7	368	2	100	1.6
	Pyrazophos	Positive	17.4	374	2	100	0.8
18.0–19.9	Chlorpyriphos methyl	Negative	19.0	302	2	100	1.1
20.0–22.5	Profenofos	Positive	21.6	374	2	100	0.8
22.6–30	Pirimiphos ethyl	Negative	23.7	304	2	100	1.6
	Temephos	Negative	23.3	451	2	100	1.2
	Bromophos ethyl	Negative	24.7	351	2	100	1.4
	Chlorpyrifos ethyl	Negative	25.3	330	2	100	1.0

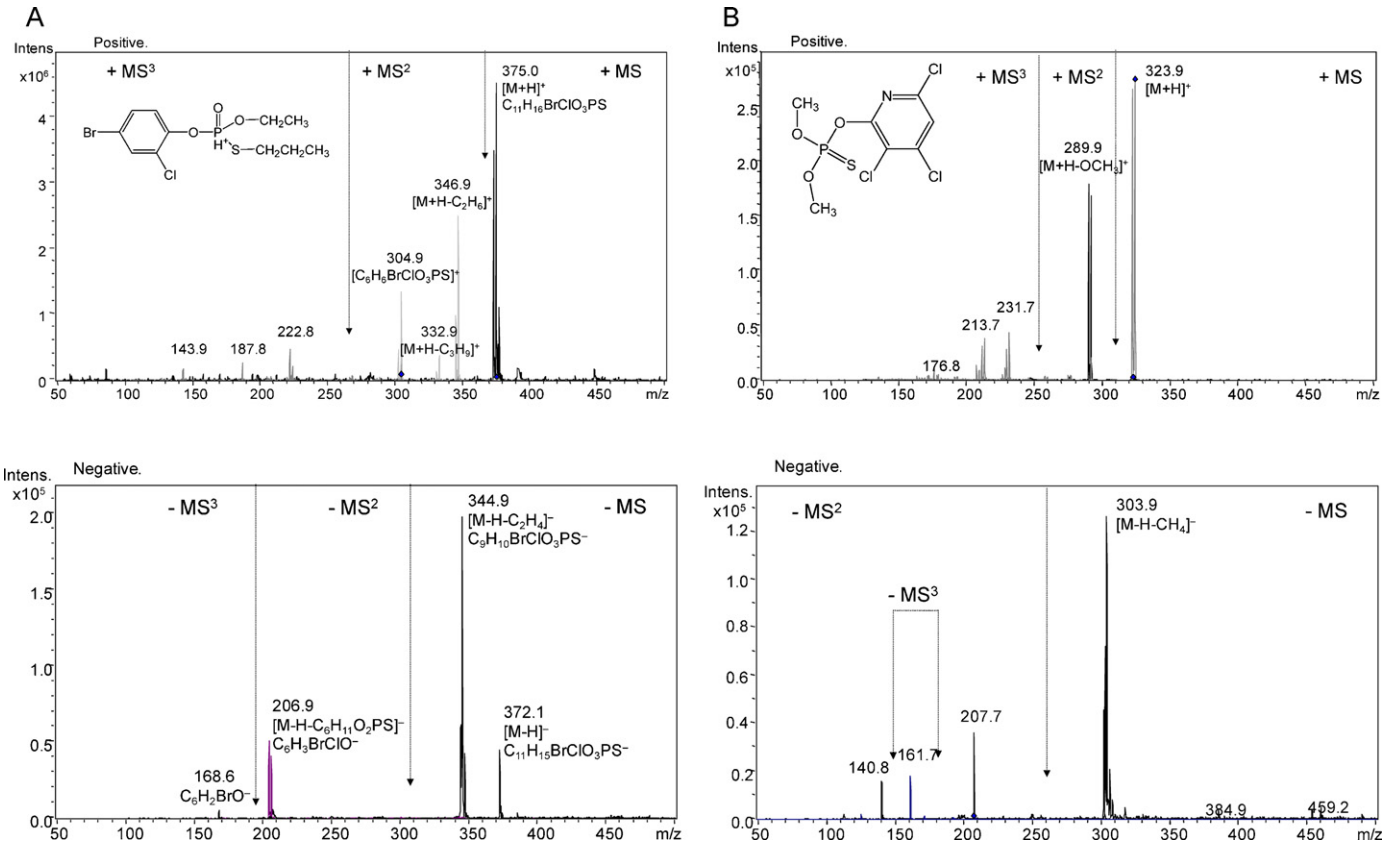

Fig. 1. Several stages of the mass spectra obtained for (A) profenofos and (B) chlorpyriphos methyl.

as precursor and most sensitive ion mode. Fig. 1 illustrated the fragmentation of some of the studied pesticides in both positive and negative ionization. In this figure the isotopic abundance due to the chlorine and bromide atoms, which is not reflected in Table 1, can be observed. Only one compound, fenoxycarb, did not give response in negative mode whereas bromophos ethyl and pirimiphos ethyl did not show any signal in positive ionization mode. The simultaneous analysis of the 12 insecticides in a single run can only be performed if the APCI interface was programmed to change the mode of operation between negative and positive ionization mode, along the chromatographic analysis. The change of polarity during the acquisition method was performed without any damage in the intensity of the peaks observed.

Most of the target pesticides are separated by LC prior to MS detection. Chromatographic separation on Luna C18 column using a methanol/water gradient revealed that six of the insecticides (fenoxycarb, phenthoate, fonofos, diazinon, phosalone and pyrazophos) are eluting before the chlorpyriphos methyl, which are eluting before profenofos. This way, it was possible to switch ionization polarities from positive for the first six insecticides to negative for the chlorpyriphos methyl, back to positive for profenofos and back to negative for pirimiphos ethyl, temephos, bromophos ethyl and chlorpyriphos ethyl in the same LC run, resulting in maximum intensities. The silica phase of this HPLC column ensures two important chromatographic properties: resolution and peak shape. The high efficiencies and bonded phase surface coverage provide for sharp peaks. This is allowed to cover a broad range of chemically different compounds. LC gradient has been optimized to distinguish the 12 pesticides keeping in mind that coeluted compounds showing different masses could be separated by the mass spectrometer using multiple reaction monitoring (MRM) mode. In order to achieve the best compromise between time analysis and sensitivity, the number of pesticides isolated and fragmented in a single window has been limited to 4 (see Fig. 1S of the Supporting material). The total LC cycling (separation and return to start conditions) program was 45 min.

The standards and spiked samples prepared to optimize and validate the method as well as samples were analyzed by LC–MS^2, according to the conditions listed in Section 2.5 and LC–MS^2 parameters comprising precursor ions, collision energies, scan times, and data acquisition time-windows for the pesticides listed in Table 2. Five compounds, bromophos ethyl, chlorpyrifos methyl, chlorpyriphos ethyl, pyrimiphos ethyl, and temephos were determined in negative ionization mode. The remaining pesticides, diazinon, fenoxycarb, fonofos, phentoate, phosalone, profenofos and pyrazophos where determined in positive ion mode. The MS^2 is a powerful technique that minimises the potential for interferences from co-extracted matrix constituents, and reduces the need for extra confirmation of the residues identity. The MS^2 reduces (or eliminates) interferences which translates into cleaner chromatograms containing fewer extraneous peaks. In addition to this gain of selectivity, the use of the MS^2 mode substantially increases sensitivity by limiting the high background noise related to the honey matrix. Each precursor ion was fragmented by collision-induced dissociation and the full product ions mass spectra were monitored in the range of m/z 100–500. The possibilities to use positive and negative ionization for the same compound and to perform MS^3 were applied to obtain a further confirmation of the identity of the compounds in real sample. According to this, it is easy to achieve 4 identification points (1 point for the precursor ion and 1.5 points for each product ion)as required for confirmation of contaminants in food of animal origin, which are listed in group A of Annex I to Directive 96/23/EC.

3.2. Optimization of the extraction methods

Fig. 2 presents the recoveries achieved for honey samples with the 4 extraction methods. All the methods allowed the recovery of all the pesticides. Absolute recoveries for the PLE, SPE and QuEChERS methods were between 75 and 100% whereas for the SPME recoveries were from 25 to 92%. In preliminary tests with SPE, the influence of the amount of honey (1.5, 3, 5 and 8 g) and the volume of water in which it is dissolved (30, 100 and 200 mL) were tested at different levels and conclude that the differences between 1.5 and 5 g as well as between 30 and 100 mL were insignificant since the experiments produced comparable results (data not shown). SPE was further carried out with the parameters given in Section 2.4.2. SPME and PLE methods were optimized in previous works [30,39]. The SPME was optimized for application in honey using a single quadrupole LC–MS, including the selection of best values for the different variables such as amount of sample, volume of water that dissolves fiber, fiber type and time of turmoil, were those reported in Section 2.4.1. PLE method optimized for fruits and vegetables in the previous work [39]. Because of this the influence of the amount of sample (1.5, 3, and 5 g) and the volume of water in which dissolves (5, 10 and 15 mL) were tested. The smaller the quantity of honey and the less the volume of water to dissolve it, the best are recoveries (data not shown). According to that indicated in Section 2.4.2, 1.5 g of honey was dissolved in 3 mL of hot water (this is the maximum amount of honey and the minimum water volume to dissolve it) and extracted setting the other parameters as already reported [39]. Among the different options that could be modified in the QuEChERS method, the selected one was that which does not acidifies the acetonitrile extractant solution, since the pesticides analyzed in this study do not require it and the clean-up was designed considering that honey is a type of sample with low lipid content. The optimum amount of honey and the volume of water to dissolve it were the same as in the previous method. In this case the relation to acetonitrile and the salting out effect was also taken into account.

3.3. Validation of the method

3.3.1. CC_α and CC_β

In the 2002/657/EC European Decision, the CC_α was defined as the limit at and above which it can be concluded with an error probability of α (α = 1% for forbidden substances) that a sample is non-compliant, and CC_β as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β (β = 5% for not permitted substances). CC_α and CC_β of the method were calculated as reported in Section 2 and are listed in Table 3. As can be seen, this method enables detection of pesticides in honey at ng g^{-1} level.

3.3.2. Recovery and precision

Fig. 2 shows the comparison between the recoveries obtained by the four methods at the CC_β and 50 μg kg^{-1}. The recoveries were also calculated to an intermediate level 10 μg kg^{-1} (data not shown), the results do not show differences with the previous ones. The lowest recoveries were obtained with the SPME (between 28 and 91% with a median recovery of 44% and a mean value of 50%). On the contrary the highest recoveries were obtained with the QuEChERS method (between 87 and 98% with a median recovery of 91.5% and a mean value of 91.7%). The remaining methods provided similar results: PLE (between 79 and 93%, with a median recovery of 89 and a mean value of 87.5) and SPE (between 81 and 101% with a median recovery of 89.5 and a mean value of 90.2).

The repeatability was similar and acceptable below 20% for all the methods: SPME (RSDs between 6 and 19% with a median

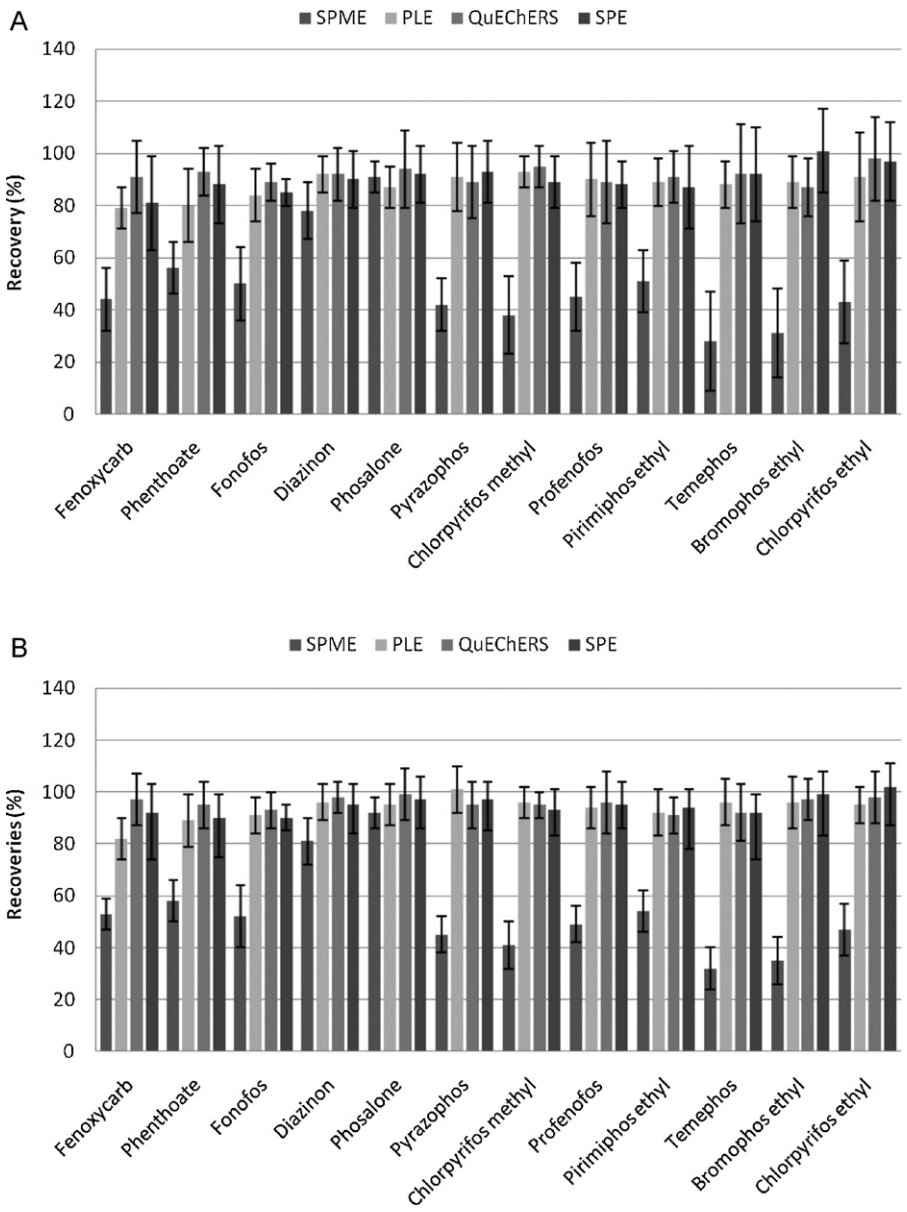

Fig. 2. Pesticide recoveries (%) with error bars representing the standard deviation ($n = 5$) obtained by SPME, PLE, QuEChERS and SPE in a multifloral honey (A) spiked at the LOQ level of each method and (B) spiked at 0.1 mg kg^{-1}.

Table 3
CC_α and CC_β obtained for the selected pesticides in honey by SPME, PLE, QuEChERS and SPE with LC–MS^2.

Pesticides	SPME		PLE		QuEChERS		SPE	
	CC_α ($\mu g\,g^{-1}$)	CC_β ($\mu g\,g^{-1}$)	CC_α ($\mu g\,g^{-1}$)	CC_β ($\mu g\,g^{-1}$)	CC_α ($\mu g\,g^{-1}$)	CC_β ($\mu g\,g^{-1}$)	CC_α ($\mu g\,g^{-1}$)	CC_β ($\mu g\,g^{-1}$)
Fenoxycarb	0.011	0.012	0.150	0.165	0.264	0.290	0.160	0.176
Phenthoate	0.002	0.002	0.039	0.042	0.065	0.072	0.037	0.040
Fonofos	0.001	0.001	0.016	0.018	0.021	0.024	0.015	0.017
Diazinon	0.001	0.001	0.023	0.025	0.042	0.047	0.024	0.026
Phosalone	0.005	0.006	0.141	0.155	0.242	0.266	0.137	0.151
Pyrazophos	0.001	0.001	0.006	0.007	0.022	0.024	0.006	0.007
Chlorpyrifos methyl	0.055	0.060	0.541	0.595	1.050	1.155	0.587	0.646
Profenofos	0.005	0.006	0.068	0.074	0.134	0.148	0.070	0.077
Pirimiphos ethyl	0.001	0.001	0.016	0.017	0.021	0.024	0.017	0.019
Temephos	0.005	0.006	0.043	0.048	0.089	0.098	0.045	0.049
Bromophos ethyl	0.001	0.001	0.010	0.011	0.022	0.024	0.009	0.010
Chlorpyrifos ethyl	0.001	0.001	0.013	0.014	0.022	0.024	0.013	0.014

Table 4
Calibration slopes obtained for the selected pesticides preparing the standard in blank honey by PLE, QuEChERS and SPE with LC–MS^2. The concentration range for each compound was from CC_β to 1000 CC_β.

Pesticides	PLE			QuEChERS			SPE		
	Slope	R^2	% matrix effect	Slope	R^2	% matrix effect	Slope	R	% matrix effect
Fenoxycarb	12,857	0.9989	40	6937	0.9999	35	10,267	0.9987	25
Phenthoate	40,013	0.9988	36	23,332	0.9993	30	41,813	0.9992	22
Fonofos	58,099	0.9994	31	44,474	0.9992	25	61,517	0.9990	17
Diazinon	62,499	0.9993	28	33,244	0.9989	27	60,096	0.9991	15
Phosalone	89,348	0.9997	27	50,005	0.9990	25	91,714	0.9998	14
Pyrazophos	220,660	0.9998	24	65,987	0.9993	20	220,660	0.9996	12
Chlorpyrifos methyl	3003	0.9990	17	1628	0.9986	15	2766	0.9996	4
Profenofos	161,152	0.9989	14	82,487	0.9986	10	154,874	0.9994	1
Pirimiphos ethyl	89,342	0.9992	12	64,298	0.9989	10	79,938	0.9997	2
Temephos	315,793	0.9997	8	154,674	0.9993	5	309,349	0.9995	1
Bromophos ethyl	150,816	0.9996	8	69,124	0.9994	10	165,898	0.9992	2
Chlorpyrifos ethyl	109,990	0.9995	10	64,161	0.9990	5	109,990	0.9992	3

repeatability of 12.5%); QuEChERS (RSDs between 7 and 19% with a median repeatability of 12.5%); PLE (RSDs between 6 and 17% with a median repeatability of 9.5%) and SPE (RSDs between 5 and 18% with a median repeatability of 13.5%).

3.3.3. Linearity and matrix effects

The linearity, plotted as MS response area vs concentration estimated for the matrix matched standards (PLE, SPE and QuEChERS) are presented in Table 4. Calibration curves have been produced for quantification. They were built using blank honey matrix spiked after the extraction step at five different pesticide levels, including the zero point (Table 4). Linearity has been observed all along the area of concentration studied depending on the chemicals. These ranges of concentrations were selected in function of the sensitivity of the mass spectrometer towards each pesticide from the CC_β to 1000× CC_β together with correlation coefficient (R^2) of the linear regression. None of the compounds showed residual level or background signal in the unfortified honey matrix. On the other hand, however, even if co-extractives do not interfere in the analysis of the pesticides, they can build up in the LC–MS^2 system and cause problems related to ruggedness of the analytical method such as signal enhancement, or conversely suppression of some susceptible pesticides. To evaluate these effects, the % of matrix effect (percentage of the quotient between the slopes of the standards in methanol and matrix matched standards) was evaluated. This calibration procedure permits to avoid matrix effect in the APCI source, such as ion enhancement or suppression.

In the case of the SPME method, the whole analytical procedure using SPME combined with LC–MS^2 was tested for linearity in the range of CC_β to 1000× CC_β. Linear relationship was obtained for each pesticide in this range (six points calibration) from water and honey (see Table 1S supplementary material). Great differences in the regression equations between water and honey were found, which is reasonable as demonstrated in the previous optimization of the SPME method by LC–MS [30].

Additionally to criteria required by the 2002/657/EC, a second calibration curve was run at the end of each sample series to check the stability of the detector response after unknown sample data acquisition. The requirement set was that the end curve had to show a bias lower than 20%, compared to initial calibration.

3.3.4. Ruggedness and specificity

Analyses of 20 blank honey samples from different origins indicated that no interferences for the product ion of each individual analyte. Over the time period employed for developing this method, variations in the retention times of the analytes never exceeded 2.5% and over 4 months, the tolerance of the relative ion abundances of the product ion mass spectrum did not vary more than 20%. These figures are in compliance with the maximum permitted tolerance for relative ion transitions (see Section 2.3.3.2 of the 2002/657/EC European Decision) [38].

3.4. Analysis of real honey samples

In order to compare SPME, QuEChERS, SPE and PLE, the data obtained by analyzing 25 honey samples, were compared (Table 5). As can be observed, of the 25 analyzed samples, only six contained pesticide residues. Fig. 3 presents the chromatograms obtained from a honey sample (orange blossom 2) obtained by SPME and QuEChERS. Compounds were identified by the two methods, however, higher quantity of residues was quantified by QuEChERS (Table 5). In addition, fenoxycarb, phentoate, phosalone, chlorpyriphos ethyl, profenofos and chlorpyriphos methyl were not found in real honey samples by any extraction methods. It should be taken into account that pesticide metabolites of the studied compounds have not been selected as target analytes of the method and they cannot be detected if present in the samples. This fact could cause an underestimation of the pesticide residues in the sample. In this study, honey samples were directly provided by the beekeepers, just after harvesting, and samples were kept at 4 °C in darkness and analyzed within a week. It is highly improbable that degradation

Table 5
Pesticide levels found in honey samples (values obtained were not corrected by the recoveries).

Type of honey sample	Pesticide	Level μg kg^{-1} ($X \pm$ SD, $n = 3$)			
		SPME	PLE	QuEChERS	SPE
Orange blossom 1	Temephos	100.2 ± 14.2	225.6 ± 15.3	236.7 ± 12.5	296.3 ± 12.8
Orange blossom 2	Fonofos	15 ± 2.2	25.2 ± 1.4	27.9 ± 7.2	25.5 ± 6.4
	Pyrazophos	12.4 ± 1.8	26.8 ± 2.9	26.2 ± 8.2	26.6 ± 8.2
Rosemary	Diazinon	34.2 ± 1.5	40.3 ± 2.1	40.3 ± 3.2	39.8 ± 4.4
Lemon 1	Diazinon	42.4 ± 1.3	54.6 ± 1.6	54.6 ± 3.6	52.4 ± 6.8
Lemon 2	Pirimiphos ethyl	19 ± 5.0	33.2 ± 3.5	33.9 ± 4.7	33.5 ± 3.7
Lavender	Bromophos ethyl	11.5 ± 1.3	33.0 ± 3.4	32.3 ± 5.2	33.7 ± 4.8

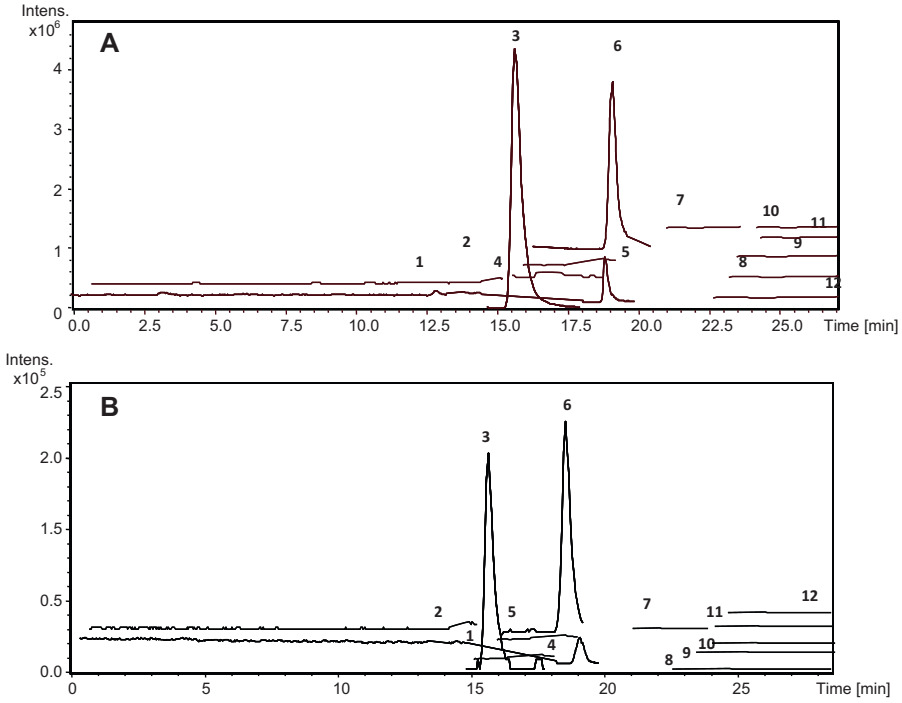

Fig. 3. Chromatogram of a real sample (orange blossom 2) extracted by the different extraction methods (A) SPME, (B) QuEChERS.

of pesticides by the storage occurs. The differences in the concentrations of each pesticide according to the extraction method can be explained by the low recoveries obtained by SPME resulting in lower efficiency of extraction than the other three extraction method.

SPME applied to honey analyses presents advantages as compared to conventional methods regarding the organic solvent saving and time consumption (60% less, after optimization of extraction conditions). Concerning the quantitative results, the detection limits reached using SPME were at least one order of magnitude better than those achieved with the other extraction methods, while the reproducibility obtained clearly presented RSD lower than 6%, due to the SPME's better precision, which reduces the number and magnitude of mistakes.

4. Conclusions

The lowest CC_α for the analysis of the selected pesticides from honey samples were achieved with the SPME method and the highest with the QuEChERS method. The CC_β achieved by LC–MS^2 were between 0.001 $\mu g\,g^{-1}$ (for fonofos, diazinon, pyrazophos, pirimiphos ethyl, bromophos ethyl and chlorpyriphos ethyl) and 0.060 $\mu g\,g^{-1}$ (for chlorpyriphos methyl) for the SPME against values in the range of 0.024 $\mu g\,g^{-1}$ (fonofos, pyrazophos, pirimiphos ethyl, bromophos ethyl and chlorpyrifos ethyl) to 1.155 $\mu g\,g^{-1}$ (chlorpyriphos methyl) for QuEChERS. The comparison of the four methods showed that all of them recover all the selected pesticides with a good repeatability. Nevertheless, QuEChERS method presented the highest recoveries (mean recovery 91.67%) followed by the SPE (mean recovery 90.25%) and the PLE (mean recovery 90.25%) whereas the SPME showed the lowest recovery (mean recovery of 49.75%) of the four selected methods at the CC_β fortification level. The QuEChERS method was the most adapted method with around 58% of recoveries higher than 90%. The SPE and PLE were proven also to be adapted for the extraction of these pesticides. The SPME is accurate as monitoring method for the extraction of the selected pesticides from honey but cannot be implemented as currently applied as quantification method due to its low recovery for pyrazophos, chlorpyriphos methyl, temephos and bromophos ethyl. The application of the internal standard should be considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.045.

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