# ANALYSIS OF PESTICIDE RESIDUES IN HONEYBEE (*Apis mellifera L.*) AND IN CORBICULAR POLLEN. EXPOSURE IN CITRUS ORCHARD WITH AN INTEGRATED PEST MANAGEMENT SYSTEM.

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## ABSTRACT

In the last years, the honeybee population is facing growing threats such as expansion of pathogens, the incorrect use of phytosanitary products and environmental contaminants, loss or fragmentation of habitat, invasive species and climate change. In Spain, the citrus cultivation in integrated pest management (IPM) attempts the most available use of strategies for the control of pests populations by means of taking actions that prevent problems, remove levels of damage and use of chemical control only when and where is necessary. The purpose of this work is to develop a simple analytical method that permits to evaluate the pesticide residue levels in honeybees and corbicular pollen when honeybees are exposed to plant protection products (PPPs) used in integrated management fields of citrus orchards. The proposed method is based in an ultrasound assisted extraction procedure followed by a dispersive solid phase extraction (d-SPE) clean-up with alumina and LC-MS/MS determination. The method was validated in samples of honeybee and corbicular pollen for the 10 pesticides mostly used in citrus orchards with IPM. This procedure was compared with QuEChERS methodologies for these matrices. The developed method was applied to the determination of these pesticides in both matrices in a two -year study in citrus orchards.



## KEYWORDS

Citrus orchard, Integrated pest management, Pesticides, Corbicular pollen, Honeybee, Ultrasound assisted extraction.


## INTRODUCTION

The citrus cultivation has a great socio-economic importance in the Spanish agricultural sector. Spain is a large producer of citrus fruits in the EU with about 6 million tons of production in nearly 285,000 ha, and the first exporting country in the world [1]. The majority of citrus varieties cultivated in Spain are parthenocarpic so that fertilization is not necessary to obtain the fruit. Citrus agrosystem is very rich and varied in pests and natural enemies. The most important pest in citrus are sucking pests (especially, aphids, whitefly and leaf miner) that feed on tender shoots.

The system of integrated pest management (IPM) relies on a combination of strategies to manage pest damage based on comprehensive information on the life cycles of pests, their interaction with the environment and the available pest control methods. Instead of trying of eradicate pests, IPM strives to prevent the development or abatement of pest populations to levels that reduce or minimize risks to human health, to the environment, and it is economically justified. The inevitable use of pesticides in

citrus orchard with an IPM program may be a potential stressor for the honeybee colony when hives are emplaced in orchards. The flowering period concurs with that of spring flushing period, so the chemical interventions during this period can coincide with the presence of bees and other pollinators in the crop.

In the Mediterranean zone, large cultivated areas of citrus represent an important source of pollen and nectar for bees during the blooming period from April to May. For instance, the regions of Valencia and Murcia provide a high honey yield in 2016, being approx. 23% of the total production in Spain [2]. The production of monofloral honey from orange blossom is valued commercially, reaching the highest price among the different types of honey, including both multi-floral and monofloral honey.

There is evidence that bees and other pollinator populations are declining [3-6]. The habitat loss, the loss of flora diversity, the action of pathogens, or the use of pesticides might be threats for honeybees and wild pollinators. To assess the impact of the use of phytosanitary products in agriculture, it is necessary develop analytical methods that provides a monitoring control through the identification of pesticides present in the agricultural environment and the quantification of concentration levels.

The QuEChERS method, with some modifications, is now the method more used in the preparation of samples for analysis of pesticides in bees ([7-9] and in bee products, such as pollen [10-11], honey [9, 12-13], beebread [14] and beeswax [15-16]. The method consists in an acetonitrile extraction/partition followed by a dispersive solid-phase extraction (d-SPE). The QuEChERS method has the advantage of covering the analysis of a wide range of pesticides (polar and no polar) in the same extraction. Modification of QuEChERS method has been carried out by changing the extraction solvent, the salt of salting-out process and the adsorbent of the clean-up. The CEN Standard Method[17] use citrate buffering, whereas the AOAC Official Method [18] use acetate buffering to extract pesticides that are sensitive in acidic or basic medium. The ruggedness characteristics of the QuEChERS approach have been thoroughly evaluated, nevertheless, it is very difficult to obtain a high degree of clean-up without reducing recoveries for some pesticides depending on the scope of a multiclass or multiresidue method. A greater clean-up can be achieved by using different sorbents than PSA (primary–secondary amine) in the original d-SPE step, obtaining acceptable recoveries. The modifications in the d-SPE clean-up step, widely applied, consist in different combinations of PSA with C18, PSA with GCB (graphitized carbon black) or PSA with C18 and GCB [12, 19-20]. Recently, novel absorbents such as zirconium oxide, the EMR-lipid (enhanced matrix removal-lipid) or chitin are used [21-22] in sample preparation for honeybee and its products.
Others techniques of sample preparation such as MSPD (matrix solid phase dispersion) [23-25]), SPE (solid phase extraction) [26-27], OC-LLE (on-column liquid–liquid extraction) [28]; SFME (solid phase microextraction [29-30]; and UAE (ultrasound assisted extraction) [31] have also been tested for the extraction of pesticide residues in samples of honeybees and in hive products.

Regarding the analytical procedures the LC-MS/MS or GC-MS/MS are the most employed techniques in the multiresidue analysis of pesticides in samples of bee

products and honeybees after the sample preparation indicated above. These techniques are subject to strong matrix effects that can lead to erroneous quantification. Effect that can be reduced by an appropriate sample preparation, including extraction and clean-up.

In this work, the QuEChERS methods based in the AOAC [18] and CEN [19] official methods, were carried out for analysis of pesticide residues in pollen collected by bees (corbicular pollen) and in bees. These sample preparation methods were compared with an UAE (ultrasound assisted extraction) method set-up in our laboratory; applied for the first time for the extraction and quantitative analysis of residues of spinosad, spirodiclofen, spirotetramat, acetamiprid, fenpyroximate, chlorpyrifos, clofentezine, etoxazole, hexythiazox, pyriproxifen in honeybees and corbicular pollen.

The developed method, after validation, was applied to the determination of these pesticides belonging to a variety of chemical families (carboxamides, phenoxypyrazoles, tetronic/tetramic acids, neonicotinoids, tetrazines, organophosphates and spinosyn) in samples of honeybees and corbicular pollen collected from hives sited in an extensive orchard of citrus trees growing in integrated management for a period of two consecutive years.

## MATERIALS AND METHOD

### Instrumentation

Residue analysis was performed on a liquid chromatography (HPLC, 1200 Series) coupled to mass spectrometry with triple quadrupole analyzer (TripleQuad 6410 Series) (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and processing were carried out by using MassHunter software (B.01.04). The triple quadrupole mass spectrometer was operated in selected reaction monitoring (SRM) in positive ionisation mode. The chromatographic column was F5 (pentafluorophenyl propyl (PFP) core-shell phase with trimethylsilane TMS endcapping) of 100 x 3 mm i.d. and 2.6µm, 100Å particle size (Kinetex F5, Phenomenex, Torrance, CA, USA). The mobile phase consisted of A (LC-grade water and 0.1 % formic acid) and B (0.1% of formic acid in acetonitrile, ACN), the flow rate was of 0.3mL $min^{-1}$ and the injection volume was 10µL. The gradient elution program used was as follows, at the start 95% of solvent A, maintained during 0.5min, decreased to 50% in 4,5min, to 30% in 2min to 10% in 5min and to 5% in 3min. Return to initial condition in 1min. Post-run was of 10min. To improve chlorpyrifos selectivity, the same gradient elution was used with the mobile phase A (LC-grade water and ammonium formiate 5mM) and B (methanol) in the quantification of this pesticide.
The electrospray ionization source (ESI) operated in positive mode in the following condition, gas temperature, 300ºC, gas flow 9L/min, nebulizer pressure 35psi and capillary voltage 3500V. Nitrogen was used in the nebulizer and in the collision cell. Selection of mass ions was carried out by direct flow injection of standard solutions; the optimised conditions for SRM transitions are shown in Table 1S. Identification of pesticide residues in samples was based on the detection of two SRM transitions, a

retention time tolerance of ± 0.1 min with the standard; and an ion ratio (a relationship between abundance of the selected transitions for identification and quantification, SRM2/SRM1) compliance of ± 30 % of the average of the calibration standards from the same sequence.
For sample preparation, a Branson 38000, CPXH series (Branson ultrasonic BV, Utrecht, The Netherland) ultrasound bath (with a tank capacity of 1.9L to 20 ; frequency of 40 KHz and 110W) and a centrifuge Selecta Medifriger (Barcelona, Spain), were used.

**Chemicals and reagents**

Methanol (MeOH) and acetonitrile (ACN) LC-MS grade were purchased from Riedel-de-Häen (Barcelona, Spain). Formic acid and ammonium formate of LC-MS grade, were purchased from Fluka (Buchs, Switzerland). Ultrapure water was provided by a MilliQ purification apparatus (Millipore Direct-Q UV, Bedford, MA). Analytical standard of acetamiprid (99.0%), chlorpyrifos (99.9%), clofentezine (98.09%), etoxazole (98.0%), fenpyroximate (99.5%), hexythiazox (98.0%), pyriproxyfen (99.0%), (spinosad, 94.8% with 84% of spinosyn A and 16% of spinosyn), spirodiclofen (99%) and spirotetramat (98.58%) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).
Standard stock solutions of 100 mg/L were prepared in ACN for each pesticide and stored at -20 °C in amber glass vials. A standard solution of 10 mg/L containing all pesticides was prepared in ACN.

Polypropylene (PP) tubes of 10mL used for the sample preparation were provided by (Deltalab, Madrid, Spain). Ceramic homogenizer were provided by Agilent (Palo Alto,CA, USA). Magnesium sulphate, sodium chloride, sodium citrate tribasic dihidrate, sodium citrate dibasic sesquihydrate and sodium acetate were from Merk (Stheim, Germany). Primary secondary amine (PSA), graphitised carbon black (GCB) and C18 were purchased from Scharlab (Barcelona, Spain).

**Field trial with an integrated pest management system**

The field trial was carried out in two citrus orchards (Plot 1 and 2) with 85 and 20 ha, respectively, located in the southern of Catalonia (Spain) during 2016 and 2017. Plot 1 was surrounded by rice field crops fundamentally and Plot 2 was in a large growing-area of citrus orchard, Clementine mandarin, fundamentally.

Both orchards include different citrus varieties, plot 1: 46% Satsuma mandarin, 29% sweet orange, 25% clementine mandarin and plot 2: 75% clementine mandarin, 25% sweet orange. Table 1 shows the pesticides applied in citrus orchards with an IPM program. The treatments were applied by foliar spray using an air-blast sprayer adjusted to standard conditions; with two application volumes, 1000-1500 and 2000-2500 L/ha, depending on the pests, except for the bait treatments, with 10L/ha.
Bee hives were installed in the orchards; in the plot 1, on 1st of April 2016 and 2nd of May 2017, and in plot 2, on 9th of May 2016 and 17th of May 2017.

Samples of corbicular pollen and honeybees were taken from the hives three times during the flowering period; from the beginning of citrus blooming to the end of

flowering (approx. 10% (S1), 35% (S2) and 75% (S3) of the flowering) in 2016 and 2017. An additional sampling was conducted in the plot 2 at the end of flowering period (100% of the flowering (S4)) Table 2S (supporting information).

The corbicular pollen, was collected after honeybee pass by a grid located to the hive entrance. The corbicular pollen load falls in a box sited below the grid. The boxes were emptied each 2-3 days. In each sampling time, honeybees were captured as well, at the hive entrance. An integrated sample of pollen and forager bees from the beehives (3 beehives in each plot) installed in the orchards were taken for analysis.

**Sample preparation.**

**QuEChERS-based extraction method**

1 g of corbicular pollen, previously homogenised in a mortar or 1 gr of adult honeybee, was weighed in a 30 mL PP tube. Two pieces of ceramic homogenizer (Agilent Technologies) and 4 mL of pure water (Mili-Q) were added into the tube and vigorously shaken in a vortex during 30 s or 120 s (for the samples of pollen and honeybees, respectively). A 5 mL volume of a 0.1% acetic acid in acetonitrile solution was added and shaken again by vortex during 1min or introduced in an ultrasonic bath during 10min. Two mixtures of salts were tested: 4g of a) acetate buffer consistent in magnesium sulphate: sodium acetate (in the proportion 4:1 w/w) following the AOAC procedure and, b) citrate buffer consistent in magnesium sulphate: sodium chloride: sodium citrate dehydrate: disodium citrate sesquihidrate (in the proportion 8:2:2:1 w/w) following the CEN procedure. The tube was immediately shaken in a vortex mixer for 20 s for preventing the coagulation of $MgSO_4$ .The mixture was centrifuged at 4500 rpm for 10 min at 4ºC. An 1 mL aliquot of the supernatant (ACN phase) was transferred to a 10 mL centrifuge tube containing 150 mg of $MgSO_4$, 50 mg of PSA and 50mg of C18 or 150mg of $MgSO_4$, 50mg PSA and 50mg of GCB, then swirled on a vortex mixer for 30 s and centrifuged (4500 rpm for 5 min). The supernatant was filtrated in a 0.22 µm nylon filter before injection in LC-MS/MS.

**Ultrasound assisted extraction.**

To 1 g of homogenized pollen with 0.5mL of water or to 1 g of honeybee (7-10 bees), 2mL of a solution of 0.1% of formic acid in acetonitrile was added. The samples were shaken by vortex during 1 min using 2 ceramic bars homogenizer in a PP tube. The mixture was sonicated in an ultrasonic bath operating at 290W, 40 kHz, at ambient temperature for 10 min. After, the samples were centrifuged at 4500 rpm and 4ºC during 5min, the supernatant extract was transferred to a tube. This extraction procedure was repeated with 2 mL of 0.1% of formic acid in acetonitrile and the yielded extracts were combined. Extract (1.0 mL) was clean-up by d-SPE using one of following adsorbents, 200 mg of alumina, 200 mg of PSA, 200 mg of C18 or a mixture of 100 mg PSA and 100 mg C18. Finally, the clean extracts were filtered through a 0.22 µm nylon filter before LC–MS/MS analysis.

**Method validation**

The method was validated in both matrices. Linearity was determined by using matrix matched standards, in pesticide-free samples of adult honeybees and pollen (commercial multi-floral pollen). Linearity was checked with correlation coefficients better than 0.990 in the range from MQL (method quantification limit) to 50 ng/g (in honeybees) or 100 ng/g (in pollen). Recovery rates were evaluated at four different concentration levels by spiking three blank samples at 1, 2, 10 or 25 and 50 or 100 ng/g depending on the matrix. Precision of the method was calculated by determining the average coefficient of variation of the replicate analysis of a spiked extract, during the same day for repeatability and on different days for reproducibility. Matrix effect was evaluated by comparison of the slopes obtained from the standard calibration in net solvent and matrix matched standard calibration. The MQL was evaluated as the minimum concentration of analyte that can be quantified with acceptable trueness and precision by spiking sample at 1 or 2 ng/g.

## RESULTS AND DISCUSSION

### Sample preparation

### QuEChERS-based extraction

For the QuEChERS procedure, ACN was used as solvent extraction due to their compatibility with LC and selectivity for a wide range of pesticides reducing the amount of matrix co-extractives. A percentage of acid solution is added in order to prolong the stability of certain pesticides which degrade more readily as pH increases (Table1). In addition miniaturization of the method using 1 g of sample (pollen or honeybees) was performed, which reduces cost and it is friendly with the environment because of an smaller size of sample and a reduced use of solvents in comparison with the original QuEChERS method. In order to avoid the possible degradation of sensitive pesticides, buffer salts (citrates or acetate) were added in the partition procedure. Comparison of pesticide recoveries using citrate salts according to the CEN Standard Method EN [17] and acetate salts according to the AOAC Official Method [18] was done.

Ultrasound assisted extraction was performed before salting-out with the aim of improving homogenisation of the samples. Comparison of recoveries of analytes were carried out in samples employing the same procedure with or without using sonication.

In relation to the clean-up, a comparison of adsorbent was done by combining PSA with C18 or PSA with GCB. These clean-up sorbents were assayed in acetonitrile extracts from acetate [18]) and citrate [17] salts used in the salting out procedure. Results in Figure 1 and 2 shown recoveries of the target pesticides in fresh bee pollen obtained after the application of QuEChERS methods above indicated, without (Fig 1) or with ultrasound assistance (Fig2). Appreciable differences were not observed in the recoveries of target pesticides by applying any of the QuEChERS methods, with values between 70 and 120% and RSD <20% for the majority of studied pesticides. Nevertheless, RSD values for fenpyroximate were >20% in some cases and the ultrasound assistance does not improve these values enough. On the other hand,

recoveries of spinosad were lower (66-69%) when ultrasound assisted extraction was applied along with C18 as clean-up sorbent versus no application of ultrasound. In pollen samples, any of the QuEChERS versions tested obtained acceptable recoveries results and in general, ultrasound assisted extraction does not provide any advantage.

When these QuEChERS modifications were used in honeybee samples (Fig 3 and 4), the recoveries results were not good enough in some cases. The efficiency of extraction for clofentezine was limited when GCB was used as sorbent in the purification step, regardless of the buffer salt used in the partition procedure and the application or no of ultrasound. Recoveries of fenpyroximate were low when acetate and GCB were used in the extraction procedure. For spinosad, only acceptable recoveries, 70±1.6% and 79±18.2%, were obtained when citrate salts and both clean-up procedures without the ultrasound assistance were used. In addition, spirodiclofen and spirotetramat extracted using the CEN method with further sonication gave high (C18 clean-up) or low (GCB clean-up) recoveries, respectively. These results indicate that, honeybee is a more complex matrix in comparison with corbicular pollen with regard to the extraction of these target compounds by QuEChERS methods. The QuEChERS procedures applied in this study are not adequate for the extraction of spinosad in honeybee samples and not acceptable recoveries for chlorpyrifos, clofentezine, fenpyroximate and piriprofixen were obtained, when the AOAC Official Method along with GCB in the purification step were used in the extraction of honeybee. Likely the co-extractives from honeybee matrix, depending on the reagents and sorbents used in the sample preparation, intercept with target pesticide being retained in the clean-up sorbent (GCB in the case of clofentezine and fenpyroximate) or the ultrasound assistance which may produce a more exhaustive extraction, pull out some honeybee component that in buffered solution with citrates salts interfere with the target pesticide as could be the case of spirodiclofen and spirotetramat. Then, for honeybee samples, the best QuEChERS procedure, with recoveries between 70 and 123 and RSD<20%, was the use of citrate buffer and clean-up with a mixture of magnesium sulphate, PSA and C18 without using ultrasound assisted extraction.
These results show that not always all QuEChERs procedures are suitable for the extraction of any pesticides in any matrix. Therefore, testing of the methodology must be carried out before extraction of real samples.

**Ultrasound assisted extraction**

Table 2 and 3 show the recoveries of the target pesticides in corbicular pollen and honeybee, respectively, after the application of the proposed ultrasound assisted extraction method. Except when the mixture of PSA with C18 in the extract of honeybee was used in the clean-up procedure, acceptable recoveries (between 79.1% and 118.5%) with RSD<20% were obtained for all pesticides in both matrices except for spinosad. Recoveries of spinosad were low when Florisil, C18 or a mixture of PSA with C18 were employed in extracts from trap pollen or honeybees. Therefore, the most appropriate clean-up sorbents for all analytes and matrices are PSA and alumina. We selected alumina in the d-SPE clean-up after extraction by ultrasound assistance because it is more economic than PSA.

## Method validation

Table 4 shows the parameters of the method validation for honeybee samples using the UAE method proposed. Recoveries were in the range of 70-120% with RSD<20% for the levels of concentration assayed (1, 2, 10 and 50 ng/g). MQL was 1ng/g for all the pesticides except for clofentezine and spirodiclofen that was 2ng/g. No or very low matrix effect (ME<20%) was found for all the pesticides in honeybee samples, except for hexythiazox where an enhancement of signal, versus the calibration in net solvent, was observed.

For corbicular pollen (Table 5), recoveries were in the range of 70-120% with RSD<20% for the levels of concentration assayed (1, 2, 25 and 100 ng/g). MQL was 1ng/g except for clofentezine, hexythiazox, spinosad and spirodiclofen (MQL=2 ng/g). No or very low matrix effect was found for all the target pesticides assayed in pollen (< 11.4%).

The proposed method provides MQL values lower than the values corresponding to toxic effects, both contact and oral LD50 reported for honeybees (Table 1) and facilitates quantification of pesticide residues in samples of honeybees and in pollen. Therefore, the proposed method was used for the determination of the target pesticides in samples of pollen and honeybees collected from hives installed in citrus orchards.

## RESIDUES LEVELS IN SAMPLES FROM FIELD EXPOSURE

To determine pesticide residue levels in honeybee samples, a matrix matched standard calibration was carried out by spiking the extract of free-pesticides honeybee whereas for pollen samples, a standard addition method was done to take into account the possible matrix effect due to the different botanical origin of pollen collected by honeybees. Samples of pollen can be of different origins depending of the accessibility and preferences of the honeybee for different types of flowers. The standard addition method in pollen samples was made by addition to a determined volume of pollen extract to the same volume of standard at different concentration. Standard concentrations were added to the pollen extract until the chromatographic response of pollen extract without fortified was at least 4 times lower than the fortified extract inside a lineal curve.

The findings of pesticide residues in samples of honeybees and corbicular pollen using as sample preparation the ultrasound assisted extraction procedure followed of a dispersive solid phase extraction (d-SPE) with alumina, are presented in Table 6.

The pesticides fenpyroximate, spinosad, spirodiclofen and spirotetramat were not found in any of analysed samples. Whereas spirotetramat (insecticide) and spirodiclofen (acaricide) have a high LD50 for honeybee and they do not pose risk for the honeybee, spinosad, and fenpyroximate could entail risk. Spinosad is applied for *Ceratitis capitata* control in citrus orchards. The employed formulation is Spintor Cebo, an spinosad-based insecticide formulated with a *C. capitata* attractant, and it is applied as a bait, about 1-1.5 L/ha (0.024%p/v) diluted in 10 L of water. Mangan and Moreno [32] suggested that some spinosad formulations (such as GF-120) are repellent for honeybee, and thus, honeybees would avoid any pollen or nectar mixed with this

product. Further, treatments against *C. capitata* are performed in September, when mandarins start ripening [33] and coinciding with higher adult medfly population [34], at least 6 months before citrus flowering epoch. Thus, these could be the causes of the absence of spinosad in the samples analysed in this study. Yáñez et al [35] also did not find spinosad in samples of corbicular pollen collected from apiaries located near of fruit orchards. Fenpyroximate is an acaricide also used in hive to control of the varroa mite. No fenpyroximate was neither detected in any of honey samples assayed by Kim and Myung, 2017[36]. The brief half-life of this pesticide, 3.5 days in grapes [37], could be the cause of the absence of fenpyroximate in the samples of honeybee and pollen analyzed in this work. Residue levels of clofentezine (28.5 ng/g) and etoxazole (8.2 ng/g) were only found in plot 2 in 2016, in a pollen sample at the end of citrus bloom, whereas they were not detected in honeybees in any year and plot. These last three compounds are acaricides usually used in citrus groves to control spotted spider mites, *Tetranychus urticae* mainly in mandarin varieties, and they usually are applied in mid-summer [38] outside the citrus flowering period. In spite of that, in some occasions and when *T. urticae* populations are very high, an acaricide can be used together with the aphicides in spring. This must be the case of hexythiazox, an acaricide used in citrus to control tetraniquid mites that was quantified in fresh pollen from plot 2 at all flowering period with higher amount in 2016 than in 2017. Hexythiazox was also found in honeybees, from plot 2, during all blooming in 2016 but only at the middle of flowering in 2017.

Pyriproxyfen is a juvenile hormone mimic and an insect growth regulator that prevents nymphs from developing into adulthood and thus rendering them unable to reproduce. It is used in citrus mainly to control California red scale, *Aonidiella aurantii*, and is recommended to apply it to control the first nymph generation of the insect, from mid-May to mid-June [39]. In our work, it was only found in the plot 2 at the end of flowering period in both matrices, pollen and honeybee, and the two years 2016 and 2017, coinciding with the moment of its typical use, Nevertheless, piriproxifen residues in plot 1 were also found in pollen at the beginning and at the end of blooming period, whereas it was not detected in honeybees.

Chlorpyrifos and acetamiprid were the pesticides more frequently detected in the analysed samples of fresh pollen and honeybee and the highest residues levels of these pesticides were found in the middle or end of flowering period. Both are used to control aphids that feed on new flushes mainly in spring, very close to citrus bloom. Low or no detectable residue levels of acetamiprid were found in honeybee samples during 2016 and 2017, whereas residue levels in fresh pollen varied from 1.1 ng/g to 54.7 ng/g, with appreciable differences between amounts of acetamiprid in pollen during 2016 and 2017 in plot 2. Chlorpyrifos was present in both matrices being detected in all the samples analysed with values from <MQL to 21.7 ng/g in honeybees and from <MQL to 398.2 ng/g in pollen. These high values in pollen were semiquantified because the highest spiked recovery level validated was 100 ng/g. This organophosphate pesticide of high toxicity for honeybees has been detected in other bee matrices (honeybee wax, beebread, pollen and adult honeybees [40-43]. In a study carried out by Calatayud-Vernich et al [44], the analysis of samples of dead honeybees from Spanish Mediterranean areas where the main corps are citrus revealed that chlorpyrifos was the most frequent, both in percentage and in number of positive

cases, reaching in April the maximum concentration of 140 ng/g wet honeybee that corresponded to the citrus blooming period. In addition, it has been found that chlorpyrifos and acetamiprid in pollen collected by bees from Spanish intensive farming land areas were the pesticides more prevalent with a 50% and 19% of positive cases respectively and concentration levels ranged between 7 and 104 ng/g [41].

The characteristics of the areas selected in this study to place the hives, have influenced the amount of pesticide residues in corbicular pollen and honeybees. Hives located in plot 2 are more contaminated than those located in plot 1. Plot 1 is bordered by a river, and the surrounding vegetation is mainly rice, while plot 2 is sited in a large producing area of citrus.
The detection of residues in pollen and honeybees of pesticides not directly applied in the plots or applied at least 7 months before carrying out the samplings indicate that, although the IPM areas selected are large (20 and 80 ha) and there is sufficient pollen and nectar in the citrus orchards, the honeybees forage beyond to other attractive flora or crops.

## CONCLUSION

The proposed ultrasound assisted extraction method followed of a SPE-d clean-up with alumina and LC-MS/MS determination was validated for the analysis of 10 pesticides in corbicular pollen and honeybee samples. The procedure, at four concentration levels, give acceptable recoveries in the range of 70-120% and RSDs (precision) below 20%. Linearity and matrix effects were also established and MQL were of 1 or 2 ng/g for honeybees and corbicular pollen. The proposed method has the advantage of be simple and cost effective, allowing the simultaneous extraction of several samples, requiring low reagent consumption under milder conditions of temperature and pressure, which diminishes laboratory waste, and minimizes the handling of the sample making it a simple and easy method to be carried out.

Of the 10 pesticides analyzed, six have been quantified in corbicular pollen and four in honeybees. Chlorpyrifos and acetamiprid, related to the aphids control in spring, very close to citrus blooming, were the pesticides mostly detected in the analysed samples of fresh pollen and honeybee.

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## REFERENCES

[1] FAO (Food and Agriculture organization of the United Nations) 2017. Citrus Fruit Fresh and Processed Statistical Bulletin 2016. Market and Policy Analysis of Raw Materials, Horticulture and Tropical (RAMHOT). Products Team Trade and Markets Division. Rome, 2017. http://www.fao.org/3/a-i8092e.pdf

[2] MAGRAMA. Ministerio de Agricultura, Pesca y Alimentación. El sector Apícola en Cifras. Principales indicadores económicos. (2018) Subdirección General de Productos Ganaderos. Dirección General de Producciones y Mercados Agrarios. Catálogo de Publicaciones de la Administración General del Estado: http://publicacionesoficiales.boe.es/

[3] S. G. Potts, S. P. M. Roberts, R. Dean, G. Marris, M.A. Brown, R. Jones, P. Neumann, J. Settele. Declines of managed honey bees and beekeepers in Europe. J Apic Res 49 (2010): 15-22. https://doi.org/10.3896/IBRA.1.49.1.02

[4] E. Genersch. Honey bee pathology: current threats to honey bees and beekeeping. Appl Microbiol Biotechnol 87 (2010):87–97. DOI 10.1007/s00253-010-2573-8

[5] M. Henry, M. Béguin, F. Requier, O. Rollin, J-F. Odoux, P. Aupinel, J. Aptel, S. Tchamitchian, A. Decourtye. A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. Science 336 (2012) 6079:348-350. DOI: 10.1126/science.1215039

[6] F. Requier, J.F. Odoux, T. Tamic, N. Oreau, M. Henry, A.Decourtye, V. Bretagnolle, Honey bee diet in intensive farmland habitats reveals an unexpectedly high flower richness and a major role of weeds. Ecol Appl, 25 (2015) 4: 881–890. https://doi.org/10.1890/14-1011.1

[7] K.M. Kasiotis, C. Anagnostopoulos, P. Anastasiadou, K. Machera. Pesticide residues in honeybees, honey and bee pollen by LC–MS/MS screening: Reported death incidents in honeybees. Sci Total Environ 485–486 (2014) 633–642. DOI: 10.1016/j.scitotenv.2014.03.042

[8] P. Calatayud-Vernich, F. Calatayud, E. Simó, Y. Picó. Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees. MethodsX 3 (2016) 452–458. http://dx.doi.org/10.1016/j.mex.2016.05.005

[9] C. A. Mullin, M. Frazier, J. L. Frazier, S. Ashcraft, R. Simonds, D. van Engelsdorp, J. S. Pettis, High Levels of Miticides and Agrochemicals in North American Apiaries: Implications for Honey Bee Health. (2010) PLoS ONE. Volume 5, Issue 3, e9754. DOI:10.1371/journal.pone.0009754

[10] K. A. Stoner, B. D. Eitzer. Using a Hazard Quotient to Evaluate Pesticide Residues Detected in Pollen Trapped from Honey Bees (Apis mellifera) in Connecticut PLoS ONE (2013) Volume 8, Issue 10, e77550. DOI: 10.1371/journal.pone.0077550

[11] P. Parrilla Vázquez, A. Lozano, S. Uclés, M.M. Gómez Ramos, A.R. Fernández-Alba. A sensitive and efficient method for routine pesticide multiresidue analysis in bee pollen samples using gas and liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr A, 1426 (2015) 161–173. DOI: 10.1016/j.chroma.2015.11.081

[12] L. Wiest, A. Buleté, B. Giroud, C. Fratta, S. Amic, O. Lambert, H. Pouliquen, C. Arnaudguilhem. 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, 1218 (2011) 5743– 5756. DOI: 10.1016/j.chroma.2011.06.079

[13] D. Paradis, G. Bérail, J.M. Bonmatin, L.P. Belzunces, Sensitive analytical methods for 22 relevant insecticides of 3 chemical families in honey by GC-MS/MS and LC-MS/MS. Anal Bioanal Chem 406 (2014) 621–633. DOI: 10.1007/s00216-013-7483-z

[14] B. Giroud, A. Vauchez, E. Vulliet, L. Wiest, A. Buleté, Trace level determination of pyrethroid and neonicotinoid insecticides in beebread using acetonitrile-based extraction followed by analysis with ultra-high-performance liquid chromatography–tandem mass spectrometry, J. Chromatogr. A, 1316 (2013) 53– 61. DOI: 10.1016/j.chroma.2013.09.088

[15] G. Daniele, B. Giroud, C. Jabot, E. Vulliet. 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. DOI 10.1007/s11356-017-9227-7

[16] M.D. Gil García, S. Uclés Duque, A.B. Lozano Fernández, A. Sosa, A. R. Fernández-Alba. Multiresidue method for trace pesticide analysis in honeybee wax comb by GC-QqQ-MS. Talanta 163 (2017) 54-64. http://dx.doi.org/10.1016/j.talanta.2016.10.083

[17] European Committee for Standardization (CEN) Standard Method EN 15662. Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE. Modular QuEChERS-method (2008) 84pp

[18] AOAC Official Method 2007.01. AOAC Official Method 2007.01. Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate Gas Chromatography/Mass Spectrometry and Liquid Chromatography/Tandem Mass Spectrometry First Action 2007 (2007 AOAC INTERNATIONAL)

[19] K. M. Kasiotis, Z. D. Tzouganaki, K. Machera. Chromatographic determination of monoterpenes and other acaricides in honeybees: Prevalence and possible synergies. Sci Total Environ 625 (2018) 96–105. DOI: 10.1016/j.scitotenv.2017.12.244

[20] S. Walorczyk, B. Gnusowski, 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 1216 (2009) 37: 6522-6531. DOI: 10.1016/j.chroma.2009.07.045

[21] P. Kaczynski, I. Hrynko, B. Lozowicka. Evolution of novel sorbents for effective clean-up of honeybee matrix in highly toxic insecticide LC/MS/MS analysis. Ecotox Environ Safe 139 (2017) 124–131. DOI: 10.1016/j.ecoenv.2017.01.033

[22] T. Kiljanek, A. Niewiadowska, S. Semeniuk, M. Gaweł, M. Borzęcka, A. Posyniak, 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 incidents. J. Chromatogr A 1435 (2016) 100-14 DOI: 10.1016/j.chroma.2016.01.045

[23] B. Morzycka, Simple method for the determination of trace levels of pesticides in honeybees using matrix solid-phase dispersion and gas chromatography. J. Chromatogr A 982 (2002) 2: 267-273. DOI: 10.1016/S0021-9673(02)01505-4

[24] S. Totti, M. Fernandez, S. Ghini, Y. Pico, F. Fini , J. Manes, S. Girotti Application of matrix solid phase dispersion to the determination of imidacloprid, carbaryl, aldicarb, and their main metabolites in honeybees by liquid chromatography–mass spectrometry detection. Talanta 69 (2006) 724–729. DOI: 10.1016/j.talanta.2005.11.012

[25] A.I. Garcia-Valcarcel, E. Molero, J.L. Tadeo, M.D. Hernando, Determination of selected environmental contaminants in foraging honeybees. Talanta 148 (2016): 1-6. DOI: 10.1016/j.talanta.2015.10.064

[26] E. Karazafiris, U. Menkissoglu-Spiroudi, A. Thrasyvoulou, New multiresidue method using solid-phase extraction and gas chromatography-micro-electron-capture detection for pesticide residues analysis in royal jelly. J. Chromatogr A 1209 (2008) 1-2: 17-21. DOI: 10.1016/j.chroma.2008.09.018

[27] P.G. Balayannis, Gas chromatographic determination of coumaphos and tau-fluvalinate residues in royal jelly produced under commercial conditions. J. Apic. Res**.** 40(2001) 2: 71-78. DOI: 10.1080/00218839.2001.11101054

[28] C. Pirard , J. Widart, B.K. Nguyen, C. Deleuze, L. Heudt, E. Haubruge, E. De Pauw, J.-F. Focant. Development and validation of a multi-residue method for pesticide determination in honey using on-column liquid–liquid extraction and liquid chromatography–tandem mass spectrometry. J. Chromatogr A, 1152 (2007) 116–123. DOI: 10.1016/j.chroma.2007.03.035

[29] M. Fernández , C. Padrón , L. Marconi , S. Ghini , R. Colombo , A.G. Sabatini ,S. Girottic, Determination of organophosphorus pesticides in honeybees after solid-phase

microextraction. J. Chromatogr A, 922 (2001) 257–265. DOI: 10.1016/S0021-9673(01)00880-9

[30] N. Campillo, R. Penalver, M. Hernandez-Cordoba, Evaluation of solid-phase microextraction conditions for the determination of chlorophenols in honey samples using gas chromatography. J. Chromatogr A 1125 (2006) 1: 31-37. DOI: 10.1016/j.chroma.2006.05.051

[31] J. Zhou, X. Xue, Y. Li, J. Zhang, F. Chen, L. Wu, L. Chen, J. Zhao. Multiresidue determination of tetracycline antibiotics in propolis by using HPLC-UV detection with ultrasonic-assisted extraction and two-step solid phase extraction. Food Chem. 115 (2009) 1074–1080. DOI: 10.1016/j.foodchem.2008.12.031

[32] R. L. Mangan, A. T. Moreno. Honey bee foraging preferences, effects of sugars, and fruit by toxic bait components .J. Econ. Entomol. 102 (2009) 1472-1481. DOI: 10.1603/029.102.0411

[33] M. T. Martínez-Ferrer, J. M. Campos-Rivela, J. M. Fibla Queralt,.. Field Efficacy of Ceratitis capitata (Diptera: Tephritidae) Mass Trapping Technique on Clementine Groves in Spain. J Appl Entomol. 136 (2012) 181-190. DOI: 10.1111/j.1439-0418.2011.01628.x

[34] M. T. Martínez-Ferrer, C.; Navarro, J. M. Campos, C. Marzal, J. M Fibla.; L. Bargues, F. Garcia-Marí. Seasonal and annual trends in field populations of Mediterranean fruit fly, Ceratitis capitata, in Mediterranean citrus groves: comparison of two geographic areas in eastern Spain. Span. j. agric. res. 8: (2010) 757-765. DOI: 10.5424/sjar/2010083-1275

[35] K.P Yáñez; M.T. Martín; J.L Bernal, M.J. Nozal, J. Bernal (2014). Determination of spinosad at trace levels in bee pollen and beeswax with solid–liquid extraction and LC–ESI-MS. J. Sep. Sci. 37 (2014) 204–210. DOI: 10.1002/jssc.201301069

[36] J. M. Kim, S-W. Myung Determination of Fenpyroximate from Four Types of Honey by Liquid Chromatography/Tandem Mass Spectrometry. Bull. Korean Chem. Soc. 39 (2018)65–70. DOI: 10.1002/bkcs.11346

[37] F. Malhata, A. El-Mesallamyb, M. Assyb, W. Madianb, N. M. Loutfyc, M. Tawfic Ahmedc. Residues, half-life times, dissipation,and safety evaluation of the acaricide fenpyroximate applied on grapes. Toxicol Environ Chem 95 (2013) 1309–1317. DOI: 10.1080/02772248.2013.877245

[38] S. Pascual-Ruiz, E. Aguilar-Fenollosa,V. Ibáñez-Gual, M.A. Hurtado-Ruiz, M.T. Martínez-Ferrer, J.A. Jacas, Economic Threshold for Tetranychus urticae (Acari: Tetranychidae) in clementine mandarins Citrus. Exp. Appl. Acarol. 62 (2014):337–362. DOI: 10.1007/s10493-013-9744-0

[39] J. M. Campos-Rivela, M. T. Martínez-Ferrer, J. M. Fibla-Queralt, Population dynamics and seasonal trend of California red scale (Aonidiella aurantii Maskell) in citrus in Northern Spain. Span. j. agric. res. 10 (2012).198-208. DOI: 10.5424/sjar/2012101-105-11

[40] S. Tosi, C. Costa, U. Vesco, G. Quaglia,G. Guido, A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by gricultural pesticides. Sci. Total Environ. 615 (2018) 208–218. DOI: 10.1016/j.scitotenv.2017.09.226

[41] P. Calatayud-Vernich, F. Calatayud, E. Simó, Y. Picó. Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure. Environ. Pollut. 241 (2018)106-114. DOI: 10.1016/j.envpol.2018.05.062

[42] R. Manning, Chemical residues in beebread, honey, pollen and wax samples collected from beehives placed on canola crops in Western Australia. J. Apic. Res**.**57 (2018) 696-708 https://doi.org/10.1080/00218839.2018.1494889

[43] P. Calatayud-Vernich, F. Calatayud, E. Simó, Y. Picó Pau Calatayud-Vernich, Occurrence of pesticide residues in Spanish beeswax. Sci. Total Environ. 605–606 (2017) 745–754. DOI: 10.1016/j.scitotenv.2017.06.174

[44] P. Calatayud-Vernich, F. Calatayud, E. Simó, M.M. Suarez-Varela, Y. Picó, Influence of pesticide use in fruit orchards during blooming on honeybee mortality in 4 experimental apiaries. Sci. Total Environ. 541 (2016) 33–41. DOI: 10.1016/j.scitotenv.2015.08.131

**FIGURE CAPTIONS**

Fig 1. Recoveries (20 ng/g) of pesticides by using QuEChERS-based extraction in corbicular pollen

Fig 2. Recoveries (20 ng/g) of pesticides by using QuEChERS-based extraction with UAE (ultrasound assisted extraction) in corbicular pollen.

Fig 3. Recoveries (20 ng/g) of pesticides by using QuEChERS-based extraction in honeybee

Fig4. Recoveries (20 ng/g) of pesticides by using QuEChERS-based extraction with UAE (ultrasound assisted extraction) in honeybee

**Table 1**

Table1. Characteristics of selected pesticides

| Pesticide | Trade name | Chemical group | Mode of Action (IRAC classification) | Field rate (kg or L/ha) | Target pest in citrus | Physic-chemical properties[1] | | | | Contact –Oral LD50 [2] (µg/bee) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | MW (g/mol) | logKow | Stabil[a] (days) | Solub (mg/L) | |
| Acetamiprid | Epik Gazel Plus | Neonicotinoid | Nicotinic acetylcholine receptor (nAChR) competitive modulators (4A) | 0.5 kg | Aphids | 222,67 | 0.8 | stable | 2950 | 7.9-14 |
| Chlorpyrifos | Piritec | Organophosphate | Acetylcholinesterase (AChE) inhibitor (1B) | 0.25 L | CRS Aphids | 350.58 | 4.7 | 53.5* | 1.05 | 0.07-0.24 |
| Clofentezine | Apolo 50-SC Skunk | Tetrazine | Mite growth inhibitor (10A) | 0.2 L | TSSM | 303.15 | 3.1 | 1.43* | 0.002 | 48-71 |
| Etoxazole | Borneo | Diphenyl oxazoline | Mite growth inhibitor (10B) | 0.5 L | TSSM | 359.42 | 5.52 | 161* | 0.07 | >200->200 |
| Fenpyroximate | Flash UM | Pyrazolium (phenoxypyrazole) | Mitochondrial complex I electron transport inhibitors (21) | 2 L | TSSM | 421.49 | 5.01 | 226** | 0.023 | 11-n.a |
| Hexythiazox | Diablo | Carboxamide | Mite growth inhibitor (10A) | 1.5 L | TSSM | 352.88 | 2.67 | stable | 0.1 | >200- n.a |
| Pyriproxyfen | Discolo Alazin | Pyridine | Juvenile hormone mimic (7C) | 1.5 L | CRS | 321.37 | 5.37 | Stable | 0.367 | >100-n.a |
| Spinosad | Spintor-Cebo | Spinosyn A (95%) and B (5%) (macrocyclic lactones) | Nicotinic acetylcholine receptor (nAChR) allosteric modulators (5) | 1.5 L | Medfly | 731.98 | 2.8 | >30 | 89 | 0.003-0.057 |
| Spirodiclofen | Envidor | Tetronic acid | Inhibitors of acetyl CoA carboxylase (23) | 0.6 L | TSSM | 411.32 | 5.83 | 52.1* | 0.05 | 256-252 |
| Spirotetramat | Movento | Tetramic acid | Inhibitors of acetyl CoA carboxylase (23) | 1.5 L | CRS | 373.48 | 2.51 | 8.6* | 29.9 | 242-195 |

[a]Acuoso hidrólisis DT50 (days) at 20°C and pH 7 *pH sensitive, ** slow but pH sensitive (1)PPDB Pesticide Properties Database Agriculture & Environment Research Unit (AERU) at the University of Hertfordshire https://sitem.herts.ac.uk/aeru/footprint/es/index.htm and www.fao.org/fileadmin/templates/agphome/documents/Pests

(2) Toxicity data for honey bees were obtained from the Pesticide Manual (Tomlin CDS (2009) The e-Pesticide Manual. In: Tomlin CDS, editor. 12 ed. Surrey, U.K.: British Crop Protection Council.), the ECOTOX database of the U.S. Environment Protection Agency (http://cfpub.epa.gov/ecotox/) and the AgriTox Database of the Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail in France (http://www.agritox.anses.fr/index.php). n.a: no available. TSSM: Two-spotted spider mite; CRS: California Red Scale.

**Table 2**

Table 2. Recoveries of pesticides (20 ng/g) by using ultrasound assisted extraction in corbicular pollen.

| | **Clean-up sorbent** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Alúmina | | Florisil | | PSA:C18 | | PSA | | C18 | |
| | % Rec | %RSD | % Rec | %RSD | % Rec | %RSD | % Rec | %RSD | % Rec | %RSD |
| **ACETAMIPRID** | 90.4 | 3.8 | 90.0 | 7.8 | 90.7 | 4.9 | 88.2 | 8.9 | 94.3 | 8.3 |
| **CHLORPYRIFOS** | 98.5 | 9.8 | 101.3 | 9.7 | 97.5 | 5.3 | 97.2 | 3.9 | 88.5 | 4.1 |
| **CLOFENTEZINE** | 95.4 | 7.3 | 84.3 | 9.2 | 83.3 | 2.4 | 91.0 | 10.3 | 89.3 | 7.6 |
| **ETOXAZOLE** | 102.8 | 8,1 | 93.9 | 9.1 | 89.4 | 3.4 | 100.0 | 8.8 | 85.0 | 6.2 |
| **FENPYROXIMATE** | 104.0 | 16.8 | 94.9 | 14.2 | 88.6 | 3.3 | 104.0 | 18.9 | 91.4 | 7.7 |
| **HEXYTHIAZOX** | 94.0 | 11.6 | 91.8 | 7.2 | 85.9 | 1.0 | 92.8 | 11.3 | 88.3 | 6.2 |
| **PYRIPROXYFEN** | 94.6 | 5.2 | 97.4 | 5.8 | 90.1 | 2.3 | 97.5 | 5.6 | 88.4 | 5.4 |
| **SPINOSAD** | 76.9 | 4.4 | 8.7 | 8.5 | 51.1 | 4.1 | 81.4 | 5.5 | 59.7 | 5.2 |
| **SPIRODICLOFEN** | 104.9 | 7.9 | 97.4 | 6.3 | 104.8 | 4.5 | 99.9 | 9.8 | 88.5 | 9.6 |
| **SPIROTETRAMAT** | 102.2 | 11.5 | 99.5 | 17.2 | 92.3 | 10.0 | 81.4 | 15.9 | 102.6 | 19.7 |

**Table3**

Table 3. Recoveries of pesticides (20 ng/g) by using ultrasound assisted extraction in honeybee.

| | **Clean-up sorbent** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Alúmina | | Florisil | | PSA:C18 | | PSA | | C18 | |
| | % Rec | %RSD | % Rec | %RSD | % Rec | %RSD | % Rec | %RSD | % Rec | %RSD |
| **ACETAMIPRID** | 99.8 | 3.7 | 90.1 | 8.4 | 119.9 | 5.5 | 111.2 | 8.4 | 117.6 | 5.2 |
| **CHLORPYRIFOS** | 93.2 | 17.7 | 99.7 | 14.4 | 107.9 | 2.7 | 124.9 | 7.4 | 80.8 | 10.6 |
| **CLOFENTEZINE** | 89.9 | 6.9 | 82.5 | 7.3 | 132.8 | 6.6 | 118.5 | 4.9 | 114.9 | 4.7 |
| **ETOXAZOLE** | 86.7 | 3.9 | 80.0 | 6.8 | 96.6 | 6.9 | 103.7 | 4.5 | 89.9 | 3.9 |
| **FENPYROXIMATE** | 83.7 | 9.4 | 117.4 | 31.7 | 126.0 | 24.2 | 116.2 | 19.0 | 99.3 | 8.3 |
| **HEXYTHIAZOX** | 91.9 | 2.7 | 91.9 | 14.8 | 121.6 | 12.7 | 114.2 | 12.8 | 86.1 | 11.7 |
| **PYRIPROXYFEN** | 91.8 | 7.5 | 84.3 | 4.1 | 92.9 | 1.4 | 101.0 | 1.7 | 84.5 | 4.4 |
| **SPINOSAD** | 95.8 | 2,8 | 46.6 | 6.1 | 44.0 | 5.4 | 105.5 | 4.1 | 61.5 | 1.8 |
| **SPIRODICLOFEN** | 86.9 | 10.8 | 81.3 | 10.4 | 102.5 | 15.1 | 99.9 | 6.2 | 79.1 | 9.0 |
| **SPIROTETRAMAT** | 95.3 | 14.7 | 99.2 | 14.8 | 116.2 | 7.0 | 105.8 | 3.7 | 119.9 | 4.7 |

**Table 4**

Table 4. Validation parameters of the LC-QqQ-MS/MS method in honeybee using the ultrasound assisted extraction method. R (%) %Recoveries, RSD % precision, ME % matrix effect

| Pesticide | Linearity | | 1 ng/g | | 2 ng/g | | 10 ng/g | | 50 ng/g | | ME (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (ng/g) | r2 | R (%) | RSD(%) | R(%)…RSD(%) | | R% | RSD(%) | R% | RSD(%) | |
| Acetamiprid | 1-50 | 0.9919 | 89.3 | 12.1 | 82.14 | 7.6 | 98.7 | 6.0 | 99.5 | 7.9 | -1.0 |
| Chlorpyrifos | 1-50 | 0.9979 | 107.7 | 8.9 | 107.4 | 5.7 | 95.7 | 6.1 | 99.8 | 3.6 | -32.0 |
| Clofentezine | 2-50 | 0.9912 | | | 84.7 | 20.2 | 97.6 | 5.6 | 77.9 | 20.0 | -1 |
| Etoxazole | 1-50 | 0.9987 | 115.5 | 11.4 | 94.8 | 6.4 | 91.6 | 5.4 | 103.5 | 7.6 | -0.1 |
| Fenpypoximate | 1-50 | 0.9957 | 99.4 | 13.5 | 95.0 | 0.4 | 112.6 | 16.0 | 95.5 | 7.6 | -8 |
| Hexythiazox | 1-50 | 0.9985 | 98.3 | 18.0 | 76.8 | 6.7 | 95.6 | 9.8 | 99.4 | 10.0 | 24.7 |
| Pyriproxyfen | 1-50 | 0.9945 | 110.7 | 15.7 | 90.3 | 5.7 | 96.7 | 7.6 | 99.1 | 5.8 | 2.5 |
| Spinosad | 1-50 | 0.9979 | 107.2 | 5.0 | 102.3 | 6.8 | 99.6 | 5.7 | 95.9 | 3.0 | 0 |
| Spirodiclofen | 2-50 | 0.9929 | | | 86.1 | 13.8 | 74.4 | 9.7 | 98.7 | 6.4 | -10 |
| Spirotetramat | 1-50 | 0.9950 | 113.9 | 20.5 | 98.2 | 17.3 | 100.1 | 19.6 | 119.9 | 19.8 | -4 |

**Table 5**

Table 5. Validation parameters of the LC-QqQ-MS/MS method in corbicular pollen using the ultrasound assisted extraction method. R (%) %Recoveries, RSD % precision, ME % (matrix effect)

| Pesticide | Linearity | | 1 ng/g | | 2 ng/g | | 25 ng/g | | 100 ng/g | | ME (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | (ng/g) | r2 | R(%) | RSD(%) | R(%) | RSD(%) | R% | RSD(%) | R% | RSD(%) | |
| Acetamiprid | 1-100 | 0.9985 | 99.1 | 28.4 | 108.5 | 17.1 | 91.0 | 7.8 | 104.4 | 5.2 | -1.1 |
| Chlorpyrifos | 1-100 | 0.9975 | 100.3 | 10.0 | 109.8 | 9.2 | 94.4 | 3.7 | 99.8 | 2.7 | -0.4 |
| Clofentezine | 2-100 | 0.9993 | | | 102.8 | 18.8 | 72.1 | 11.9 | 88.4 | 9.5 | -6.8 |
| Etoxazole | 1-100 | 0.9994 | 106.8 | 6.5 | 107.1 | 6.2 | 92.9 | 6.1 | 100.1 | 6.5 | -0.6 |
| Fenpypoximate | 1-100 | 0.9984 | 120.0 | 6.8 | 110.3 | 7.0 | 87.3 | 19.3 | 95.0 | 10.3 | 0.4 |
| Hexythiazox | 2-100 | 0.9966 | | | 104.1 | 12.4 | 85.0 | 10.9 | 98.8 | 8.5 | -1.3 |
| Pyriproxyfen | 1-100 | 09995 | 112.6 | 20.1 | 120.3 | 8.8 | 94.8 | 0.9 | 98.6 | 3.7 | -11.0 |
| Spinosad | 2-100 | 0.9981 | | | 81.0 | 17.9 | 75.2 | 3.1 | 69.9 | 6.1 | -11.4 |
| Spirodiclofen | 2-100 | 0.9969 | | | 103.2 | 3.8 | 99.9 | 6.6 | 97.1 | 3.9 | 11.3 |
| Spirotetramat | 1-100 | 0.9983 | 107.4 | 9.9 | 101.9 | 15.8 | 72.1 | 10.7 | 70.0 | 15.8 | 1.6 |

**Table 6**

Table 6. Levels of pesticide residues in honeybees and fresh pollen collected in IPM citrus orchards after sample preparation with the ultrasound assisted extraction procedure.

| Pesticide | Sample period[1] | PLOT 1 | | | | PLOT 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2016 | | 2017 | | 2016 | | 2017 | |
| | | Honeybee (ng $g^{-1}$) | Pollen (ng $g^{-1}$) | Honeybee (ng $g^{-1}$) | Pollen (ng $g^{-1}$) | Honeybee (ng $g^{-1}$) | Pollen (ng $g^{-1}$) | Honeybee (ng $g^{-1}$) | Pollen (ng $g^{-1}$) |
| Acetamiprid | S1 | n.d | n.d | n.d | 6.4 | n.d | 23.4 | n.d | <MQL |
| | S2 | 1.1 | 23.0 | n.d | 1.1 | 4.5 | 54.7 | n.d | 1.7 |
| | S3 | n.d | 19.0 | 1.9 | 21.4 | <MQL | 19.8 | n.d | <MQL |
| | S4 | --- | --- | --- | --- | n.d | 11.8 | --- | --- |
| Chlorpyrifos | S1 | <MQL | 75.3 | <MQL | 7.1 | 1.4 | 40.1 | 3.4 | 160.1 |
| | S2 | 5.0 | 81.6 | <MQL | 6.0 | <MQL | 17.7 | <MQL | 71.5 |
| | S3 | 1.0 | 19.4 | 4.7 | 12.5 | 1.5 | 8.3 | 21.7 | 398.2 |
| | S4 | --- | --- | --- | --- | 12.2 | 388.5 | --- | --- |
| Clofentezine | S1 | n.d | n.d | n.d | <MQL | n.d | n.d | n.d | <MQL |
| | S2 | n.d | n.d | n.d | <MQL | n.d | n.d | n.d | <MQL |
| | S3 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d |
| | S4 | --- | --- | --- | --- | n.d | 28.5 | --- | --- |
| Etoxazole | S1 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d |
| | S2 | n.d | n.d | n.d | n.d | n.d | <MQL | n.d | n.d |
| | S3 | n.d | n.d | n.d | n.d | n.d | <MQL | n.d | n.d |
| | S4 | --- | --- | --- | --- | <MQL | 8.2 | --- | --- |
| Hexythiazox | S1 | n.d | n.d | n.d | n.d | 2.1 | 6.4 | n.d | 4.8 |
| | S2 | n.d | n.d | n.d | n.d | 54.4 | 41.1 | 3.4 | 24.4 |
| | S3 | n.d | n.d | n.d | n.d | 17.8 | 7.7 | n.d | 2.3 |
| | S4 | --- | --- | --- | --- | 11.1 | 27.3 | --- | --- |
| Pyriproxifen | S1 | n.d | n.d | n.d | 10.2 | n.d | n.d | n.d | n.d |
| | S2 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | n.d |
| | S3 | n.d | n.d | n.d | 1.0 | n.d | n.d | 3.4 | 6.4 |
| | S4 | --- | --- | --- | --- | 2.5 | 43.2 | --- | --- |

[1] See Table 2S

**Figure 1**

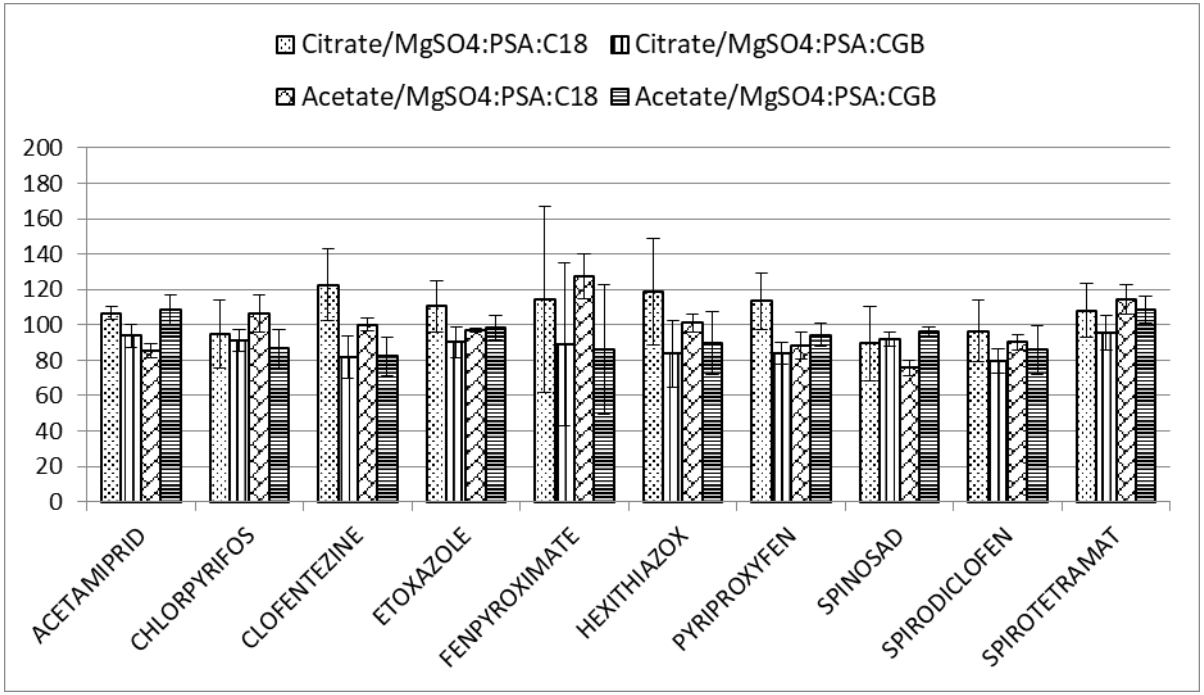


**Fig 1**.

**Figure 2**

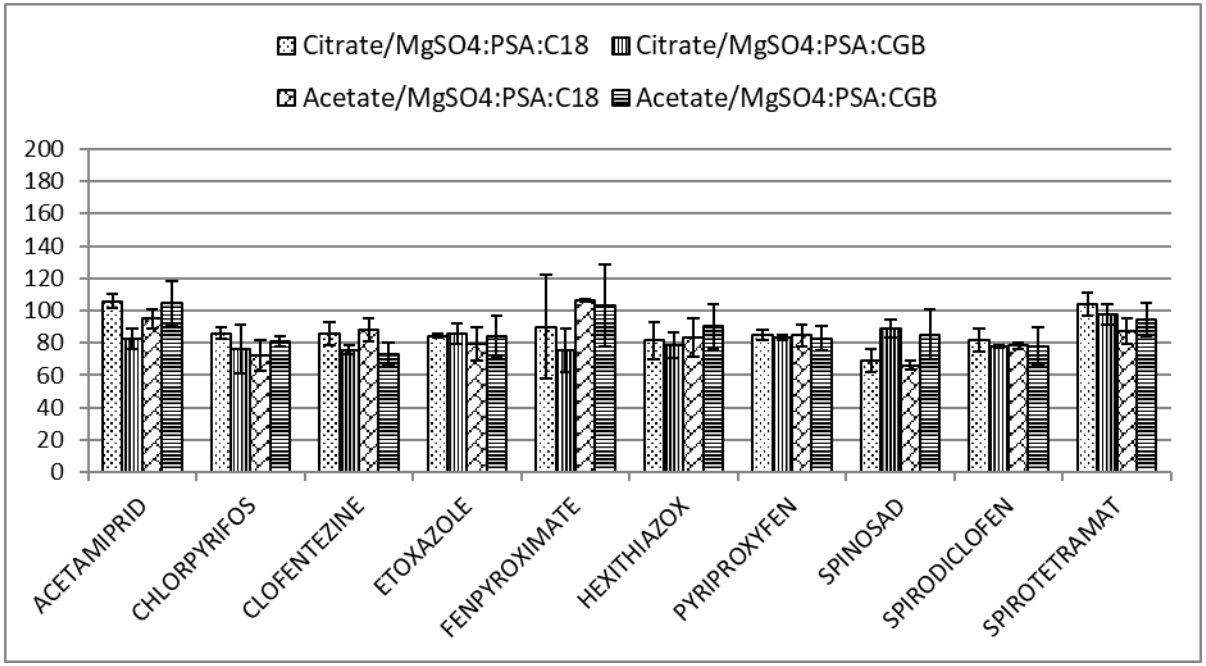


**Fig 2**.

**Figure 3**

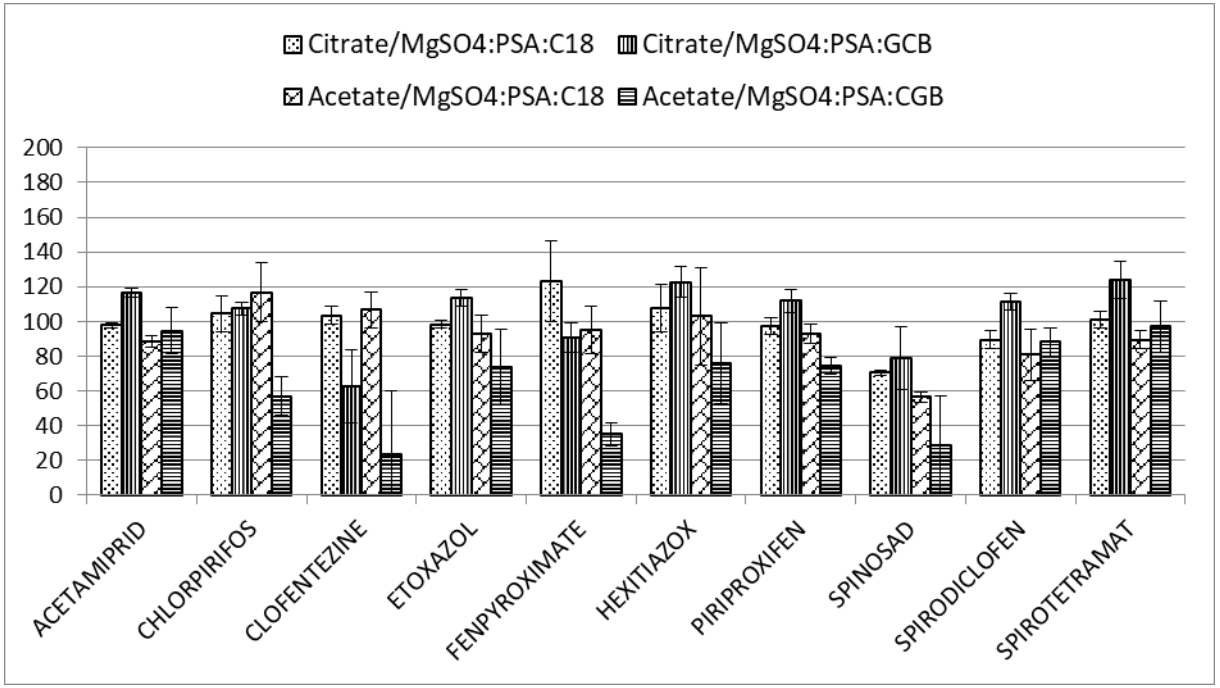


**Fig 3**.

**Figure 4**

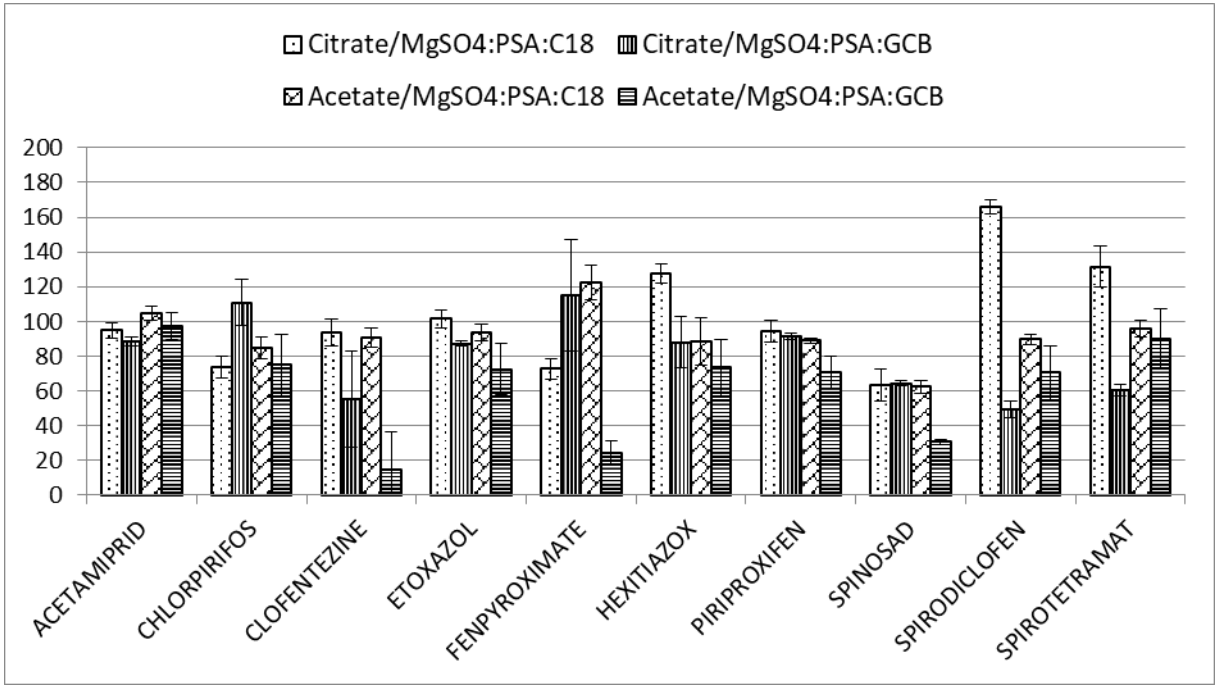


**Fig 4**.

**Checklist**

1. Cover letter
2. Novelty Statement
3. Highlights
4. Graphical abstract
5. Tables
6. Figures
7. Supporting information
8. Manuscript
9. List of three potential reviewers
10. Checklist

*Graphical Abstract (for review)

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