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Ultrasound-assisted extraction based on QuEChERs of pesticide residues in honeybees and determination by LC–MS/MS and GC-MS/MS

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Abstract

In this work, 260 pesticide residues, including insecticides, acaricides, fungicides and herbicides, were extracted from honeybees using the QuEChERS methodology modified by applying an ultrasonic probe, which avoided the homogenization step and reduced the extraction time. Gas and liquid chromatography, both coupled to triple-quadrupole mass spectrometry, allowed the determination of the pesticide residues extracted from the samples. The optimization of the main ultrasonic conditions (sonication amplitude, number of cycles and time of each cycle) was performed using a Box-Benhken Experimental Design involving 15 experimental samples. The results obtained with this approach showed that the recoveries were not affected by these experimental parameters for 95 pesticide residues whereas the sonication amplitude was the main factor affecting the recoveries of 107 pesticide residues. The extraction time and the number of cycles affected 4 and 1 pesticide residues, respectively. The effectiveness of the ultrasonic-assisted extraction without homogenization of the honeybee samples compared favorably with those for the conventional QuEChERS methodology applied to the same previously homogenized samples. The proposed methodology was validated according to the SANTE/11945/2015 guidelines, with a 5 μg/Kg limit of quantitation. Recoveries between 70-120% and relative standard deviations lower than 20% were obtained for most analytes. Thirty honeybee samples taken from Spanish apiaries were analyzed using this new methodology. The results revealed the presence of 30 different pesticide residues in the honeybee samples, the highest concentration levels corresponding to certain insecticides/acaricides used by beekeepers to control *Varroa destructor*. Permethrin, thiabendazol, carbendazim and coumaphos were the most frequently detected pesticide residues in the selected samples.

Keywords: Honeybees, pesticide residues, ultrasound-assisted extraction, LC-QqQ-MS/MS, GC-QqQ-MS/MS

Introduction

Bees contribute to ecosystem services and their decline threatens pollination of both wild and cultured plants, threatening biodiversity and food production [1]. Indeed, one third of the world's food depends on these pollinators [2]. One hypothesis explaining the high bee mortality rate is the combination of several factors such

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as pathogens, parasites, climate change, the lack of biodiversity and floral abundance, and the increasing use of pesticides in agricultural and apicultural practices [3,4]. In the latter, bees are subjected to unintentional exposure during their foraging activities to pesticides applied to crops and/or they are exposed in their hives to pesticides that have been intentionally introduced to suppress pests such as the *Varroa destructor* mite. The European Food Safety Authority (EFSA) [5,6] has initiated a series of actions, involving scientists from different areas, to establish a risk assessment of the multiple factors affecting bees. Recently, Rortais et al. [4] reviewed the principles for risk assessment of multiple stressors in bees, pesticides amongst them, to collate high quality data for use as a regulatory tool.

With the aim of examining honeybee contamination, several pesticide multi-residue methods have been described that include numerous pesticides. Sample preparation is a challenging issue because this matrix contains large amounts of beeswax, proteins and other substances that are readily extractable by organic sorbents. To date, several multi-residue analytical methods have been reported for determining pesticides in honeybees using gas chromatography-tandem mass spectrometry (GC-MS/MS) [7-9] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [9-11] involving different sample treatments. For instance, S. Walorczyk et al. [7] developed a sample preparation approach for 150 pesticides, based on acetonitrile:water extraction followed by hand shaking with citrate, anhydrous magnesium sulfate and sodium chloride. Subsequently, low-temperature precipitation clean-up (the freezing-out procedure) was applied to remove the long-chain aliphatic hydrocarbons and esters of aliphatic fatty acids coming from the beeswax; finally, a dispersive solid-phase extraction (d-SPE) with PSA (primary secondary amine), C18 (octadecylsilane)and GCB (Graphitized Carbon Blacks) was used to eliminate the remaining matrix constituents. These authors found that dispersive sorbents had a notable impact on the recovery of pesticides that were susceptible to adsorption, in such a way that a careful optimization of each sorbent amount was necessary. To find a sample extraction procedure with only one protocol that gives acceptable recoveries for analytes belonging to different chemical classes, L. Wiest et al. [8] extracted 80 pesticides from honeybees using a QuEChERS-based method modified by adding 3 mL of hexane to the acetonitrile solvent to eliminate lipids that interfere with the MS analysis. The authors found that volumes smaller than 3 mL do not sufficiently remove apolar interferences, while higher volumes resulted in low recoveries of apolar pesticides. The same strategy was used by Z. Bargańska et al. [10] for the simultaneous screening of 19 pesticides in honeybees. In this work, the recovery of some analytes decreased by adding hexane, the lowest being 22% for diazinon. Likewise, K.M. Kasiotis et al. [11] extracted 150 pesticides from honeybees using acetonitrile (with and without 2% triethylamine), deionized water and hexane. The use of triethylamine improved the extraction-elution steps for neonicotinoids because it provides basic pH conditions, thus preventing the protonation of basic or weak basic compounds, while extraction without trietylamine was used for the rest of the pesticides. Recently, the buffered QuEChERS method has been modified using new sorbents in the clean-up step. In this way, T. Kiljanek et al. [9] developed a novel method to determine 200 pesticide residues (98% of them approved for use in the European Union) and pesticide metabolites in honeybees. Bee samples were extracted with acetonitrile containing 1% acetic acid and then subjected to clean-up by dispersive solid phase extraction (dSPE) using PSA and a new Z-sep+ sorbent. These authors concluded that the use of both sorbents showed excellent honeybee extract clean-up with about 99.6% matrix removal efficiency.

In all these methods, the extraction of pesticide residues requires sample homogenization, this prior step being difficult when only small amounts of sample are available. A valuable alternative in solid sample treatment is the use of ultrasound irradiation. Ultrasound energy exerts its chemical effects through the cavitation phenomenon, in such a way that (i) surface particles can occur causing an increase in the surface area available for extraction, (ii) bulk sample particles can enhance the ability of the extraction solvent to leach analytes, (iii) extremely high temperatures and pressures generated during the bubble collapse (implosion) result in increased solubility and diffusivity of analytes, as well as in the penetration and transport at the interface

between the extraction solvent and the solid matrix and (iv) leaching is facilitated by the oxidative energy of radicals [12,13]. Although ultrasonic baths have been more widely used in ultrasound-assisted solvent extraction (UASE), ultrasonic probes are more efficient as they focus the ultrasonic energy on the sample zone, which results in more experimental reproducibility and repeatability [14]. In any case, ultrasound-assisted extraction is usually faster and more efficient than conventional extraction, providing high efficiencies with reduced extraction solvent consumption, along with similar or better yields [15].

The main goal of this study was to assess the exposure of honeybees to pesticide residues within a monitoring program framework involving a wide range of honey production and agricultural sites in Spain. In addition, the use of ultrasonic-assisted extraction, rather than the homogenization step used in the QuECHERS method, was applied for the first time as sample treatment procedure and it was followed by the clean-up of extracts with PSA and zirconium dioxide-based (Z-sep) sorbents and detection by GC-MS/MS and LC-MS/MS.

Experimental

Chemicals and materials

Analytical-grade standards of pesticides and metabolites (260 pesticides in total) of high purity (> 98%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and from Sigma–Aldrich (Steinheim, Germany). Isotope–labeled internal standards of dichlorvos-d6, malathion-d10 and TTP were used as surrogate standards and were purchased from Dr. Ehrenstorfer and from CDN Isotopes (Quebec, Canada).

Acetonitrile (ACN) and methanol (MeOH), both of HPLC-grade, were obtained from Sigma–Aldrich and ethyl acetate was obtained from Fluka (Steinheim, Germany). Formic acid and trisodium citrate dihydrate were purchased from Fluka, sodium chloride was purchased from J.T Baker (Deventer, Netherlands), disodium hydrogencitratesesquihydrate was purchased from Sigma-Aldrich and anhydrous magnesium sulfate was supplied by Panreac (Barcelona, Spain). Primary-secondary amine (PSA) bonded silica and Z-Sep bulk sorbents were supplied by Supelco (Bellefonte, PA, USA).

A Sonopuls HD 3100 ultrasonic system was used, supplied by Bandelin Electronic GmbH & Co. KG (Germany); it was equipped with a GM 3100 high intensity generator (100 W), a UW 3100 ultrasonic converter, an SH 70G standard horn and a 3 mm-diameter titanium MS73 probe for 2-50 mL volumes along with an AGYTAX® automatic axial extractor supplied by Cirta Lab. S.L. (Spain).

Pesticide standard solutions

Individual pesticide stock solutions (1000–2000 mg/L) were prepared in HPLC-grade acetonitrile and ethyl acetate for LC and GC analysis, respectively. All individual stock solutions were stored in amber screw-capped glass vials in the dark at −20 °C. For optimization and calibration, working solutions were prepared daily by appropriate dilution of the stock standard solutions, which were kept at -20 °C. For optimization of the ion-source-dependent parameters for LC-MS/MS and GC-MS/MS operations, individual standard solutions of each pesticide were prepared at 100 µg/L in methanol and 1 mg/L in ethyl acetate, respectively. For the calibration studies, working standard solution mixtures were prepared at different concentration levels in ACN:H_2O (20:80, v/v) and ethyl acetate for LC and GC, respectively.

Honeybee samples and spiking procedure

Honeybee samples were collected by beekeepers from 30 apiaries which were randomly selected across different Spanish regions during 2015. Any disorder or high mortality was reported by the beekeepers. All samples were transported at low temperature (in a cooler) to the laboratory and were frozen at -20ºC until analysis.

A honeybee sample from an ecological apiary was analyzed to confirm the absence of pesticide residues and used as the blank for optimization and validation studies according to the SANTE11945/2015 guidelines [16].

Honeybees were spiked with a standard mix of pesticides in methanol (100 μL of the standard per gram of honeybees) and after 30 min, 30 honeybees (approximately 2 g) were weighed in a 50 mL Falcon tube for UASE and extracted as described below.

Sample preparation procedure

Pesticide residues were extracted from the honeybees using UASE with acetonitrile, followed by a d-SPE clean-up step with PSA and Z-Sep. In the first step, 30 honeybees were weighed in a 50 mL PTFE centrifuge tube (approximately 2 g) and 5 mL of ultrapure water was added. After waiting for 5 min, 5 mL of ACN and 25 μL of the 10 mg/L internal standard solution mix (surrogated) were added. The sample-extractant mixture was sonicated at a 75% amplitude for 140 s (ten extraction cycles of 12 s each plus a 2 s pause between them). After that, 2g of anhydrous magnesium sulfate, 0.5 g of sodium chloride, 0.5 g of trisodium citrate dihydrate and 0.25 g of disodium hydrogen citrate sesquihydrate were added and the mixture was automatically shaken for 5 min and then centrifuged at 3500 rpm at ambient temperature for 5 min. In the second step (clean up), 2 mL of the supernatant extract were collected in a 15 mL PTFE centrifuge tube and 750 mg of anhydrous magnesium sulfate, 125 mg of PSA and 125 mg of Z-Sep were added. The mixture was shaken in a vortex for 30 s and afterwards it was centrifuged at 3500 rpm for 5min. Finally, 1 mL of cleaned extract was transferred into a screw-cap vial and 10 μL of acetonitrile with 5% formic acid were added.

For GC analysis, 125 μL of the final extract was evaporated to dryness under N_2, reconstituted in the 50 μL of ethyl acetate (corresponding to 1gr of honeybee matrix in 1 mL of extract) and 2 μL were directly injected, whereas for LC analysis, 50 μL of the final extract were diluted with 450 μL of ultrapure water (corresponding to 0.04 gr of bee matrix in 1 mL of extract) and 5 μL were injected into the LC system.

Analytical procedures

GC-QqQ-MS analysis

A total of 153 pesticide residues were analyzed in a 7890 GC equipped with a 7693B autosampler and a 7000 series GC-QqQ-MS system (Agilent Technologies, Palo Alto, CA, USA). The separation of these compounds was performed on an HP-5MS UI (15m×0.25mm×0.25μm) column from Agilent. Samples were injected into a 7890A GC multimode inlet using the splitless-injection mode through an inlet liner filled with a glass wool frit (Ultra Inner liner) from Agilent. 2 μL of sample extract was injected under the following operating conditions: the injector temperature was kept at 80 °C during the solvent evaporation stage, then ramped up to 300 °C at 600 ºC/min and, finally, this temperature was maintained for 20 min.

Helium (high purity) was used as both the carrier gas and the quenching gas, and nitrogen (high purity) as the collision gas. The oven temperature program was set as follows: 70 °C for 1 min, then up to 150 °C at 50 °C/min; next to 200 °C at 6 °C/min and finally to 280 °C at 16 °C/min. The total run time was 23 min, including backflushing at 280 ºC for 3 min in order to shorten the analysis time and to reduce system maintenance.

The triple quadrupole mass spectrometer (QqQ-MS) was operated in the multiple reaction monitoring (MRM) mode using electron impact ionization (EI) as the ionization source. The temperatures of the transfer line, ion source and the first quadrupole (Q1) and second quadrupole (Q2) were 280 °C, 280 °C and 150 °C, respectively. The electron multiplier voltage was set at 1592 V and mass peak widths were set to “wide” in the first and third quadrupoles (Q1 and Q3).

The analysis was performed with a solvent delay of 2 min to prevent instrument damage. Retention Time Locking (RTL) was used to eliminate the need for adjusting the time segment windows of the multiple reaction

monitoring (MRM) groups, using trifluralin as the reference compound at a retention time (t_R) of 5.81 min. For control and data analysis, MassHunter QQQ Acquisition and Quantitative Analysis B.07.00 software (Agilent) was used.

The MS/MS detection was optimized firstly with individual injections in full-scan mode of each pesticide at 1 mg/L in order to obtain their t_R and to select the optimal precursor ions, the most intense ion with the highest m/z value being selected in most cases. Next, the product-ion scan methods were automatically created by the Mass Hunter software with different collision energies (CE) ranging from 5 to 30 V; this was done to select the product ions with the best analytical response. Table S1, included in the Electronic Supplementary Material (ESM), shows the t_R, the two most intense product ions for each pesticide and their optimal CE, with the most intense selected as the quantifier ion (SRM1) and the second as the qualifier ion (SRM2).

The collision gas flow was 1.5 mL/min and the quenching gas flow was 2.25 mL/min, the optimal values recommended by the manufacturer. A 4-time-segment method was created to obtain adequate sensitivity and signal-to-noise ratio (S/N), and the cycle time for each segment was set between 200 and 250 ms.

LC-QqQ-MS analysis

An Agilent UPLC 1290 Series coupled to an Agilent 6490 TripleQuad-LC/MS from Agilent Technologies was used for the analysis of 107 pesticide residues (including their metabolites). The chromatographic separation was performed on a Zorbax Eclipse Plus C8 column of 1.8 μm × 2.1 mm × 100 mm (Agilent). The temperature of the LC column was maintained at 35 ºC and the analytes were separated using a gradient program of 0.1% formic acid in ultrapure water as solvent A and 0.1% formic acid in acetonitrile:ultrapure water (95:5, v/v) as solvent B at a constant flow rate of 0.3 mL/min. The optimized gradient program was as follows: 20% of B (initial conditions) for 2 min, then a linear gradient up to 100% of B in 13 min plus 2 min more under these conditions (100% B); finally, the mobile phase came back to the initial conditions (20% B) in 2.5 min. The total run time was 17.5 min and the injection volume was 5 μL.

The UPLC was coupled to a QqQ-MS with an electrospray interface (ESI), operating in both positive and negative ionization modes (PI and NI), with 380 V selected as the fragmentor voltage and 3000 V for the capillary voltage of both the PI and NI. The ESI source parameters were: 120 ºC for the drying gas temperature at a flow rate of 13 L/min, 375 ºC for the sheath gas temperature at a flow rate of 10 L/min and 45 psi for the nebulizer pressure. Nitrogen (high purity) was used as the nebulizer gas and collision gas.

For the optimization of the MS parameters, individual pesticide standard solutions of 100 μg/L in acetonitrile:water (1:1, v/v) were infused directly into the MS system in full-scan mode with a mass range of 50–800 m/z, and the most intense ion was selected as the precursor ion for each analyte. Next, optimal CE were selected for the two most intense transitions of each analyte in product ion mode; the most intense of them being the quantifier ion (SRM1) and the second, the qualifier ion (SRM2). Table S2 (in the ESM) shows the t_R, the precursor ion, the two transitions (SRM1 and SRM2) for each pesticide and their corresponding CEs.

For control and data analysis, the MassHunter QQQ Acquisition and Quantitative Analysis B.07.00 software (Agilent) using Dynamic MRM software with a retention time window of 0.8 min was used.

Experimental design and statistical analysis

To optimize the main factors affecting the extraction of pesticide residues from the honeybees using UASE, the Box-Behnken experimental design (BBD) was applied [17]. The three factors optimized were the amplitude of the ultrasonic probe (between 50% and 100%), the number of extraction cycles (between 10 and 20 cycles) and the time for each extraction cycle (between 4s and 12s). These factors were studied at three previously selected levels, involving a total of 15 experiments, which included three replicates for the central

value. The BBD method was optimized, using as the response the bias obtained for honeybee samples spiked at 10 μg/Kg for all pesticides, applying UASE for pesticide residue extraction and d-SPE for extract cleaning.

Results and discussion

Optimization of the ultrasonic extraction method

Box-Benhken Experimental Design

For the extraction of pesticide residues from honeybees, the QuEChERS method was modified by using ultrasonic probe extraction, avoiding the pre-homogenization and sample shaking steps.

The sequential optimization study in the UASE method requires a great number of experiments that depend on several factors associated with the probe parameters. Therefore, an experimental Box-Benhken design was applied for the optimization of the UASE step.

The main factors affecting UASE were sonication amplitude and extraction time, the later including two parameters: the number of sonication cycles and the sonication time in each cycle. For this, we considered sonication amplitude (Factor A), cycle numbers (Factor B) and cycle time (Factor C). All factors were evaluated at 3 value levels: Factor A (50, 75 and 100% of sonication amplitude), Factor B (10, 15 and 20 cycles) and Factor C (4, 8 and 12 s for each cycle). Thus, the BBD for the 3 factors and the 3 levels consisted of 15 experiments, including 3 central points. Table S3 (in the ESM) shows the value levels for the 3 factors and their corresponding code values for the 15 experimental runs.

Using the ultrasonic probe increases the temperature during the extraction step, reduces the viscosity and surface tension and improves the solubility of the pesticides, allowing a higher rate of extraction. However, too high a temperature can lead to losses or a degradation process in some pesticides. Therefore, the maximum temperature was fixed in the ultrasonic probe system as 60 ºC in such a way that this value was maintained through each UASE experiment.

The optimization of the UASE step was performed using honeybee blank samples spiked at 10 μg/Kg for all the pesticides and extracted according to the experimental conditions of each BBD run and following the whole extraction method, including the QuEChERs salts addition and clean up step with PSA and Z-Sep. Recoveries were obtained for all the pesticide residues in each experiment and were included in the experimental design as a response for optimization.

Determination of the significant factors in the pesticide extraction

To identify the factors that influence the efficiency of the extraction process, an analysis of variance (ANOVA) test was applied to the experimental data. The results showed that factors with a p-value ≤ 0.1 affected the extraction of the pesticide residues from the honeybees (Table S4 in the ESM).

The analysis of the obtained results shows that the extraction of 95 pesticide residues was not affected by the experimental conditions of the sonication probe, whereas 112 pesticide residues were affected by one factor. Thus, the sonication amplitude affects the extraction of most pesticides (107 pesticide residues), the extraction time in each cycle affects the extraction of 4 of them (famoxadone, fenthion sulfoxide, imazalil and trifloxystrobin) and the number of cycles affects the recovery of methoxyfenozide. On the other hand, 31 pesticide residues were affected by two factors, 10 of them by both the sonication amplitude and the number of cycles, and 21 by both the sonication amplitude and the extraction cycle time. Lastly, 22 of the pesticide residues were affected by all the factors.

Optimization of the significant factors using Response Surface Methodology

Response Surface Methodology (RSM) was applied in order to select the optimum values for the UASE variables (Table S4, in the ESM). The recovery of more than 65 % of the pesticide residues depends on the experimental conditions of the ultrasonic probe. Thus, for some of them, when the sonication amplitude and/or the extraction time increases (more cycles and more extraction time in each cycle), the recoveries are higher than 120%. This may be because working with high cycle numbers and extraction times per cycle also increases the background of the chromatogram consequence of a high concentration of coextracted compounds, which negatively affects the analyte quantification. Therefore, the negative bias was selected as a response to determine the optimum extraction parameter values and to minimize the coextraction of matrix components, in such a way that error 0 would be the optimum response.

Table S4 shows the optimum values of the three studied variables to achieve the best recovery for those pesticide residues that are affected by the sonication conditions (P-value ≤ 0.10). One can see that the extraction of 70 pesticide residues improved when increasing the probe's sonication amplitude and, in addition, for 27 of them the extraction increased with the sonication time per cycle. Only in the case of the diphenyl and dimethipin residues were the optimum conditions for extraction achieved at the higher values for the three sonication factors (100% sonication amplitude, 12s time cycling and/or 20 extraction cycles). However, the best recoveries for 59 of the pesticide residues were found at the lower values for the three considered factors. Thus, the optimum conditions for pesticide residue extraction were selected as a compromise, taking the intermediate values for the UASE method (75 % amplitude sonication and 10 extraction cycles of 12s each).

Comparison of the UASE method with conventional QuEChERS

The QuEChERS-based method has been one of the most widely used for extracting pesticide residues in various matrices, honeybees amongst them [8,9].

To establish the effectiveness of the optimized UASE method, it was compared with conventional QuEChERS in terms of recovery (Fig. 1). Using UASE, acceptable recoveries within the SANTE range (70-120 %) were obtained for more pesticide residues than when using conventional QuEChERS (237 and 185 pesticide residues, respectively).

Furthermore, when using UASE, 18 pesticides showed recoveries lower than 70% while 39 pesticides presented recoveries in the same range as using the conventional QuEChERS method without sonication. On the other hand, only 4 pesticides (chlorfenapyr, fenthion, phentoathe and prometryn) showed recoveries higher than 120% when using UASE vs. 35 pesticides when using conventional QuEChERS (with previous sample homogenization); this is because ultrasonic-assisted extraction is more selective and the level of matrix interferences extracted with the first method is low.

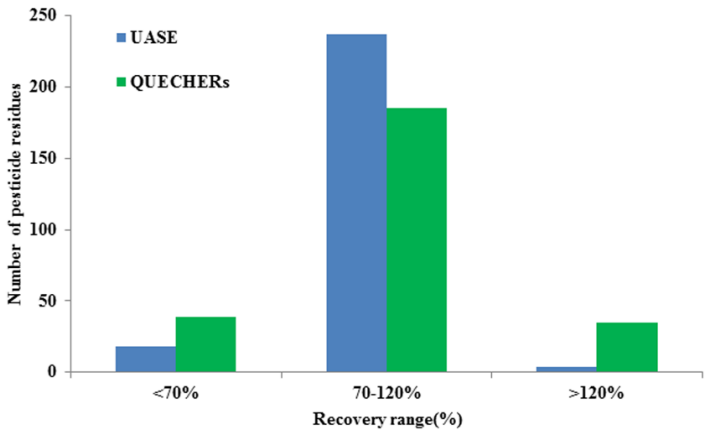

Fig. 1 Recovery ranges of pesticide residue in honeybees by UASE and conventional QuEChERS

Method Validation and Quality Control

To ensure the quality of the analytical results, the proposed method was validated in accordance with Document SANTE/11945/2015 [16] established for pesticide residue analysis in food and feed.

The linearity was determined using calibration standards in triplicate at five different concentration levels, between 0.5 and 100 μg/L in acetonitrile:ultrapure water (1:9, v/v) for LC-MS/MS, and between 5 and 500 μg/L in ethyl acetate for GC-MS/MS.

Good linearity was found for most pesticides in the concentration range considered in this study ($R^2 > 0.99$), except for some in which the linear range was lower depending of their sensibilities.

Subsequently, calibration standards of all the pesticides in blank honeybee extract (matrix matched), at the same concentration as in pure solvent, were analyzed by LC-MS/MS and GC/MS/MS. Tables 1 and 2 show the linear range for each pesticide in matrix-matched and their corresponding R^2, which were higher than 0.99 in all cases. To determine the matrix effect, the slopes of both calibration curves (in pure solvent and in matrix-matched) were compared. For GC analysis (Table 1), an enhancement of the signal was found for all pesticides (the exception being folpet, which had a low signal suppression, ME(%) = -17) while for LC analysis (Table 2) more than 60% of the pesticides showed low or medium signal suppression (the exception being aldicarb sulfoxide, ME(%) = -64) and for the rest an enhancement of the signal was found. In general, 31% of all pesticides showed a low matrix effect (ME(%)< $|20|$, 25% presented a medium matrix effect ($|20| < ME(\%) < |50|$) and the rest (44%) experienced a strong matrix effect (ME(%) > $|50|$), the ME for 36 pesticides being higher than 100%. These values are lower that those obtained by using conventional QuEChERs method. For this reason, the quantification of pesticide residues in real samples should be carried out using matrix-matched calibration or standard addition method.

Recovery and precision studies were carried out in honeybee blank samples, by replicate (n=5), spiked at two concentration levels, corresponding to the quantitation limit (LOQ) and 50 μg/Kg. Tables 1 and 2 show the mean recovery (%) and RSD (%) for GC/MS and LC/MS analysis, respectively. One can see that the recovery for most pesticide residues (97% of the pesticides in GC-MS/MS and 99% of the pesticides in LC-MS/MS) ranged between 60-120% at the two concentration levels, except for biphenyl, butylate, mevinphos and ethirimol, which had lower recoveries (40-57%) and for prometryn and merphos with recoveries slightly higher than 120% at the lowest level. The lowest recoveries for some pesticide residues can be explained because the experimental conditions for the ultrasonic extraction were not the optimal ones obtained for them in the experimental design (100% amplitude and 20 sonication cycles) but a compromise.

The precision of both multi-residue methods was validated at the two concentration levels (LOQ and 50 μg/Kg) with the results obtained for the five fortified honeybee samples, the RSDs in most cases being lower than 20% and similar for the two concentration levels (12.2% for the LOQ concentration level and 9.9% for 50 μg/Kg). The LOQ of each pesticide was established as the lowest validated concentration level in honeybee samples: 5 μ/Kg for 239 pesticides, 10 μg/Kg for 12 pesticides, 15 μg/Kg for 7 pesticides and 40 μg/Kg for chlordane (the highest LOQ value).

Taking into account the validation parameters (accuracy and precision) of the two analytical methods, the expanded uncertainty (U) associated to each pesticide residue concentration was calculated. The expanded uncertainty (U) associated to each pesticide concentration was calculated taking into account the validation parameters (accuracy and precision) of the two analytical methods. In general, U(%) was between 25% and 47% at the two concentration levels considered (LOQ and 50 mg/Kg), being lower than 50% for all pesticide residues according to the recommendation of the SANTE document [16].

Table 1. Validation parameters of the multi-residue GC-QqQ-MS/MS method in honeybee

Pesticide	Linearity (µg/Kg)	R^2	LOQ (µg/Kg)		50 µg/Kg		ME (%)
			R(%)	RSD(%)	R(%)	RSD(%)	
2.4'-DDE	5-500	0.9997	88.4	17.1	79.0	6.9	26
2.4'-DDT+4.4'-DDD	5-500	0.9997	91.7	8.8	78.4	7.0	43
3.5-Dichloroaniline	5-500	0.9967	82.6	17.2	77.4	11.4	36
3-Chloroaniline	5-500	0.9908	69.6	7.9	82.3	10.0	54
4.4'-DDE	5-500	0.9995	79.6	12.1	74.5	9.8	13
4.4'-DDT	5-500	0.9997	86.7	16.6	73.9	18.1	58
Acrinathrin	10-250	0.9980	60.3	7.9	67.3	12.8	379
Alachlor	5-500	0.9999	90.4	12.2	85.1	5.5	49
Aldrin	5-500	0.9991	80.8	16.4	77.1	11.5	21
Ametryn	5-500	1.0000	92.2	6.8	91.7	12.4	57
Anthraquinone	5-500	0.9997	82.5	11.6	86.0	11.0	47
Atrazine	5-500	1.0000	81.4	7.0	81.4	13.1	68
Benalaxyl	5-500	0.9999	93.5	12.2	118.5	11.5	28
Bifenox	5-500	0.9950	92.9	11.5	102.3	13.1	47
Bifenthrin	5-500	1.0000	90.7	14.4	85.6	9.3	49
Biphenyl	5-500	0.9992	56.3	18.5	50.3	18.3	15
Bixafen	5-500	0.9999	99.8	10.7	89.5	12.0	108
Boscalid	5-500	0.9999	87.2	5.5	89.3	10.2	85
Bromopropylate	5-500	0.9997	79.2	9.8	79.5	9.6	94
Butralin	10-250	0.9956	81.5	11.1	79.4	9.2	77
Butylate	5-500	1.0000	57.1	6.8	62.6	15.2	26
Cadusafos	5-500	0.9999	88.8	9.0	73.4	6.7	86
Carbophenothion	5-500	0.9998	90.2	13.0	87.0	11.9	79
Chlordane	40-500	1.0000	71.4	13.6	83.1	13.3	20
Chlorfenapyr	10-500	0.9992	137.2	17.8	93.4	11.8	35
Chlorfenvinphos	5-500	1.0000	90.2	17.1	86.3	10.3	54
Chlorobenzilate	5-500	0.9999	87.8	11.4	85.4	8.8	53
Chlorpropham	5-500	0.9999	92.6	11.9	84.0	9.0	61
Chlorpyrifos	5-500	0.9998	78.8	12.5	85.9	14.6	32
Chlorpyrifos-Methyl	5-500	0.9994	84.6	14.6	81.9	6.9	75
Chlorthal-Dimethyl	5-500	1.0000	90.7	11.9	83.9	9.3	23
Chlozolinate	5-500	0.9998	89.5	13.8	72.4	8.5	27
Coumaphos	5-500	0.9998	110.9	12.0	87.5	9.7	246
Cyfluthrin	5-500	0.9978	93.5	9.0	75.5	7.4	173
Cypermethrin	5-500	0.9975	97.5	13.7	76.9	8.1	154
Deltamethrin	5-500	0.9985	105.2	10.8	77.7	17.4	230
Dichlorvos	5-500	1.0000	79.0	15.2	75.0	16.6	28
Diclobutrazole	5-500	1.0000	99.0	7.4	81.9	9.2	135
Dicofol	5-500	0.9990	73.8	8.4	88.9	11.1	59
Dieldrin	10-500	0.9996	84.5	19.8	83.6	11.0	18
Dimethenamid	5-500	0.9999	89.2	18.8	85.7	6.6	54
Dimethipin	5-500	0.9995	92.2	9.9	84.1	7.9	43
Diphenylamine	5-500	0.9998	93.1	14.2	77.2	7.0	35
DMST	5-500	0.9990	101.2	10.0	111.3	12.7	90
Dodemorph	5-500	0.9997	101.6	17.3	106.3	15.8	62
Endosulfan Alpha	5-500	0.9999	86.3	15.2	84.0	10.9	23
Endosulfan Beta	5-500	0.9994	101.1	8.4	81.9	10.7	18
Endosulfan Sulfate	5-500	1.0000	90.8	15.1	79.0	14.0	35
Endrin	5-500	0.9998	95.3	19.7	81.9	7.1	42
EPN	5-250	0.9936	95.7	13.6	75.7	7.0	205
Ethofenprox	5-500	0.9999	92.5	8.1	90.1	13.1	80
Ethofumesate	5-500	0.9999	95.4	13.5	89.0	9.8	41
Ethoxyquin	5-500	1.0000	84.8	16.1	81.3	14.9	159
Etrimfos	5-500	0.9998	88.4	13.2	79.6	4.0	86
Fenamidone	5-500	0.9996	83.7	11.6	89.6	12.1	55
Fenchlorphos	5-500	1.0000	96.9	16.0	83.1	10.4	67
Fenitrothion	5-250	0.9979	86.4	12.2	76.2	6.7	155

Table 1 (cont.).

Pesticide	Linearity (µg/Kg)	R^2	LOQ (µg/Kg)		50 µg/Kg		ME (%)
			R(%)	RSD(%)	R(%)	RSD(%)	
Fenpropathrin	5-500	0.9993	87.2	10.0	79.6	7.6	73
Fenvalerate/Esfenvalerate RR/SS	5-250	0.9982	100.1	10.4	78.1	7.2	235
Fenvalerate/Esfenvalerate RS/SR	5-250	0.9987	97.9	12.7	72.0	5.4	286
Fipronil	10-500	0.9997	92.0	18.7	79.7	9.9	92
Fipronil-Desulfinil	10-500	0.9987	92.1	8.2	86.2	14.4	67
Fipronil-Sulfone	10-500	0.9918	100.1	14.2	82.4	14.8	85
Flamprop-Isopropyl	5-500	1.0000	103.7	12.6	82.9	9.7	45
Flamprop-Methyl	5-500	1.0000	96.6	9.5	78.6	8.6	34
Flonicamid	5-500	0.9997	92.5	11.5	79.7	7.2	72
Fluacrypyrim	5-500	0.9998	100.4	9.2	85.1	15.9	70
Fluazifop-p-Butyl	5-500	0.9999	101.2	15.2	86.3	11.4	82
Flucythrinate	5-500	0.9998	92.3	12.9	79.8	6.4	146
Fludioxonil	5-500	0.9992	82.7	9.8	83.8	9.6	71
Fluopicolide	5-500	0.9999	84.4	10.3	82.1	11.6	89
Fluquinconazole	5-500	0.9998	78.5	5.7	84.3	7.3	77
Flutolanil	5-500	1.0000	86.2	2.9	83.7	8.6	82
Fluvalinate-tau	5-250	0.9989	81.2	7.9	113.1	13.3	274
Folpet*	10-500	0.9979	79.0	15.4	81.4	10.5	-17
Fonofos	5-500	0.9996	92.4	13.0	86.7	6.7	91
Formothion	5-500	0.9998	70.4	12.0	75.8	15.0	122
HCB	5-500	0.9996	66.3	9.0	64.7	15.1	39
HCH-alpha	5-500	0.9993	83.8	14.3	78.8	5.6	27
HCH-beta	5-500	0.9956	82.5	7.9	87.0	9.4	31
Heptachlor	5-500	0.9998	73.3	13.3	71.4	4.4	49
Heptachloroepoxide-cis	5-500	0.9991	79.7	17.6	82.5	14.1	65
Heptachloroepoxide-trans	5-500	0.992	107.4	10.7	89.2	10.2	76
Heptenophos	5-500	1.0000	73.8	3.9	69.2	12.5	165
Iprodione	5-500	0.9998	72.3	8.2	91.0	15.4	132
Isazofos	5-500	0.9999	100.9	13.3	87.1	6.9	85
Isocarbophos	5-500	0.9999	110.8	14.2	98.3	12.4	127
Isofenphos-Ethyl	5-500	0.9998	111.6	15.2	97.2	9.2	62
Isofenphos-Methyl	5-500	1.0000	97.5	17.8	94.6	8.7	61
Isoprothiolane	10-500	1.0000	94.0	17.3	82.8	5.5	52
Isopyrazam	5-500	0.9999	95.6	8.0	84.9	6.5	83
Lambda-Cyhalothrin	5-500	0.9994	115.1	12.1	79.3	6.9	159
Lindane	5-500	0.9999	89.8	15.8	81.3	6.9	61
Malathion	5-500	0.9998	86.4	12.8	81.6	9.2	97
Mecarbam	5-500	0.9998	95.4	13.7	84.1	8.2	85
Mepanypirim	10-500	0.9999	71.5	10.4	83.1	8.7	101
Merphos	5-500	0.9999	126.3	4.6	83.9	13.7	80
Metazachlor	5-500	0.9998	101.4	11.4	85.2	9.3	56
Metconazole	5-500	0.9997	82.4	11.9	78.9	9.9	84
Methidathion	5-500	0.9999	90.2	13.7	80.0	6.3	142
Methiocarb	5-500	0.9998	94.2	17.7	101.0	14.0	158
Methiocarb Sulfone	5-500	0.9998	76.4	17.6	89.9	11.7	42
Metolachlor	5-500	0.9999	83.0	12.5	85.0	6.2	48
Methoxychlor	5-500	0.9974	77.7	14.5	77.0	12.8	75
Mevinphos	5-500	1.0000	46.8	19.5	51.2	9.9	85
Molinate	5-500	1.0000	85.3	16.2	76.3	12.1	41
Napropamide	5-500	0.9999	90.7	12.8	85.5	6.9	59
Nuarimol	5-500	0.9999	91.7	9.5	90.7	9.5	62
Ofurace	5-500	0.9997	100.3	14.3	85.8	18.8	60
Ortophenylphenol	5-500	0.9999	100.4	8.9	94.6	8.9	87
Oxadixyl	5-500	0.9999	100.9	12.4	84.0	13.0	59
Parathion-Methyl	10-500	0.9963	78.3	17.6	85.1	7.8	154
Pebulate	5-500	1.0000	68.9	10.2	45.5	12.3	35
Pentachloroaniline	5-500	0.9998	78.0	12.5	79.2	13.4	106

Table 1 (cont.).

Pesticide	Linearity (μg/Kg)	R^2	LCL (μg/Kg)		50 μg/Kg		ME (%)
			R(%)	RSD(%)	R(%)	RSD(%)	
Permethrin	5-500	0.9999	118.9	7.2	83.6	10.2	94
Phenothrin	10-500	0.9997	105.5	15.5	83.8	12.3	99
Phorate	5-500	0.9996	87.7	16.1	79.2	10.1	109
Phosmet	5-500	0.9990	86.3	11.1	81.1	12.9	625
Phthalimide*	5-500	1.0000	105.0	16.5	80.7	5.3	145
Picolinafen	5-500	0.9997	84.0	4.0	82.1	11.0	83
Picoxystrobin	5-500	1.0000	90.1	16.5	82.6	5.4	54
Procymidone	5-500	1.0000	97.7	13.0	85.7	11.1	36
Prometon	5-500	0.9999	95.8	13.1	87.0	7.0	79
Prometryn	5-500	1.0000	136.7	12.1	93.8	3.6	56
Propaphos	5-500	1.0000	63.1	9.5	99.2	12.3	134
Propazine	5-500	0.9999	89.0	13.6	85.0	9.0	62
Propyzamide	5-500	0.9998	84.1	12.2	81.8	8.3	101
Prosulfocarb	5-500	0.9996	88.4	10.9	82.4	9.9	78
Prothiophos	5-500	1.0000	86.1	12.0	79.0	5.1	57
Pyrazofos	5-500	0.9998	91.8	8.7	88.2	10.4	296
Pyridaben	5-500	0.9997	61.4	6.8	73.0	8.8	192
Pyrifenox	5-500	0.9999	80.8	10.0	85.0	7.5	81
Pyriproxyfen	5-500	1.0000	91.5	12.2	82.9	5.2	91
Quinalphos	5-500	0.9998	88.8	9.4	83.2	13.2	60
Quintozene	5-500	0.9983	85.6	18.0	77.5	7.4	98
Secbumeton	5-500	1.0000	100.1	11.4	89.0	9.1	54
Spirodiclofen	5-500	0.9998	87.5	13.5	73.4	10.7	55
Spiromesifen	5-500	1.0000	97.5	9.6	76.9	5.3	74
Sulfotep	5-500	0.9999	85.6	13.4	73.8	3.6	95
Tebuconazole	5-500	0.9999	76.6	12.9	81.6	11.0	112
Tebufenpyrad	5-500	0.9998	84.4	10.0	84.8	10.7	70
Tecnazene	5-500	0.9993	81.6	11.8	79.0	5.9	91
Tefluthrin	5-500	1.0000	82.6	12.9	80.3	6.2	47
Terbumeton	5-500	0.9999	101.7	3.6	81.9	9.3	82
Terbutryn	5-500	0.9998	104.3	12.6	93.7	8.4	47
Tetrachlorvinphos	5-500	0.9999	106.7	19.8	76.9	13.9	112
Tetradifon	5-500	0.9999	95.9	7.6	81.3	11.1	40
Tetramethrin	5-500	0.9995	94.8	5.1	84.5	10.4	96
Tolclofos-Methyl	5-500	0.9999	83.0	12.0	88.7	11.5	61
Triazophos	5-500	0.9998	92.4	4.6	83.7	9.9	198
Trifluralin	5-250	0.9974	80.2	11.6	86.0	2.7	101
Vinclozolin	5-500	0.9999	83.2	5.8	79.1	8.0	67
*Dichlorvos-d6***	5-500	0.9983	77.5	15.2	79.5	11.1	
*Malathion-d10***	5-500	0.9995	84.3	8.6	85.1	9.4	
*TPP***	5-500	0.9984	90.3	17.2	90.1	12.5	

**Folpet plus phatalimide residues; ** Isotope–labeled internal standards (IL-IS)*

To control the effectiveness of the full extraction procedure, isotope–labeled internal standards (IL-IS of dichlorvos-d6, malathion-d10 and TTP) were added to all honeybee samples, at a concentration level of 10 μg/Kg, after adding the acetonitrile.

Analysis of honeybees from Spanish apiaries

The proposed UASE method was applied to the determination of pesticide residues in 30 honeybee samples taken from apiaries in Spain (samples B1 to B30) and the concentrations found are presented in Table 3**.** All honeybee samples analyzed contained between 2 and 10 different pesticide residues with a total of 30 (8 insecticide/acaricides, 4 insecticides, 14 fungicides and 4 herbicides). One can see that the total pesticide residue load was lower than 50 μg/Kg for 16 honeybee samples, between 70 and 127 μg/Kg for 8 honeybee samples, between 231 and 632 μg/Kg for 4 honeybee samples and the highest total load was found in two honeybee

samples with 3909 and 3911μg/Kg (samples B3 and B24, respectively). Some amounts of pesticides reported in Table 3 are lower than the corresponding LOQs and can only be considered as semiquantitative values as they were not evaluated for recovery and repetability. As an example, Fig. 2 shows extracted ion chromatograms (XIC) of the SRM1 and SRM2 transitions obtained for permethrin in sample AB16 and a honeybee extract spiked at 5 μg/Kg. One can see that even though the concentration level in the sample is lower than the LOQ, the identification was possible following the SANTE/11945/2015 criteria [16].

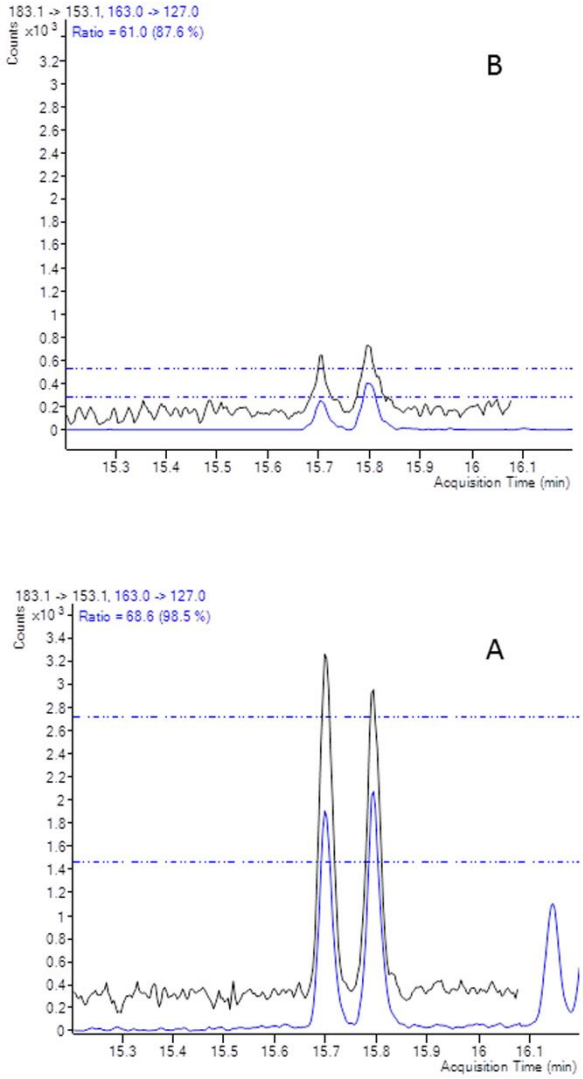

Fig. 2 *Extracted ion chromatograms (XIC) for SRM1 and SRM2 of permethrin corresponding to: (A) ecological honeybee sample spiked at a concentration of 5 μg/kg and (B) honeybee sample (AB16)*

The most frequently detected pesticide residues (Fig. 3) were permethrin, thiabendazol and carbendazim in 28, 19 and 18 samples, respectively but at concentration levels lower than their LOQs; and coumaphos in 19 samples at different concentrations, two of them at high concentration levels (360 μg/Kg in B19 and 3910 μg/Kg in B24).

Acrinathrin was found in 10 honeybee samples at concentration levels between 2 and 570 μg/Kg, fluvalinate-tau in 6 honeybee samples (between 1 and 276 μg/Kg), cholpyriphos in 5 honeybee samples at lower concentration levels (≤ 5 μg/Kg) and pyridaben and terbuthylazine were found in 4 honeybee samples at concentration levels lower than 12 μg/Kg with the exception of one sample (B3) that had a very high concentration level of pyridaben (3900 μg/Kg). The rest of pesticides were found in 1 or 2 honeybee samples,

with concentrations levels lower than their corresponding LOQ in most cases, the exception being sample B4 with 48 and 146 μg/Kg of iprodione and metalaxyl, respectively.

Table 2. Validation parameters of the multi-residue LC-QqQ-MS/MS method in honeybee

Pesticide	Linearity (μg/Kg)	R^2	LOQ (μg/Kg)		50 μg/Kg		ME (%)
			R(%)	RSD(%)	R(%)	RSD(%)	
3-Hidroxycarbofuran	5-500	0.9983	76.2	1.3	81.2	12.1	-32
Acetamiprid	5-500	0.9989	73.5	4.4	86.3	6.8	-47
Aldicarb	5-500	0.9993	93.8	12.4	84.3	11.3	-13
Aldicarb Sulfone	5-500	0.9989	65.6	15.2	73.5	9.4	-7
Aldicarb Sulfoxide	15-500	0.9991	64.0	15.4	70.5	11.0	-64
Azinphos-methyl	5-500	0.9932	99.7	17.8	115.9	9.8	-34
Azoxystrobin	5-500	0.9969	84.5	8.4	92.9	4.2	-11
Bitertanol	5-500	0.9998	68.9	18.0	79.8	12.4	9
Bromuconazole	5-500	0.9999	78.0	9.6	77.9	4.6	-4
Bupirimate	5-500	0.9997	78.2	10.1	75.2	13.1	-2
Buprofezin	5-500	0.9999	75.4	8.1	78.6	13.9	1
Carbaryl	5-500	0.9999	84.8	11.0	90.6	10.7	-22
Carbendazim	5-500	0.9998	95.3	9.0	78.1	12.2	-42
Carbofuran	5-500	0.9996	92.5	12.2	105.7	9.6	-43
Chlorantraniliprole	5-500	0.9998	87.6	7.3	84.3	5.9	-5
Clofentezine	5-500	0.9986	105.5	7.7	88.8	6.1	-16
Cymoxanil	15-500	0.9992	84.1	14.2	72.6	10.7	-7
Cyproconazole	5-500	0.9999	80.0	14.6	74.8	12.0	0
Cyprodinil	5-500	0.9991	75.9	15.4	79.4	12.1	-22
Diazinon	5-500	0.9987	79.4	16.4	81.2	8.3	-16
Dicrotophos	5-500	0.9983	71.9	12.2	68.0	9.5	202
Diethofencarb	5-500	0.9992	89.3	11.8	84.4	11.3	9
Difenoconazole	5-500	0.9990	83.5	11.5	80.5	14.3	1
Diflubenzuron	5-500	0.9963	104.8	9.1	85.4	7.3	1
Dimethoate	5-500	0.9998	93.4	11.7	102.8	6.4	-15
Dimethomorph	5-500	0.9999	77.2	11.2	88.1	7.1	0
Diniconazole	5-500	1.0000	80.7	12.8	78.9	11.2	-5
Ethion	5-500	0.9943	83.1	11.9	91.2	7.2	-10
Ethirimol	5-500	0.9990	48.5	13.8	40.0	19.3	-15
Ethoprophos	5-500	0.9997	85.1	7.9	79.0	7.5	-3
Famoxadone	5-500	0.9995	82.3	14.2	88.0	13.9	44
Fenamiphos	5-500	0.9984	72.0	6.5	71.4	11.0	4
Fenamiphos Sulfone	5-500	0.9990	79.4	11.7	73.9	9.5	-10
Fenamiphos Sulfoxide	5-500	0.9997	72.4	12.9	77.3	12.4	-7
Fenarimol	5-500	0.9992	75.9	8.8	79.8	9.1	-2
Fenazaquin	5-500	0.9975	79.7	10.2	74.1	16.3	-32
Fenbuconazole	5-500	0.9998	82.9	10.2	75.0	8.6	10
Fenhexamid	5-500	0.9995	72.7	12.1	79.3	2.8	5
Fenoxycarb	5-500	0.9999	93.4	18.6	87.8	6.9	4
Fenpropimorph	5-500	0.9999	79.3	10.1	83.1	12.1	-8
Fenpyroximate	5-500	0.9968	81.9	12.2	75.6	10.5	-17
Fenthion	5-500	0.9990	120.5	18.1	84.5	9.5	11
Fenthion Oxon	5-500	0.9986	86.2	10.6	77.2	4.1	-15
Fenthion Oxonsulfone	5-500	0.9946	77.6	16.9	70.7	8.6	-8
Fenthion Sulfone	5-500	0.9931	66.3	1.0	71.9	4.0	-14
Fenthion Sulfoxide	5-500	0.9989	88.1	8.5	88.0	3.8	14
Flubendiamide	15-500	0.9997	96.4	14.8	70.2	13.5	37
Flufenoxuron	5-500	0.9934	85.9	16.2	81.9	9.3	53
Fluopyram	5-500	0.9993	84.9	15.4	84.3	7.9	-15
Flusilazole	5-500	0.9995	86.0	14.3	91.2	18.7	-4
Flutriafol	5-500	0.9980	83.7	8.6	67.1	7.9	5
Fosthiazate	5-500	0.9999	82.3	13.4	79.8	10.6	-11
Hexaconazole	5-500	0.9997	83.1	7.4	75.2	9.0	-4
Hexythiazox	5-500	0.9937	83.7	13.8	86.1	6.8	-5
Imazalil	5-500	0.9999	68.8	15.8	75.2	8.2	2
Imidacloprid	5-500	0.9998	88.4	12.7	92.0	7.1	-24
Indoxacarb	5-500	0.9983	84.0	12.4	86.4	7.7	18

Table 2 (cont.).

Pesticide	Linearity (µg/Kg)	R^2	LOQ (µg/Kg)		50 µg/Kg		ME (%)
			R(%)	RSD(%)	R(%)	RSD(%)	
Iprovalicarb	5-500	0.9991	80.5	11.7	84.1	14.3	5
Isoprocarb	5-500	0.9986	82.8	10.8	85.9	7.4	-17
Kresoxim-Methyl	5-500	1.0000	89.8	18.7	94.7	15.0	-4
Linuron	5-500	0.9917	86.4	8.0	89.0	5.4	36
Lufenuron	5-500	0.9907	92.3	14.3	75.8	11.7	61
Malaoxon	15-500	0.9981	74.6	17.4	77.3	5.8	43
Mandipropamid	5-500	0.9969	80.6	7.9	88.7	9.7	-5
Meptyldinocap	5-500	0.9951	71.2	15.6	81.2	18.7	21
Metalaxyl	5-500	0.9919	87.7	10.1	94.4	7.7	-22
Methomyl	5-500	0.9999	65.4	8.6	97.9	14.7	-40
Methoxyfenozide	5-500	0.9989	78.5	13.3	80.2	5.8	5
Metobromuron	5-500	0.9988	88.0	14.3	93.0	5.6	-16
Monocrotophos	5-500	0.9982	75.4	14.7	70.4	10.1	-47
Myclobutanil	5-500	0.9997	74.9	13.6	78.7	7.6	1
Oxydemeton-methyl	5-500	0.9988	78.2	18.2	72.0	13.4	-35
Paclobutrazole	5-500	0.9999	76.8	16.3	86.6	11.4	11
Paraoxon Methyl	5-500	0.9976	64.0	15.2	54.3	9.4	34
Parathion	5-500	0.9981	82.2	13.9	99.2	8.4	-19
Penconazole	5-500	0.9999	75.2	14.7	75.6	13.4	-5
Pencycuron	5-500	0.9996	83.2	13.3	78.3	8.1	-20
Pendimethalin	5-500	0.9949	97.5	11.6	83.9	14.1	-21
Phenthoate	15-500	0.9982	132.3	18.1	90.6	4.0	-20
Phosalone	5-500	0.9990	95.2	3.0	106.2	15.0	-2
Phoxim	5-500	0.9975	86.5	3.8	94.0	8.6	-1
Pirimicarb	5-500	0.9978	79.3	13.1	85.2	6.9	15
Pirimicarb Desmethyl	5-500	0.9993	73.1	12.3	81.9	13.2	-29
Pirimiphos-Methyl	5-500	0.9998	80.7	10.2	76.4	4.4	-10
Prochloraz	5-500	0.9997	72.7	15.3	73.4	7.6	23
Profenofos	5-500	0.9962	82.4	19.7	73.7	13.0	-10
Propargite	5-500	0.9936	103.1	8.2	78.2	13.6	-33
Propiconazole	5-500	0.9999	81.2	14.7	76.0	7.3	3
Propoxur	5-500	0.9994	85.8	13.5	109.4	11.3	-7
Prothioconazole	5-500	0.9991	85.7	8.6	88.3	7.2	6
ProthioconazoleDesthio	15-500	0.9908	76.7	7.7	90.2	10.4	14
Pyrethrins	5-500	0.9929	86.6	12.9	82.3	9.6	28
Pyrimethanil	5-500	0.9996	87.9	17.2	81.4	10.0	-27
Quinoxyfen	5-500	0.9970	81.6	19.9	77.9	9.7	-32
Rotenone	5-500	0.9995	91.5	14.7	84.8	9.3	-8
Tebufenozide	5-500	0.9984	79.7	16.4	95.1	17.9	-16
Terbuthylazine	5-500	0.9999	81.7	12.9	80.9	13.0	-8
Tetraconazole	5-500	0.9978	83.5	10.7	82.7	12.6	1
Thiabendazole	5-500	0.9997	70.4	2.5	70.8	6.7	-45
Thiacloprid	5-500	0.9996	89.1	15.6	90.7	10.2	1
Thiametoxam	15-500	0.9963	93.8	18.0	88.9	9.1	-25
Triadimefon	5-500	0.9943	85.1	13.9	75.8	6.0	15
Triadimenol	5-500	0.9998	86.1	6.9	82.3	9.3	13
Trifloxystrobin	5-500	0.9999	88.7	11.0	82.9	12.0	-15
Triflumuron	5-500	0.9982	84.8	19.4	83.1	8.6	21
Triticonazole	5-500	0.9999	67.5	12.0	75.6	8.6	11
Zoxamide	5-500	0.9998	85.0	10.2	84.7	8.2	-22
*Diclorvos-d6**	5-500	0.9990	73.5	14.8	74.2	12.7	
*Malathion-d10**	5-500	0.9991	83.4	10.4	94.8	7.9	

** Isotope–labeled internal standards (IL-IS)*

The pesticide residues found in this work were similar those recorded by other authors [9,11,18-22]. A study [18] performed on adult bees in 140 North American apiaries in 2010 showed more than 45 pesticide residues in the samples with un maximum of 25 in one of them. The most detected pesticide residues were fluvalinate-tau, coumaphos and chlorpyrifos in 84%, 60% and 9% of the samples, respectively.

Table 3. Pesticide residues in honeybees from thirty Spanish apiaries

Pesticide residue	Concentration of pesticide residues (µg/Kg)																													
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**	**B18**	**B19**	**B20**	**B21**	**B22**	**B23**	**B24**	**B25**	**B26**	**B27**	**B28**	**B29**	**B30**
Acrinathrin		570		2*				12	26	2*													22		6	66	23		38	
Azoxystrobin																	0.3*	0.8*												
Bixafen																												2*		
Boscalid		37		1*										3.4*																
Carbendazim													1*	0.9*	0.9*	0.8*	0.8*	0.8*	0.7*	1*	0.7*	0.8*	0.9*	0.7*	1.1*	0.2*	0.8*	0.7*	0.8*	1.1*
Chlorpyrifos	3*							3*																	5	2*			1*	
Clomazone													13																	
Coumaphos				18	3*	5	7	13	22	23	25	6						18	360	2*			62	3910	57	51	50		30	16
Diazinon													0.4*	0.1*						0.1*										
Difenoconazole									29																					
Dimethoate		1*				3*																								
Diphenylamine																											1.3*			
Flusilazol		4*	3*	8																										
Fluvalinate-tau				1*	2*						13		101								75							276		
Iprodione		2*		48																										
Kresoxim-methyl		12	1*	3*																										
Metalaxyl				146																										
Metolachlor													0.5*																	
Omethoate		1*				3*												0.6*												
o-Phenylphenol														8					2*											
Pendimethalin										15																		4*		
Permethrin	4*	3*	5	3*	4*	4*	4*	4*	3*	2*	3*	6	2*	3*		2*	2*	2*	2*	2*	2*	1*	2*	1*	2*	2*	2*		1*	2*
Pyraclostrobin														0.8*																
Pyridaben		2*	3900	1*																					1*					
Pyriproxyfen																											2*			
Spinosad									12																		3*			
Spiroxamine													0.1*																	
Terbuthylazine													9		1*												1*	6		
Tetramethrin																					4*									
Thiabendazol	1*												1*	0.5*	0.5*	1*	0.7*	1.5*	1*	1*	1.4*	1*	0.4*	0.4*	0.4*	0.3*	0.7*	0.1*	0.3*	0.4*
Total Load (µg/Kg)	8	632	3909	231	9	15	11	32	92	42	41	12	127	11	1	3	2	20	365	6	81	2	86	3911	71	121	81	288	70	18
Nº Pesticides	3	9	4	10	3	4	2	4	5	4	3	2	9	7	3	3	4	6	5	5	5	3	5	4	7	6	9	6	6	4

** Concentration level lower than LOQ*

The presence of pesticide residues in honeybees in European countries has also been studied. Chauzat et al. [19] analyzed a total of 309 honeybee samples from different locations in continental France and 25 pesticide residues were found in 44% of the samples, the highest mean concentrations being for coumaphos (545.6 mg/Kg), carbaryl (214.3 mg/Kg), fluvalinate-tau (65.5 mg/Kg) and tebuconazole (218.2 mg/Kg). Recently, pesticide residues were detected in 48 honeybee samples from French beehives [20], the fungicide boscalid being the one most often detected in the samples but generally at concentration levels below 1 μg/Kg. In Greece, the most detected residues were clothianidin, chlorpyrifos, thiamethoxam, imidacloprid and coumaphos in 50% of the honeybee samples collected from 2011 to 2013 [11]. Pohorecka et al. [21] detected 5 insecticides (acetamiprid, dimethoate, imidacloprid, pyridaben and thiacloprid) and 3 fungicides (carbendazim, fenpropimorph and propamocarb) in 21% of the honeybee samples analyzed from a hive in Poland. In other studies, live and poisoned honeybees collected in Poland were analyzed; 48 pesticide residues were found in 151 samples of living honeybee samples [22] and 57 pesticides detected in a total of 74 poisoned honeybee samples [9]. Chlorpyrifos was detected in 12.2% of the living honeybees and in more than 50% of the dead honeybees, amongst them insecticides (thiacloprid and acetamiprid) and some fungicides (tebuconazole, boscalid and trifloxystrobin).

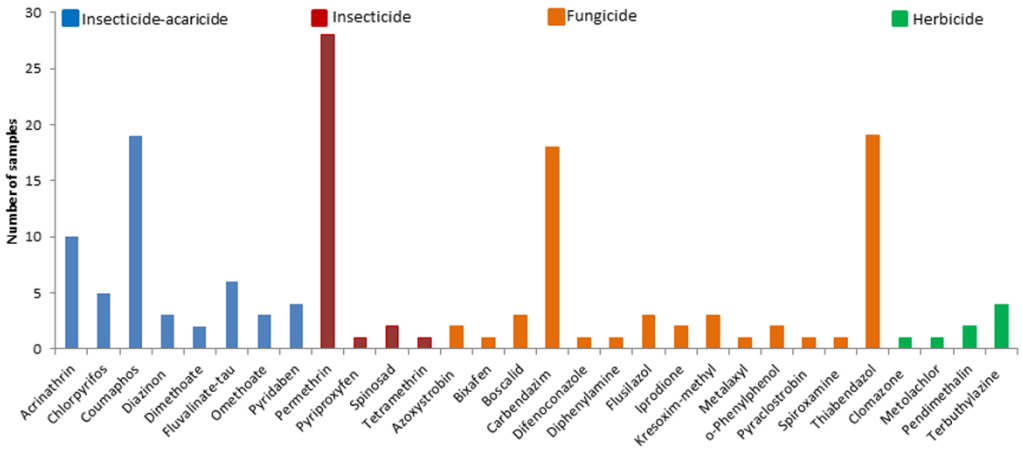

Fig. 3 *Pesticide residues found in 30 honeybee samples from different Spanish apiaries*

Conclusions

An ultrasonic-assisted extraction method based on QuEChERS has been developed and compared with conventional QuEChERS, for pesticide residues in honeybees. A multi-response optimization study based on a Box-Benhken design allowed us to select the optimal conditions: sonication amplitude, number of cycles and cycle time) for UASE extraction in just 15 experiments. The main advantages of UASE compared to conventional extraction techniques are the reduced extraction time, sample handling and matrix effect. In this work, 2 min and 20s were needed to directly extract the pesticide residues from the bees' bodies, whereas conventional QuEChERS required previous homogenization of the honeybees (in a blender or a manual mortar) followed by shaking for 4 or 5 min. All these advantages additionally reduce analysis costs. The proposed method was validated according to SANTE guidelines and, for most analytes, validation parameters complied with this guidance (recoveries between 70-120%, RSD ≤ 20% and U ≤ 50%). Finally, thirty honeybee samples collected in Spanish apiaries at different locations (areas) were analyzed. Thirty types of pesticide residues were detected but at different concentration levels and frequency in the samples (between 2 and 10 pesticides), the most frequently found being permethrin, thiabendazol, carbendazim and coumaphos. The last, an insecticide/acaricide used by beekeepers to control *Varroa destructor*, showed the highest concentrations levels.

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CRediT authorship contribution statement

M.D. Gil García: Guarantor of integrity of the entire study, Methodology, Supervision, Writing – original draft, S. Uclés Duque: Validation, Laboratory work, A.B. Lozano Fernández: Study concepts and design, Laboratory work. M. Martínez Galera: Literature research Conceptualization, Visualization, Writing – original draft Amadeo R. Fernández-Alba: Guarantor of integrity of the entire study, Supervision. , Project administration and Funding acquisition.

Electronic Supplementary Material

The online version of this article https://doi.org/10.1007/s00216-018-1167-7) contains supplementary material, which is available to authorized users.

Compliance with Ethical Standards

The authors declare no conflict of interest with any of the instruments or materials referred to in this work.

References

[1] Simon-Delso N, San Martin G, Bruneau E, Minsart LA, Mouret C, Hautier L. Honeybee Colony Disorder in Crop Areas: The Role of Pesticides and viruses. PLOS ONE. 2014;9 e103073.

[2] Klein A., Vassiére BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. B. 2007;274:303-313.

[3] Goulson D, Nicholls E, Botías C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 2015;374(6229):1255957.

[4] Rortais A, Arnold G, Dorne JL, More SJ, Sperandio G, Streissl F, Szentes C, Verdonck F. Risk assessment of pesticides and other stressors in bees: Principles, data gaps and perspectives form the European Food Safety Authority. Sci. Total Environ. 2017;587-588:524-537.

[5] European Food Safety Authority (EFSA). Towards holistic approaches to the risk assessment of multiple stressors in bees. Summary Report of the EFSA Scientific Colloquium XVIII. Parma, Italy. 2013;76.

[6] European Food Safety Authority (EFSA). Towards an integrated environmental risk assessment of multiple stressors on bees: review of research projects in Europe, knowledge gaps and recommendations. EFSA J. 2014;12(3):3594:1-102.

[7] Walorczyk S, Gnusowski B. Development and validation of a multi-residue method for the determination of pesticides in honeybees using acetonitrile-based extraction and gas chromatography-tandem quadrupole mass spectrometry. J. Chromatogr. A. 2009;1216:6522-6531.

[8] Wiest L, Buleté A, Giroud B, Fratta C, Amic S, Lambert O, Pouliquen H, Arnaudguilhem C. 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. J. Chromatogr. A. 2011;1218:5743– 5756.

[9] Kiljanek T, Niewiadowska A, Semeniuk S, Gaweł M, Borzecka M, Posyniak A. Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry—Honeybee poisoning incident., J. Chromatogr. A. 2016;1435:100-114.

[10] Bargańska Ż, Ślebioda M, Namieśnik J. Determination of Pesticide Residues in Honeybees using Modified QUEChERS Sample Work-Up and Liquid Chromatography-Tandem Mass Spectrometry. Molecules. 2014;19(3):2911-2924.

[11] Kasiotis KM, Anagnostopoulos C, Anastasiadou P, Machera K. Pesticide residues in honeybees, honey and bee pollen by LC–MS/MS screening: Reported death incidents in honeybees. Sci. Total Environ. 2014;485-486:633-642.

[12] Filgueiras AV, Capelo JL, Lavilla I, Bendicho C. Comparison of ultrasound-assisted extraction and microwave-assisted digestion for determination of magnesium, manganese and zinc in plant samples by flame atomic absorption spectrometry. Talanta. 2000;53:433–441.

[13] Priego-Capote F, Luque de Castro MD. Analytical uses of ultrasound I. Sample preparation. Trends Anal. Chem. 2014;23:644-653.

[14] Morales-Muñoz S, Vreuls RJJ, Luque de Castro MD. Dynamic ultrasound-assisted extraction of environmental pollutants from marine sediments for comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection. J. Chromatogr. A. 2005;1086:122–127.

[15] Roldán-Gutiérrez JM, Ruiz-Jiménez J, Luque de Castro MD. Ultrasound-assisted dynamic extraction of valuable compounds from aromatic plants and flowers as compared with steam distillation and superheated liquid extraction. Talanta. 2008;75:1369–1375.

[16] SANTE/11945/2015. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed. 2015.

[17] Box GEP, Behnken DW. Some New Three Level Designs for the Study of Quantitative Variables. Technometrics. 1969;2:455-475.

[18] Mullin CA, Frazier M, Frazier JL, Ashcraft S, Simonds R, vanEngelsdorp D, Pettis JS. High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. PLOS ONE. 2010;5:1-19.

[19] Chauzat MP, Martel AC, Cougoule N, Porta P, Lachaize J, Zeggane S, Aubert M, Carpentier P, Faucon JP. An assessment of honeybee colony matrices, Apis mellifera (Hymenoptera Apidae) to monitor pesticide presence in continental France. Environ. Toxicol. Chem. 2011;30:103–111.

[20] Daniele G, Giroud B, Jabot C, Vulliet E. Exposure assessment of honeybees through study of hive matrices: analysis of selected pesticide residues in honeybees, beebread, and beeswax from French beehives by LC-MS/MS. Environ. Sci. Pollut. Res. 2017; https://doi.org/10.1007/s11356-017-9227-7.

[21] Pohorecka K, Szczęsna T, Witek M, Miszczak A, Sikorski P. The exposure of honey bees to pesticide residues in the hive environment with regard to winter colony losses. J. Apic. Sic. 2017;61:105-125.
[22] Kiljanek T, Niewiadowska A, Gaweł M, Semeniuk S, Borzecka M, Posyniak A, Pohorecka K. Multiple pesticide residues in live and poisoned honeybees-Preliminary exposure assessment. Chemosphere. 2017;175:36-44.