DETERMINATION OF ACARICIDES IN HONEYS FROM DIFFERENT BOTANICAL ORIGINS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Abstract

An analytical method has been proposed and validated to determine seven acaricides (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, and τ-fluvalinate) in honeys from different botanical origins (multifloral, heather and rosemary) by means of gas chromatography-mass spectrometry. An efficient and simple sample treatment was proposed that involved a solvent extraction with an ethyl acetate and cyclohexane (50:50, v/v) mixture. Chromatographic analysis (< 25 min) was performed in a DB-5MS column under programmed temperature conditions. The method was validated in terms of selectivity, limits of detection (0.2-2.0 μg kg^{-1}) and quantification (0.5-7.6 μg kg^{-1}), linearity (limit of quantification-700 (heather) or 800 (multifloral and rosemary) μg kg^{-1}), matrix effect (< 20% in most cases), trueness (recoveries between 81% and 108%), and precision (relative standard deviation < 15%). Finally, of the seven acaricides investigated in several honey samples only τ-fluvalinate residues (< limit of quantification - 23 μg kg^{-1}) were found.

Keywords: acaricides; food analysis; food safety; gas chromatography-mass spectrometry; honey; method validation.

1. Introduction

In recent years a series of food alerts have been issued related to the detection of contaminants such as pollutants, antibiotics, or pesticides in honey from around the world (Valverde, Ibáñez, Bernal, Nozal, Hernández, & Bernal, 2018; Notardonato, Avino, Cinelli, & Russo, 2016). It should be mentioned that the worldwide emergence of the problems created by *Varroa destructor* has required the use of different compounds for its mitigation, and in particular different acaricides, the effectiveness of which is less due in large part to resistance phenomena (Martin, 2004). This leads to the frequent application of doses higher than those recommended, and it is highly likely that acaricide residues appear in the different beehive products (Nozal, Imaz, Bernal, Nieto, Higes, & Bernal, 2021) including honey. Indeed, acaricide residues have been found in honeys from different countries (Almeida, Oloris, Faria, Ribeiro, Cantini, & Soto, 2020; Bajuk et al., 2017; Calatayud-Vernich, Calatayud, Simó, & Picó, 2016; Chiesa et al., 2016; Gaweł et al., 2019; Jiménez, Bernal, & Atienza, 1996; Kamel, & Al-Ghamdi, 2006; Kumar, Gill, Bedi, & Kumar, 2018; Lasheras, Lázaro, Burillo, & Bayarri, 2021; Panseri et al., 2020; Lazarus et al., 2021; Martel., Zeggane, Aurières, Drajnudel, Faucon, & Aubert, 2007; Notardonato, Avino, Cinelli, & Russo, 2014; Notardonato et al., 2016; Panseri et al., 2020; Rafique et al., 2018; Rial-Otero, Gaspar, Moura, & Capello, 2007; Shamsipur, Yazdanfar, & Ghambarian, 2016; Shendy, Al-Ghobashy, Mohammed, Alla, & Lofty, 2016; Zheng et al., 2018), and maximum residue levels have been established by regulatory agencies like the Directorate-General for Health and Food Safety of the European Commission see **Table 1**; European Union Pesticide Database, 2022) to protect the consumer´s health.

As shown in **Table 1S** (see Supplementary Material), in recent years acaricides have been studied in honey principally by gas chromatography (GC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detectors, although other detectors such as electron capture and nitrogen-phosphorus, among others, have also been employed. The preference for GC could be attributed to the physicochemical characteristics of the acaricides; however, some of these, especially coumaphos, have been been determined by means of liquid chromatography (LC) coupled to ultraviolet-visible/diode-array detectors or MS/MS detectors. Furthermore, different sample treatments have been proposed (see Supplementary Material, **Table 1S**); these include conventional approaches such as solvent extraction (acetone, acetonitrile, ethyl acetate, hexane, or cyclohexane), and solid-phase extraction (SPE) using different sorbents like C_{18}, XAD-2 or carbograph. Sample preparation methods based on QuEChERS (quick, easy, cheap, effective, rugged, and safe) were mainly proposed. Other alternatives such as accelerated solvent extraction, solid-phase microextraction, and dispersive liquid-liquid microextraction were selected in some studies (see Supplementary Material, **Table 1S**). Thus, acaricides have been studied in honey in quite different ways, and in most cases, they were determined with many other pesticides from different families, which could explain the existence of the recurrent matrix effect. In addition, in only one study (Zheng et al., 2018) the LC-MS/MS based-method was validated for honeys of different botanical origins, although the final conditions were the same for all the honeys studied and a matrix effect was observed. This is quite an important issue, as we have previously demonstrated with other pesticides that the different physicochemical characteristics of the honey depending on its botanical origin meant that different sample treatments had to be applied (Valverde et al., 2018; Ruiz, Ares, Valverde, Martín, & Bernal, 2020).

Therefore, the main goal of the present study was to propose an alternative GC-MS-based method for determining in honeys of different botanical origins (multifloral, rosemary and heather) seven of the most frequently detected acaricides in bee products around the world: atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, and τ-fluvalinate (Gil-García, Uclés-Duque, Lozano-Fernández, Sosa, & Fernández-Alba, 2017; Mullin et al., 2010). Our aim is to develop a new method by adapting the GC-MS conditions from a recent study of our group on beeswax (Nozal et al., 2021) and proposing a novel sample treatment that should be efficient, simple, economic, fast, and as environmentally friendly as possible. We have managed to obtain optimal performance in terms of extraction efficiency (recoveries), minimizing as far as possible the matrix effect and fulfilling the principles of green analytical chemistry (Gałuszka, Migaszewski, & Namieśnik, 2013). To the best our knowledge, this is the first GC-based study that was optimized and validated in order to determine these acaricides in honeys of different botanical origins. Further goals of the present study were to validate the proposed method for all the above-mentioned aspects, and to analyze honey samples from different Spanish regions.

2. Materials and methods

2.1. Reagents and materials

Acaricide standards (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, τ-fluvalinate and chlorfenvinphos-d10; see structures in **Table 2S**, Supplementary Material), all of analytical-grade and with purity greater than 99%, were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All solvents (ethyl acetate, cyclohexane, methanol, acetonitrile, and acetic acid) were of chromatographic grade and obtained from VWR Prolabo Chemicals (Fontenay-sous-Bois, France). Ultrapure water was obtained using Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). A vortex

mechanical mixer from Heidolph (Schwabach, Germany), a thermostated ultrasound bath, a drying oven, and a vibromatic mechanical shaker, all supplied by J.P. Selecta S.A. (Barcelona, Spain), a 5810 R refrigerated bench-top centrifuge from Eppendorf (Hamburg, Germany), a R-3 rotary evaporator from Buchi (Flawil, Switzerland), and Nylon syringe filters (17 mm, 0.45 μm; Nalgene, Rochester, NY) were employed for sample treatment. In addition, QuEChERS dSPE enhanced matrix removal lipid (EMR-Lipid) sorbent was supplied by Agilent Technologies (Folsom, CA, USA), and primary secondary amine (PSA) and C_{18} dSPE sorbents were provided by Supelco (Bellefonte, PA, USA); while different SPE cartridges like Oasis™ HLB (3 mL; 60 mg; Waters, Milford, MA, USA), Florisil® (3 mL; 500 mg; Waters, Milford, MA, USA), Strata® C_{18}-E (3 mL; 500 mg; Phenomenex, Torrance, CA, USA), and Strata® X (3 mL; 60 mg; Phenomenex, Torrance, CA, USA), as well as a 10-port Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA), were used for SPE extractions.

2.2. Standards

Standard (matrix-free) stock (≈ 1000 mg L^{-1}) and working solutions of the studied acaricides were prepared in a mixture of ethyl acetate and cyclohexane (50:50, v/v). Honey samples (5 g multifloral and rosemary; 2 g heather), in which the absence of acaricide residues had been previously confirmed using GC-MS (blank samples) were spiked with variable amounts of the analytes before (BF samples) or after (AF samples) sample treatment (see subsection 2.3) to prepare the standard in matrix extracts; the spiking of the samples was done similarly to Ares et al., (2020; see Supplementary Material, **Table 3S**), and the internal standard (IS; chlorfenvinphos-d10) was always added at the same concentration (0.1 mg L^{-1} or 40 μg kg^{-1}). These samples were used for validation (spiked samples (low, medium, and high) and calibration curves), and sample treatment studies. It must be specified that three replicates for each botanical origin, which were injected three times, were prepared for all the above-

mentioned studies mentioned. Each spiked sample was prepared with 5 g (multifloral and rosemary) or 2 g (heather) of blank honey samples spiked with three different concentrations of the acaricides within the linear range. These were as follows: low-LOQ (see **Table 1**); medium-100 µg kg^{-1}; high-800 µg kg^{-1} (rosemary and multifloral), 700 µg kg^{-1} (heather). The standard stock solutions were stored in glass containers in darkness at -20 ºC; working and standard matrix solutions were stored in glass containers and kept in the dark at +4 ºC.

2.3. Sample procurement and treatment

2.3.1. Samples

Honey samples (n = 18) from different regions of Spain, in which acaricides treatment had been employed on certain crops, were kindly donated by the Center for Agroenvironmetal and Apicultural Investigation (Marchamalo, Guadalajara, Spain) or purchased in local markets (Valladolid, Spain). They were selected according to their botanical origin, different colors (light honey: multifloral and rosemary; dark honey: heather; see Supplementary Material, **Figure 1S**), and composition. Their botanical origin was confirmed by melissopalynological analysis and corresponded to rosemary, *Rosmarinus officinalis* (six samples); multifloral (six samples); heather, *Erica spp* (six samples). To homogenize each of these samples, they were individually stirred with a glass rod and subsequently stored in different tubes in darkness at 4 ºC until analysis. Three replicates (sub-samples) of each honey sample, which were injected in triplicate, were examined to determine the acaricides content.

2.3.2. Sample treatment

Briefly, 5 g (rosemary and multifloral) or 2 g (heather) homogenized honey was weighed in a 50 mL centrifuge tube, after which 10 mL of ultrapure water were added, and the tube was then shaken for 1 min in a vortex device, and during 3 min in an ultrasonic bath at 25º C. Then, 10

mL of an ethyl acetate and cyclohexane (50:50, v/v) mixture was added to the tube. The resulting mixture was shaken in the vibromatic mechanical shaker for 10 min and centrifuged (10000 rpm, 5 ºC) for 5 min. This procedure was repeated for heather honeys. Then, 5 mL (rosemary and multifloral) or 14 mL (heather) of the extract were collected and evaporated to dryness at 60ºC in a rotary evaporator. Finally, the dry extract was reconstituted with 1 mL (rosemary and multifloral) or 0.5 mL (heather) of an IS solution (0.1 mg L^{-1}), and it was passed through a 0.45 µm nylon filter before the GC-MS analysis. **Figure 1** outlines the steps of the procedures used during the present study.

2.4. GC-MS conditions

An Agilent Technologies (Palo Alto, CA, USA) 7890A gas chromatograph (GC) coupled to an Agilent Technologies 5975C mass spectrometer (MS) equipped with an ALS 7693B autosampler and a MS ChemStation E 01.00.237 software (Agilent Technologies) was employed. The chromatographic column was an Agilent DB-5MS (30 m × 0.25 mm × 0.2 µm). The GC-MS parameters were adapted from previous work (see **Table 2**; Nozal et al., 2021). Analyses were performed in selected ion monitoring (SIM) mode, with one target/quantification and two qualifier ions for each analyte (see **Table 2**). Under optimal GC-MS conditions, all compounds eluted in less than 21 min (see **Figure 2**). It should be noted that τ-fluvalinate showed two peaks, which is because this compound presents a diastereomeric pair of compounds (Frison, Breitkreitz, Currie, Nelson, & Sporns, 1999). Thus, the sum of their corresponding areas was employed for quantification purposes.

3. Results and Discussion

3.1. Optimization of the sample treatment

As mentioned in the introduction, the most used sample treatments when determining acaricides were QuEChERS, SPE, and solvent extraction. It has also been indicated that one of the objectives of the study was to propose a procedure which was simple, fast, and with as little consumption and use of reagents as possible. For this reason, we decided to start by testing solvent extraction. Optimization was carried out by analyzing spiked blank honey samples of the three different botanical origins in all cases (see Subsection 2.2). Different compounds have been employed to extract acaricides from honey, chiefly hexane, acetone, acetonitrile, ethyl acetate, and cyclohexane (see Supplementary Material, **Table 1S**). However, the current trend in sample treatment prioritizes the use of solvents that are lowest in terms of toxicity and that are compatible with the environment (green solvents), such as acetate of ethyl and cyclohexane, both of which are used in this study. Cyclohexane is greener than n-hexane (Byrne et al., 2016), and ethyl acetate is a more environmentally-friendly solvent than acetonitrile and quite similar to acetone (Capello, Fischer, & Hungerbühler, 2007). A combination of these solvents was previously employed in sample treatment proposed by Lazarus et al. (2021), yet in the present study a large amount of both solvents (200 mL) was used. As a result of the experience of our research group and previous studies (Valverde et al., 2018; Ruiz et al., 2020), our optimization process began with our decision to use 5 g of honey. This was dissolved in 10 mL of water, after 1 min of shaking time (vortex) and 3 min in an ultrasound bath at 27 ºC, 10 mL of solvent, and 10 min of agitation in a vibromatic mechanical shaker. Next, the resulting mixture was centrifuged (5 min, 5 ºC, 10000 rpm), and 5 mL of supernatant were collected and evaporated to dryness at 60 ºC. Finally, the dry residue was reconstituted with 1 mL of the extraction mixture (BF samples) or 1 mL of the IS at 0.1 mg L^{-1} (AF samples). Firstly, the influence of the combined ethyl acetate and cyclohexane mixture on analyte extraction was explored. Thus, three different mixtures (20:80, 50:50, 80:20; v/v) were tested, and the results showed in all cases that the highest recoveries were obtained with the 50:50 (v/v) mixture (see Supplementary Material,

Table 4S). However, these values were lower for heather honeys, recoveries in most cases being lower than 50%. In addition, a significant matrix effect (> 20%) was observed for some of the compounds examined in heather honeys (chlorpyrifos, α-endosulfan, bromopropylate, and coumaphos). Following this, the effect of the addition of salts in extraction efficiency was evaluated so as to remove the water that might have remained in the organic phase, favouring the salting-out process. Consequently, 0.5 g of magnesium sulfate and sodium chloride were added to the resulting mixture before the shaking procedure, despite no significant increase in the recovery percentages for heather honeys being observed (data not shown); subsequently, they were no longer used. Next, the influence of the extractant volume (5-15 mL) and shaking time (5-15 min) on extraction efficiency was studied. The highest recovery values were always obtained when using 10 mL of the selected mixture (data not shown) and 10 min of shaking time (see Supplementary Material, **Table 5S**). No significant improvements in extraction efficiency for heather honeys were observed with these modifications. Therefore, this was the point at which the optimal conditions for light honeys (multifloral and rosemary) were selected (see subsection 2.3.2 and Figure 1). The results were satisfactory in terms of recovery percentages (82%-104%) and matrix effect (< 20%), while new alternatives were evaluated for improving sample treatment performance in heather honeys. Firstly, we decided to apply the sample treatment selected for light honeys, yet with a small amount of heather honey (2 g). The results showed a reduction in matrix effect for bromopropylate and coumaphos (see Supplementary Material, **Table 6S**), but the recovery percentages were still very low (< 50% for 4 of the 7 compounds). Therefore, we varied the number of extractions, performing these in duplicate under the same conditions. The supernatants for both extractions were combined before the evaporation step. As a result, recoveries were significantly higher (> 70% for all the compounds) compared with those employing a single extraction; only the α-endosulfan signal was affected by the matrix effect (see Supplementary Material, **Table 6S**). It was also seen that an increase in the

collection of the supernatant (a total of 14 mL) improved the performance of the treatment, with recoveries greater than 80% in all cases (see Supplementary Material, **Table 6S**); however, the matrix effect was still a problem in the case of α-endosulfan.

We also examined whether the addition of compounds that we had previously employed in the QuEChERS dSPE stage (C_{18}, PSA, or EMR-lipid) would be useful to reduce the matrix effect; nevertheless, in all cases this was higher than 20% (data not shown), and subsequently the use of these compounds was abandoned. Finally, a lower amount of reconstitution solution, from 1 mL to 0.5 mL, was used to improve method sensitivity without affecting extraction efficiency. The results showed only a slight decrease in the recovery percentages for some of the compounds, but these were always above 80% (see Supplementary Material, **Table 6S**); consequently, 0.5 mL was selected as the amount of reconstitution solution. Once the optimal conditions had been selected for the solvent extraction procedure for the three different honeys, we decided to check the suitability of different SPE sorbents (Strata® X; Strata® C_{18}-E; Florisil®, and Oasis™ HLB, which were successfully employed in previous studies: Kamel et al., 2006; Rafique et al., 2018; Ruiz et al., 2020; Shamsipur et al., 2016; Valverde et al., 2018) for reducing the matrix effect that affected α-endosulfan in heather honeys. Thus, different SPE cartridges, some of which had proven to be useful in previous studies were evaluated. As shown in **Table 7S** (see Supplementary Material), the best performance (extraction efficiency and matrix effect) was obtained with the Strata® X, although with results inferior to those obtained with the solvent extraction. Therefore, the use of SPE was abandoned, and a double solvent extraction was selected as sample treatment for dark honeys.

To sum up, two solvent extraction procedures were selected in accordance with the color and botanical origin of the honeys; this is connected with the fact that honey composition is closely related to its color (Kaczmarek, Muzolf-Panek, Tomaszewska-Gras, & Konieczny, 2019; Ruiz et al., 2020). These can be considered promising and represent an alternative to existing extraction methods, since both are among the fastest, simplest, and among the greenest of those that have been used (see Supplementary Material, **Table 1S**). This is particularly significant for the procedure employed in light honeys, as it required a low consumption of reagents and only a few steps. Moreover, recoveries were satisfactory for all the compounds of the honeys (81% - 111%; see **Table 3**), and, most importantly, the matrix effect was not significant for all the acaricides with the exception of α-endosulfan in dark honeys (see **Table 3**). Nevertheless, the absence of a significant matrix effect in the majority of cases is a very important result and advantage, since in most of the existing methods it has not been possible to minimize the influence of the matrix on the signal of the acaricides. It is also essential to point out that, unlike in previous studies, this is the first time that sample treatment has been fully evaluated and optimized for honeys of different origins, which can be considered a success (see Supplementary Material, **Table 1S**).

3.2. Method validation

Validation was performed according to current legislation (EURACHEM, 2014; SANTE, 2021) and recent publications of our group (Ruiz et al., 2020; Valverde et al., 2018). In addition, several of the main elements of uncertainty (Konieczka and Namieśnik, 2010) were considered when optimizing and validating this method (amount of sample used; recovery value of the analytical procedure; precision, and repeatability). Validation was performed with blank honeys, standards in the solvent, and standards in matrix extracts obtained according to the selected sample treatments for each type of honey (see Subsection 2.3.2). The specific procedures for determining the different validation parameters are summarized in **Table 8S**

(see Supplementary Material). The main difference between this study and all previous publications except for one (Zheng et al., 2018) is that validation studies were performed on honeys of different botanical origins (multifloral, heather and rosemary), and not only of one. Thus, a comparison of the values obtained for different parameters is not entirely authentic; however, in the present study data was collected from honeys of three different origins, with the complexity that this entails.

3.2.1. Selectivity

No interferences from matrix compounds were observed at the retention times of the analytes when blank honeys and standards in solvents were compared (see Supplementary Material, **Figure 2**). Moreover, the mass spectrum of the acaricides in solvent and matrix extracts was quite similar (see Supplementary Material, **Figure 2S**). Finally, the relative intensities of the selected ions for each compound in both types of standards were compared. In all cases, these were within ± 15% of the relative intensity (data not shown), which is lower than the maximum values allowed (± 30%; SANTE, 2021).

3.2.2. Limits of detection and quantification

The limits of detection (LODs) and quantification (LOQs) are summarized in **Table 1**; they ranged from 0.2 to 1.7 µg kg-1 and from 0.5 to 7.6 µg kg-1, respectively. These values are below the MRLs established by legislation (European Union Pesticide Database, 2021) and are comparable to the best values obtained in previous publications (see Supplementary Material **Table 1S**). The values were slightly different depending on the botanical origin, with the lowest values in the present study being obtained for light honeys. On the other hand, in some studies (see Supplementary Material **Table 1S**), the limits were the same for many compounds, which represents a lack of method specificity. Our results demonstrate the excellent sensitivity of the proposed method.

3.2.3. Matrix effect

As shown in Table 3, the differences in acaricide responses at the three concentrations assayed and for the different botanical origins were in all cases ± 20% of signal suppression or enhancement (SANTE, 2021); the exception was that of α-endosulfan in heather honeys, where there was a strong signal suppression. These results were confirmed following a comparison of the confidence intervals of the slopes for calibration curves, prepared with standard in solvent and standard in matrix extracts; it was found that they overlapped in all cases but one, namely, in that of α-endosulfan in heather honeys (see **Table 1**). This could be tentatively explained by the presence of some matrix components that were not removed with the proposed sample treatment, and which require a more exhaustive clean-up of the dark honeys. Consequently, it can be concluded that the matrix did not significantly affect the MS acaricide signals except in the case of one compound, and only here does the matrix effect need to be addressed in calibration by using a standard addition in matrix calibration curves. This is an important finding, as the matrix effect was observed in most of the studies in which these compounds were determined by means of MS detectors (see Supplementary Material, **Table 1S**).

3.2.4. Linearity/Working range

Standard solvent calibration curves were used to quantify the acaricides in all the honeys, except α-endosulfan in heather honeys quantified with the standard in matrix calibration curves. The concentration of the analytical curves varied between LOQ and 2000 μg L^{-1} (LOQ, 25, 100, 250, 500, 1000, and 2000 μg L^{-1}), which corresponds to concentrations between LOQ and 720 μg kg^{-1} (heather honeys; LOQ, 10, 35, 90, 180, 360, 720 μg kg^{-1}) or 800 μg kg^{-1} (multifloral and rosemary honeys; LOQ, 10, 40, 100, 200, 400, 800 μg kg^{-1}). The graphs obtained in all the calibration curves were straight lines, with the coefficient of the determination values (R^2) higher than 0.99 in all cases (see **Table 1**). Moreover, the deviation

of back-calculation concentration from true concentration was lower than 20% (data not shown; SANTE, 2021).

3.2.5. Precision

Precision, expressed as the percentage of relative standard deviation (%RSD), was lower than 15% in all cases; see Supplementary Material, **Table 9S**), which is below the limits established by current regulation (≤ 20%; SANTE, 2021) and similar or better than the precision values reported in previous methods (see publications listed in **Table 1S**).

3.2.6. Trueness

Recoveries obtained for the acaricides studied at the different concentration levels and for all the honeys were between 81% and 112%, with %RSD lower than 15% in all cases (see **Table 3**). These results are comparable to those obtained in previous works (see Supplementary material, **Table 1S**), and they were within limits established in current legislation (recovery percentages between 70% and 120% (≤ 20%; SANTE, 2021).

3.3. Application of the method

The validated method was applied for determining potential acaricide residues in 18 honeys of three different botanical origins (see Subsection 2.3.1). Analyses were performed in triplicate, and IS was added to all the samples at the same concentration (0.1 mg L^{-1}; 40 μg kg^{-1}). Of the seven acaricides examined, residues of only τ-fluvalinate were detected in most samples (see **Table 4** and **Figure 3S**). For this acaricide, concentrations over LOQ (1.1 and 2.1 μg kg^{-1}) were found in 5 of the 6 multifloral and rosemary honeys, respectively. The highest concentration observed was 23 μg kg^{-1}, while for 4 heather honeys residues of τ-fluvalinate were lower than LOQ (3.0 μg kg^{-1}). In all cases, the concentration values observed were below the established MRL (50 μg kg^{-1}) for honey and other apiculture products (European Union Pesticide Database, 2022). It should be mentioned that τ-fluvalinate has been detected

in similar concentrations below the established MRL in certain of the studies summarized in **Table 1S** (see Supplementary Material). On the other hand, in one study (Notardonato et al., 2014) the τ-fluvalinate levels were much higher (220 - 430 μg kg^{-1}) in Italian honeys. In the latter study the concentration of other acaricides such as coumaphos and bromopropylate was also higher (60-420 μg kg^{-1}) than the corresponding MRLs (see **Table 1**). The authors only stated that these results were logical in accordance with the properties of these acaracides. In this regard, it should be noted that τ-fluvalinate has shown a great persistence in honey, and its levels do not decrease in this product after eight months in the dark at 35 ºC (Shendy et al., 2016; Tsigouri, Menkissoglu-Spiroudi, & Thrasyvoulou, 2001). It is, therefore, not surprising that this was the only acaricide detected in the samples analyzed. The fact that the other acaricides were not detected could be tentatively related with a potential breakdown/decomposition of these compounds in honey; however, more studies should be undertaken before a definitive conclusion supporting the degradation/decomposition of the acaricides studied in honey is reached. Finally, it should be emphasized that the absence of the other acaricides does not imply that a specific and sensitive methodology for such compounds is not necessary, as some of them, especially coumaphos, have previously been detected in honeys from different countries at variable concentration levels (see Supplementary Material, **Table 1S**).

4. Conclusions

An analytical method has been proposed to determine seven acaricides (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, and τ-fluvalinate) in honeys of three different botanical origins (heather, multifloral, and rosemary). Following an optimization process, an efficient and simple sample treatment with low solvent consumption was selected; this consisted of solvent extraction for light honeys (multifloral and rosemary)

and a double extraction for dark honeys (heather). The need for two different sample treatments is concerned with the different composition of honeys depending on their botanical origin. This issue is not usually considered when developing methods in relation to honey. The analysis time for light honeys is one of the fastest that has been proposed for this product, and an attempt has been made to find solvents that are as environmentally-friendly as possible, such as ethyl acetate. The recoveries obtained were satisfactory, and matrix effect was avoided in the case of all the compounds and botanical origins of the honey, with the exception of α-endosulfan in heather honeys. The proposed method has an advantage over most existing methods in terms of sample treatment specificity for the different botanical origins. Moreover, the chromatographic conditions we selected made it possible to separate the acaricides in less than 25 min. The method has been validated in accordance with current legislation, and the results have shown that its analytical performance was similar or, in many cases, better than that of previous proposals. The LODs and LOQs obtained were lower than the MRLs established for the compounds studied in honey and comparable with the best values published. Residues of τ-fluvalinate (< LOQ - 23 μg kg^{-1}) were found in most samples, while in heather honeys the concentrations were lower than LOQ. By contrast, the other six acaricides examined were not detected in any sample. Finally, this study highlighted some of the advantages of developing specific methods rather than multi-residue approaches; these include the absence of a significant matrix effect, extraction efficiency, and precision.

ABBREVIATIONS

AF, samples spiked after sample treatment; **BF,** samples spiked before sample treatment; **EMR,** enhanced matrix removal; **IS,** internal standard; **LOD,** limit of detection; **LOQ,** limit of quantification; **MRL,** maximum residue level; **m/z,** mass-to-charge; **PSA,** primary

secondary amine; **QuEChERS,** quick, easy, cheap, effective, rugged, and safe; **RSD,** relative standard deviation.

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CONFLICT OF INTEREST STATEMENT

The authors of this manuscript declare no conflict of interest

DATA AVAILABILITY

The datasets generated during the current study are included in this published article and the Supplementary Information, or they are available from the corresponding author on reasonable request.

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Figure captions

Figure 1.- Analytical procedures work-up flow charts: (**A**) light honeys; (**B**) dark honeys.

Figure 2.- Representative GC-MS chromatograms (SIM mode using the quantification/target ions; see Table 2) obtained from: (**A**) standards in solvent mixture (0.5 mg L^{-1}; IS, 0.1 mg L^{-1}), and (**B**) a blank multifloral honey sample extract. GC-MS conditions are summarized in Subsection 2.4 and Table 1.**1**, atrazine; **2**, chlorpyrifos; **3**, chlorfenvinphos-d_{10} (IS); **4**, chlorfenvinphos; **5**, α-Endosulfan; **6**, bromopropylate; **7**, coumaphos; **8**, τ-fluvalinate.

Figure 1

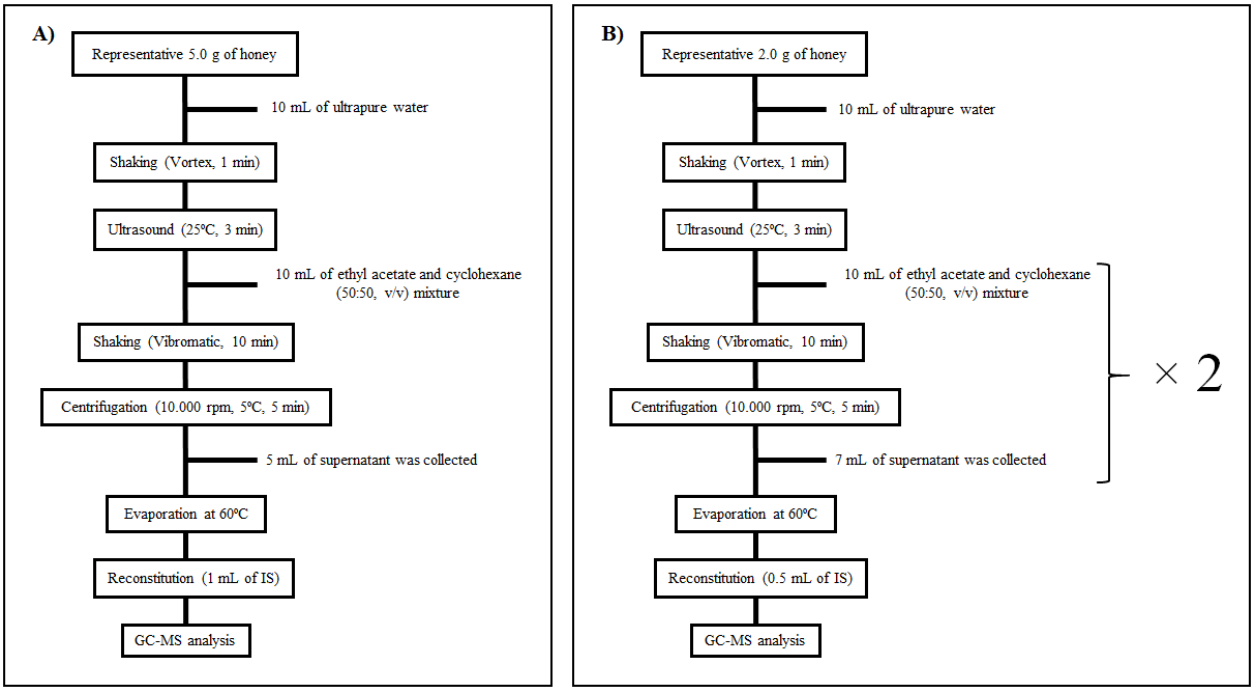
A)
Representative 5.0 g of honey
10 mL of ultrapure water
Shaking (Vortex, 1 min)
Ultrasound (25ºC, 3 min)
10 mL of ethyl acetate and cyclohexane (50:50, v/v) mixture
Shaking (Vibromatic, 10 min)
Centrifugation (10.000 rpm, 5ºC, 5 min)
5 mL of supernatant was collected
Evaporation at 60ºC
Reconstitution (1 mL of IS)
GC-MS analysis
B)
Representative 2.0 g of honey
10 mL of ultrapure water
Shaking (Vortex, 1 min)
Ultrasound (25ºC, 3 min)
10 mL of ethyl acetate and cyclohexane (50:50, v/v) mixture
Shaking (Vibromatic, 10 min)
Centrifugation (10.000 rpm, 5ºC, 5 min)
7 mL of supernatant was collected
× 2
Evaporation at 60ºC
Reconstitution (0.5 mL of IS)
GC-MS analysis

Figure 2

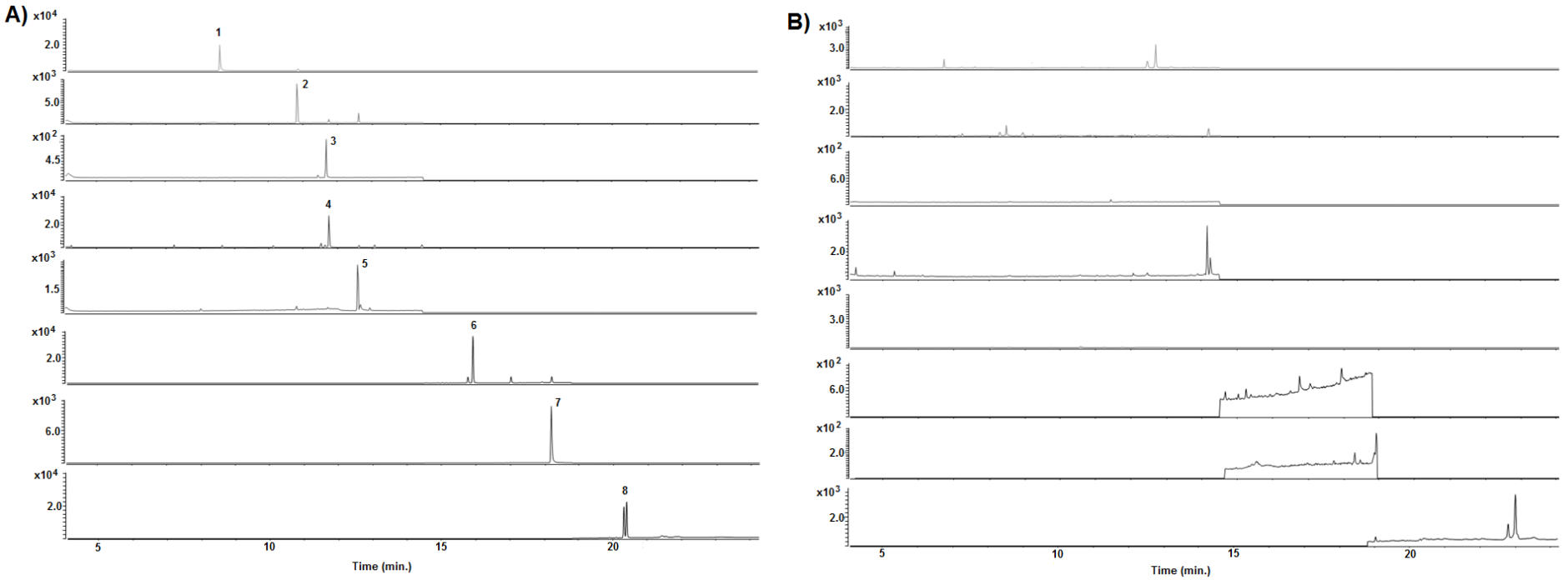
A)
x10^4
2.0
1
x10^3
5.0
2
x10^2
4.5
3
x10^4
2.0
4
x10^3
1.5
5
x10^4
2.0
6
x10^3
6.0
7
x10^4
2.0
8
5
10
15
20
Time (min.)
B)
x10^3
3.0
x10^3
2.0
x10^2
6.0
x10^3
2.0
x10^3
3.0
x10^2
6.0
x10^2
2.0
x10^3
2.0
5
10
15
20
Time (min.)

Table 1.- Calibration curve data, LOD, LOQ and MRL values.

Compounds	Standard in solvent		Multifloral honey				Rosemary honey				Heather honey				MRL
	SCI	R^2	SCI	R^2	LOD	LOQ	SCI	R^2	LOD	LOQ	SCI	R^2	LOD	LOQ	(µg kg^{-1})
Atrazine	66.8 ± 8.1	0.990	65.3 ± 5.2	0.996	0.2	0.6	64.5 ± 6.1	0.998	0.2	0.5	58.7 ± 4.7	0.990	0.5	2.1	50
Chlorpyrifos	36.4 ± 4.5	0.992	35.4 ± 5.4	0.998	0.8	2.7	37.8 ± 4.2	0.998	2.0	6.7	33.4 ± 3.3	0.994	0.4	1.9	10
Chlorfenvinphos	64.9 ± 6.4	0.995	64.0 ± 5.9	0.996	0.2	0.5	65.5 ± 4.9	0.997	0.2	0.5	66.4 ± 5.8	0.991	1.0	3.0	10
α-Endosulfan	11.3 ± 1.5	0.991	9.7 ± 1.4	0.997	0.2	0.6	10.1 ± 1.2	0.998	1.0	3.6	6.1 ± 0.8	0.992	1.7	7.6	10
Bromopropylate	90.2 ± 6.3	0.997	85.3 ± 6.1	0.999	0.5	1.5	83.2 ± 5.8	0.996	0.2	0.7	86.6 ± 8.5	0.990	0.2	0.8	10
Coumaphos	27.8 ± 3.8	0.990	30.1 ± 4.3	0.998	0.2	0.6	30.9 ± 3.5	0.995	0.2	0.7	29.4 ± 4.6	0.990	0.2	0.9	100
τ-Fluvalinate	70.7 ± 8.1	0.993	74.9 ± 7.4	0.992	0.3	1.1	72.1 ± 6.9	0.991	0.6	2.1	70.2 ± 8.9	0.990	1.0	3.0	50

SCI, slope confident intervals; **LOD,** limit of detection; **LOQ,** limit of quantification; **R^2,** determination coefficient; **MRL,** maximum residue limit.

Table 2.- GC-MS parameters. Adapted from Agronomy, 11, María J. Nozal, Edgar Imaz, José L. Bernal, José L. Nieto, Mariano Higes, José Bernal, An Optimized extraction procedure for determining acaricide residues in foundation sheets of beeswax by using gas chromatography-mass spectrometry, 804, 2021, with permission from MDPI.

HILIC parameter	**Final setting**
Programmed temperature conditions	from 60ºC (1 min) to 170ºC (0 min), at 40 C/min and then increased to 310ºC (3 min) at 8ºC/min.
Carrier gas	Helium
Flow-rate (mL/min)	1.2
Injector temperature (ºC)	280
Injection volume (µL)	1
MS parameter	**Final setting**
Operating mode	Electron impact
Ionization energy (eV)	70
Scan range (*m/z*)	50-400
Ion source temperature (ºC)	230
Quadrupole temperature (ºC)	150
Nebulizer gas (N_2) pressure (psi)	40
Target/quantification ions	Atrazine, 200 Chlorpyrifos, 197 Chlorfenvinphos, 267 α-endosulfan, 241 Bromopropylate, 345 Coumaphos, 362 τ-fluvalinate, 250 Chlorfenvinphos-d_{10}, 267
Qualifier ions	Atrazine, 173 and 215 Chlorpyrifos, 258 and 315 Chlorfenvinphos, 270 and 329 α-endosulfan, 195 and 207 Bromopropylate, 183 and 185 Coumaphos, 109 and 226 τ-fluvalinate, 181 and 207

Table 3.- Evaluation of the extraction efficiency (recovery percentages ± %RSD) of the sample treatment and the matrix effect (mean values ± %RSD). Data obtained as described in Sections 2.2, 3.3 and Table 9S, and the results were obtained from three replicates that were injected in triplicate.

Compounds	Multifloral honey						Rosemary honey						Heather honey					
	EE			ME			EE			ME			EE			ME		
	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL	LL	ML	HL
Atrazine	89 ± 4	91 ± 6	85 ± 7	5 ± 6	0 ± 7	-5 ± 4	88 ± 9	92 ± 8	97 ± 6	4 ± 3	-2 ± 5	-5 ± 6	102 ± 6	101 ± 5	107 ± 7	-5 ± 6	-12 ± 3	-10 ± 1
Chlorpyrifos	88 ± 5	82 ± 7	84 ± 9	3 ± 8	3 ± 5	-8 ± 6	88 ± 12	91 ±10	95 ± 7	9 ± 3	3 ± 4	7 ± 1	81 ± 3	85 ± 2	90 ± 5	3 ± 8	-9 ± 2	-9 ± 1
Chlorfenvinphos	81 ± 3	85 ± 2	87 ± 4	3 ± 7	0 ± 4	-4 ± 2	97 ± 3	101 ± 4	99 ± 5	1 ± 8	2 ± 9	-4 ± 5	102 ± 5	99 ± 6	107 ± 8	3 ± 7	1 ± 2	-2 ± 3
α-Endosulfan	91 ± 6	93 ± 9	86 ± 10	-17 ± 4	-11 ± 6	-4 ± 3	86 ± 8	88 ± 5	92 ± 7	-12 ± 4	-7 ± 5	-11 ± 2	98 ± 10	103 ± 8	105 ± 9	-44 ± 4	-51 ± 7	-41 ± 5
Bromopropylate	83 ± 4	88 ± 7	90 ± 8	-3 ± 8	7 ± 10	-12 ± 6	89 ± 8	93 ± 11	98 ± 9	-7 ± 3	-5 ± 2	-10 ± 5	92 ± 5	100 ± 7	95 ± 4	-3 ± 8	-3 ± 1	0 ± 2
Coumaphos	84 ± 7	89 ± 5	87 ± 6	9 ± 8	11 ± 8	5 ± 7	99 ± 6	104 ± 7	101 ± 5	8 ± 5	6 ± 7	4 ± 8	83 ± 14	89 ± 12	92 ± 10	9 ± 8	1 ± 10	6 ± 8
τ-Fluvalinate	95 ± 5	90 ± 4	94 ± 3	9 ± 6	7 ± 4	2 ± 2	92 ± 5	100 ± 3	98 ± 7	6 ± 10	-1 ± 7	1 ± 8	108 ± 9	100 ± 6	98 ± 8	0 ± 6	-2 ± 1	-5 ± 2

EE, extraction efficiency; **ME,** matrix effect; **LL,** low level (LOQ, see Table 1); **ML,** medium level (100 μg kg^{-1}); **HL,** high level (800 μg kg^{-1}, rosemary and multifloral; 700 μg kg^{-1}, heather).

Table 4.- Results (means of triplicate analyses (μg kg^{-1}); %RSD < 15% in all cases) of the investigation of the studied acaricides in honey samples. The other acaricides under study were below LOD in the samples.

Sample	τ-fluvalinate
M1	7
M2	5
M3	7
M4	6
M5	23
M6	<LOD
R1	5
R2	7
R3	<LOD
R4	6
R5	8
R6	9
H1	<LOQ
H2	<LOD
H3	<LOQ
H4	<LOQ
H5	<LOD
H6	<LOQ

Supplementary Material

DETERMINATION OF ACARICIDES IN HONEYS FROM DIFFERENT BOTANICAL ORIGINS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

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Table 1S.- Published methods for determining acaricides in honey.

Analytes (SC)	ST (time)	Reagents (g, mL[T,O])	ME[A]	Recoveries[A] (%)	LOQs (µg/kg)[A]	OSTBO /VDBO	Residues in samples SC (µg/kg)[A]	System[A]	Reference
168 (1-5)	QuEChERS + EV (> 2h)	7.5 g, 20.2 mL (7 mL ACN; 3 mL EA)	Yes	74-116	0.2-4*	No/No	2 and 6 (<LOQ-0.3)	HPLC-MS/MS[1-3] GC-MS/MS[4,5]	**(Almeida et al., 2020)**
1 (6)	SE + EV (~30 min)	5 g, 16 mL (15 mL HEX; 1 mL ACN)	No	65-70	50	No/No	No	HPLC-UV	**(Bajuk et al., 2017)**
52 (1-3,6,7)	QuEChERS (~20 min)	7.3 g, 17.5 mL (10 mL ACN)	Yes	80-95	0.5-5	No/No	NS	HPLC-MS/MS	**(Calatayud-Vernich et al., 2016)**
48 (2,4,6)	ASE + EV (NS)	7 g, NS (HEX, ISO and EA)	Yes	80-94	1-4	No/No	2, 4 and 6 (1-390)	GC-MS/MS	**(Chiesa et al., 2016; Panseri et al., 2020)**
207 (2-7)	QuEChERS + EV (~50 min)	7.5 g, 20.5 mL (9 mL ACN; 0.5 mL HEX)	Yes	65-118	1-5*	No/No	6 and 7 (<LOQ-39)	HPLC-MS/MS[6] GC-MS/MS[2-5,7]	**(Gawel et al., 2019)**
4 (5,6)	SE + EV (~30 min)	2 g, 67 mL (52 mL HEX; 15 mL AC)	No	95-97	NS	No/No	No	GC-AED/NPD/ECD	**(Jiménez et al., 1996)**
4 (6,7)	SPE (C_{18}) + EV (~20 min)	32 mL (8 mL ACN; 8.5 mLTHF)	NS	90-110	NS	No/No	6 and 7 (20-500)	GC-ECD/NPD/MS	**(Kamel et al., 2006)**
24 (2,4)	QuEChERS + EV (~20 min)	9.1 g, 22 mL (10 mL ACN; 1.8 mL HEX; 0.2 mL AC)	Yes	96-111	19	No/No	2 (60)	GC-ECD/FTD/MS	**(Kumar et al., 2018)**
399 (1-7)	QuEChERS + EV (~25 min)	7.2 g, 20 mL (10 mL ACN)	Yes	NS	NS	No/No	3 and 6 (2-37)	GC-MS	**(Lasheras et al., 2021)**

Table 1S.- Continued.

Analytes (SC)	ST (time)	Reagents (g, mL[T,O])	ME[A]	Recoveries[A]	LOQs (µg/Kg)[A]	OSTBO /VDBO	Residues in samples SC (µg/Kg)[A]	System[A]	Reference
5 (2-4,6,7)	SE + GPC+ EV (> 90 min) [Q]QuEChERS (> 1h)	40 g, > 400 mL (200 mL AC; > 100 mL EA; > 100 mL CHEX) [Q]7.5 g, 20 mL (10 mL ACN)	Yes	95-114	1-5	No/No	6 (5-31)	HPLC-MS/MS[6,7] GC-MS/MS[2-4]	**(Lazarus et al., 2021)**
3 (6,7)	SE + EV (NS)	10 g, 231 mL (100 mL HEX; 30 mL IPA; 1 mL AC)	NS	NS	10-15	No/No	6 (20-2003)[TR]	HPLC-DAD	**(Martel et al., 2007)**
5 (5-7)	SPE (XAD-2) + EV (~30 min)	NS	No	80-92	38-48	No/No	5-7 (55-427)	GC-MS/MS	**(Notardonato et al., 2014)**
5 (5-7)	SPE (CG-1) + EV (~30 min)	> 100 mL (13 mL TOL; 5 mL HEX; 5 mL AC)	No	98-102	8-10	No/No	5-7 9-36	GC-MS/MS	**(Notardonato et al., 2016)**
35 (1,2,4,6,7)	SPE (AMS, FLO and CC) + EV (~30 min)	1.5 g, NS (ACN, HEX and AC)	Yes	76-116	1-7	No/No	No	GC-MS	**(Rafique et al., 2018)**
5 (5-7)	SPME (53 min)	30 mL	Yes	100	4-39	No/No	6 (5-31)	GC-MS	**(Rial-Otero et al., 2007)**
19 (5)	SPE (C_{18}) + DLLME (~40 min)	>100 mL (4.5 mL MeOH; 20 µL CB)	NS	94-99	NS	No/No	No	GC-MS	**(Shamsipur et al., 2016)**
200 (1-5,7)	QuEChERS + EV (~25 min)	6.8 g, 22 mL (10 mL ACN; 1.8 mL HEX; 0.2 mL AC)	Yes	73-96	10[*]	No/No	7 (10)	GC-MS/MS	**(Shendy et al., 2016)**

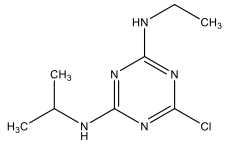
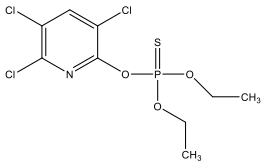
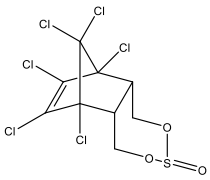
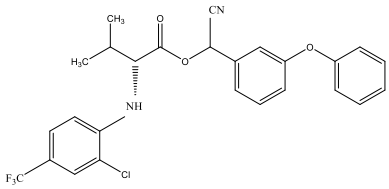
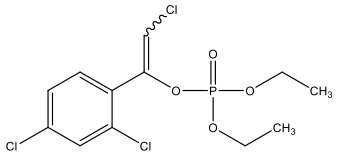
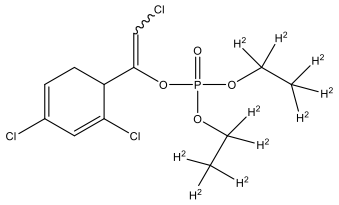
Table 1S.- Continued.

Analytes (SC)	ST (time)	Reagents (g, mL^{T,O})	ME^{A}	Recoveries^{A}	LOQs (µg/kg)^{A}	OSTBO /VDBO	Residues in samples SC (µg/kg)^{A}	System^{A}	Reference
5 (6,7)	QuEChERS + EV (> 60 min)	7.7 g, 19 mL (14 mL ACN; 5 mL PB)	Yes	68-98	1-3	Partially/Yes	<LOQ	HPLC-MS/MS	**(Zheng et al., 2018)**
7 (1-7)	SE + EV (~25 min^{MH,RH}; ~40 min^{HH})	21 mL^{MH,RH} or 31 mL^{HH} (5.5 mL or 10.5 mL EA; 5.5 mL or 10.5 mL CHEX)	No Yes (4^{HH})	81-108	0.5-7	Yes/Yes	7 (<LOQ-23)	GC-MS	**Present study**

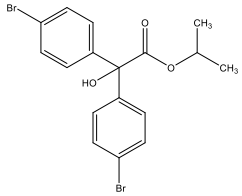
^{A}:data related only to the studied compounds; ^{O}: organic solvents; ^{Q}: QuEChERS; ^{T}: total solvents; ^{TR}: treated apiaries; *LOQs were the same for all compounds.

1, atrazine; **2,** chlorpyrifos; **3,** chlorfenvinphos; **4**, α-endosulfan; **5**, bromopropylate; **6**, coumaphos; **7**, τ-fluvalinate; **AC,** acetone; **ACN,** acetonitrile; **AED,** atomic emission detector; **AMS,** anhydrous magnesium sulfate; **CB,** chlorobenzene; **CC,** charcoal; **CG,** carbograph; **CHEX,** ciclohexane; **DAD,** diode array detector; **DLLME,** dispersive liquid–liquid microextraction; **EA,** ethyl acetate; **ECD,** electron capture detector; **EV**, evaporation; **FLO**, florisil; **FTD,** flame thermionic detector; **GC,** gas chromatography; **GPC,** gel permeation chromatography; **HEX**, hexane; **HH,** heather honey; **HPLC,** high performance liquid chromatography; **IPA,** isopropanol; **ISO,** isooctane; **LOQ,** limit of quantification; **ME,** matrix effect; **MeOH,** methanol; **MH,** multifloral honey; **MS,** mass spectrometry; **MS/MS**, tandem mass spectrometry; **NPD,** nitrogen-phosphorus detector; **NS**, not specified; **OSTBO,** optimization of sample treatment for honeys of different botanical origins; **PB,** phosphate buffer; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RH,** rosemary honey; **SC,** studied compounds; **SE**, solvent extraction; **SPE**, solid phase extraction; **SPME,** solid-phase microextraction; **ST,** sample treatment; **THF,** tetrahydrofuran; **TOL,** toluene; **VDBO,** validation for honeys of different botanical origins.

Table 2S- Chemical structure of the studied acaricides.

Atrazine CAS number: 1912-24-9 
Chlorpyrifos CAS number: 2921-88-2 
α-Endosulfan CAS number: 959-98-8 
τ-Fluvalinate CAS number: 102851-06-9 
Chlorfenvinphos CAS number: 470-90-6 
Chlorfenvinphos D-10 CAS number: 1346606-54-9 

Bromopropylate
CAS number: 18181-80-1



Coumaphos
CAS number: 56-72-4

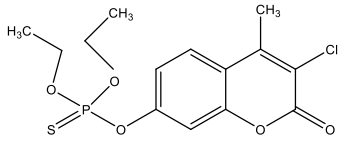


Table 3S- Spiking procedure. Reprinted from Food Research International, 130, Paola Ruiz, Ana M. Ares, Silvia Valverde, María T. Martín, José Bernal, Development and validation of a new method for the simultaneous determination of spinetoram J and L in honey from different botanical origins employing solid-phase extraction with a polymeric sorbent and liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, 108904, Copyright (2020), with permission from Elsevier.

Description of the spiking procedure	The spiking of the BF samples was done similarly to Jovanov et al. (2014) to assure that the analytes were bound to the honey matrix. Briefly, representative portions of the blank honeys were weighed and transferred to a crystallizer where they were homogeneously spiked with the working standard solutions. The mixtures were then stirred with a glass rod to assist the homogenization and left to equilibrate overnight prior to further analysis. Meanwhile, AF samples were prepared by spiking blank honey samples, which were previously treated with the proposed sample treatment, with working standard solutions that were added to the elution solvent

Table 4S.- Evaluation of the extraction efficiency (recovery percentages ± %RSD) and the matrix effect (mean values ± %RSD) when using a single extraction with 10 mL of different ethyl acetate and cyclohexane mixtures with spiked blank honey samples at medium concentration level (100 µg kg^{-1}). Data obtained as described in Sections 2.2, 3.3 and Table 9S, while experimental conditions were listed in subsection 3.1. Results were obtained from three replicates that were injected in triplicate.

Compounds	Ethyl acetate: Cyclohexane (80:20; v/v)						Ethyl acetate: Cyclohexane (50:50; v/v)						Ethyl acetate: Cyclohexane (20:80; v/v)					
	EE			ME			EE			ME			EE			ME		
	MH	RH	HH	MH	RH	HH	MH	RH	HH	MH	RH	HH	MH	RH	HH	MH	RH	HH
Atrazine	80 ± 10	77 ± 8	94 ± 12	7 ± 6	10 ± 12	-1 ± 11	91 ± 6	92 ± 8	98 ± 7	0 ± 7	-2 ± 5	-7 ± 11	72 ± 7	81 ± 3	79 ± 10	9 ± 5	-8 ± 4	-2 ± 8
Chlorpyrifos	73 ± 3	82 ± 4	23 ± 10	-7 ± 8	6 ± 10	-25 ± 8	82 ± 7	91 ±10	35 ± 9	3 ± 5	3 ± 4	-22 ± 9	70 ± 8	75 ± 7	41 ± 12	6 ± 10	-9 ± 8	-27 ± 6
Chlorfenvinphos	80 ± 7	75 ± 8	66 ± 8	10 ± 7	14 ± 9	-3 ± 7	85 ± 2	101 ± 4	64 ± 8	0 ± 4	2 ± 9	0 ± 6	69 ± 6	84 ± 5	69 ± 13	-4 ± 9	1 ± 6	1 ± 7
α-Endosulfan	83 ± 9	72 ± 10	24 ± 13	-19 ± 4	-24 ± 5	-45 ± 9	93 ± 9	88 ± 5	33 ± 11	-11 ± 6	-7 ± 5	-50 ± 8	78 ± 12	83 ± 9	44 ± 6	-23 ± 8	-17 ± 7	-48 ± 5
Bromopropylate	81 ± 11	86 ± 6	22 ± 8	-15 ± 8	-9 ± 6	15 ± 8	88 ± 7	93 ± 9	35 ± 6	7 ± 10	-5 ± 2	24 ± 10	72 ± 9	85 ± 8	42 ± 11	-12 ± 7	-11 ± 11	17 ± 12
Coumaphos	80 ± 8	87 ± 9	36 ± 9	18 ± 8	24 ± 5	33± 11	89 ± 5	104 ± 7	44 ± 8	11 ± 8	6 ± 7	27 ± 9	78 ± 4	91 ± 11	48 ± 9	13 ± 11	9 ± 5	47 ± 14
τ-Fluvalinate	79 ± 6	89 ± 11	32 ± 6	5 ± 6	14 ± 13	5 ± 6	90 ± 4	100 ± 3	39 ± 9	7 ± 4	-1 ± 7	-9 ± 7	74 ± 11	90 ± 6	36 ± 7	12 ± 9	-7 ± 3	14 ± 9

EE, extraction efficiency; **ME,** matrix effect; **MH,** multifloral honey; **HH,** heather honey; **RH,** rosemary honey.

Table 5S.- Evaluation of the extraction efficiency (recovery percentages ± %RSD) and the matrix effect (mean values ± %RSD) when using a single extraction with 10 mL of different ethyl acetate and cyclohexane mixtures and different shaking times with spiked blank honey samples at medium concentration level (100 μg kg^{-1}). Data obtained as described in Sections 2.2, 3.3 and Table 9S, while experimental conditions were listed in subsection 3.1. Results were obtained from three replicates that were injected in triplicate.

Compounds	5 minutes						10 minutes						15 minutes					
	EE			ME			EE			ME			EE			ME		
	MH	RH	HH	MH	RH	HH	MH	RH	HH	MH	RH	HH	MH	RH	HH	MH	RH	HH
Atrazine	84 ± 11	76 ± 13	87 ± 9	5 ± 8	14 ± 3	-10 ± 6	91 ± 6	92 ± 8	98 ± 7	0 ± 7	-2 ± 5	-8 ± 11	92 ± 6	95 ± 2	99 ± 14	5 ± 7	-14 ± 3	-9 ± 5
Chlorpyrifos	69 ± 9	80 ± 7	26 ± 6	-10 ± 7	12 ± 6	-30 ± 7	82 ± 7	91 ±10	35 ± 9	3 ± 5	3 ± 4	-22 ± 9	84 ± 8	89 ± 9	40 ± 10	12 ± 9	-6 ± 9	-24 ± 9
Chlorfenvinphos	70 ± 3	85 ± 5	58 ± 2	7 ± 3	8 ± 10	-8 ± 11	85 ± 2	101 ± 4	64 ± 8	0 ± 4	2 ± 9	0 ± 6	84 ± 11	104 ± 4	68 ± 11	-11 ± 4	5 ± 8	10 ± 10
α-Endosulfan	79 ± 7	77 ± 3	25 ± 5	-15 ± 7	-20 ± 9	-51 ± 5	93 ± 9	88 ± 5	33 ± 11	-11 ± 6	-7 ± 5	-50 ± 8	94 ± 8	90 ± 6	34 ± 5	-29 ± 6	-12 ± 9	-52 ± 7
Bromopropylate	73 ± 10	82 ± 8	29 ± 7	-10 ± 6	-12 ± 2	12 ± 3	88 ± 7	93 ± 9	35 ± 6	7 ± 10	-5 ± 2	24 ± 10	86 ± 10	92 ± 11	37 ± 7	-15 ± 3	-19 ± 5	14 ± 2
Coumaphos	79 ± 11	89 ± 10	36 ± 9	13 ± 9	17 ± 9	27 ± 6	89 ± 5	104 ± 7	44 ± 8	11 ± 8	6 ± 7	27 ± 9	90 ± 3	106 ± 9	47 ± 10	6 ± 7	10 ± 3	40 ± 4
τ-Fluvalinate	68 ± 2	85 ± 4	30 ± 3	10 ± 8	7 ± 8	11 ± 4	90 ± 4	100 ± 3	39 ± 9	7 ± 4	-1 ± 7	-9 ± 7	88 ± 9	103 ± 8	40 ± 9	19 ± 11	-12 ± 8	4 ± 10

EE, extraction efficiency; **ME,** matrix effect; **MH,** multifloral honey; **HH,** heather honey; **RH,** rosemary honey.

Table 6S.- Evaluation of the extraction efficiency (recovery percentages ± %RSD) and the matrix effect (mean values ± %RSD) with spiked blank heather honey samples at medium concentration level (100 μg kg^{-1}) under different conditions. Data obtained as described in Sections 2.2, 3.3 and Table 9S, while experimental details are listed in subsection 3.1. Results were obtained from three replicates that were injected in triplicate.

Compounds	Honey weight				Number of extractions				Volume of supernatant				Reconstitution volume			
	5 g		2 g		1		2		10 mL		14 mL		1 ml		0.5 mL	
	EE	ME	EE	ME	EE	ME	EE	ME	EE	ME	EE	ME	EE	ME	EE	ME
Atrazine	98 ± 7	-8 ± 11	99 ± 6	-7 ± 8	99 ± 6	-7 ± 8	103 ± 8	-10 ± 12	103 ± 8	-10 ± 12	105 ± 8	-8 ± 10	105 ± 8	-8 ± 10	101 ± 5	-12 ± 3
Chlorpyrifos	35 ± 9	-22 ± 9	44 ± 2	-21 ± 5	44 ± 2	-21 ± 5	84 ± 4	-12 ± 2	84 ± 4	-12 ± 2	88 ± 4	-10 ± 3	88 ± 4	-10 ± 3	85 ± 2	-9 ± 2
Chlorfenvinphos	64 ± 8	0 ± 6	69 ± 4	3 ± 3	69 ± 4	3 ± 3	101 ± 6	10 ± 9	101 ± 6	10 ± 9	104 ± 6	8 ± 5	104 ± 6	8 ± 5	99 ± 6	1 ± 2
α-Endosulfan	33 ± 11	-50 ± 8	42 ± 12	-46 ± 8	42 ± 12	-46 ± 8	105 ± 7	-52 ± 3	105 ± 7	-52 ± 3	106 ± 7	-47 ± 5	106 ± 7	-47 ± 5	103 ± 8	-51 ± 7
Bromopropylate	35 ± 6	24 ± 10	45 ± 10	14 ± 12	45 ± 10	14 ± 12	98 ± 10	7 ± 11	98 ± 10	7 ± 11	99 ± 10	8 ± 8	99 ± 10	8 ± 8	100 ± 7	-3 ± 1
Coumaphos	44 ± 8	27 ± 9	53 ± 7	18 ± 7	53 ± 7	18 ± 7	90 ± 9	10 ± 6	90 ± 9	10 ± 6	89 ± 9	6 ± 8	89 ± 9	6 ± 8	89 ± 12	1 ± 10
τ-Fluvalinate	39 ± 9	-9 ± 7	48 ± 11	-7 ± 9	48 ± 11	-7 ± 9	101 ± 8	-5 ± 10	101 ± 8	-5 ± 10	103 ± 8	-6 ± 11	103 ± 8	-6 ± 11	100 ± 6	-2 ± 1

EE, extraction efficiency; **ME,** matrix effect.

Table 7S.- Evaluation of the extraction efficiency (recovery percentages intervals) and the matrix effect (mean values intervals) when using different SPE cartridges with spiked blank heather honey samples at medium concentration level (100 μg kg^{-1}). Data obtained as described in Sections 2.2, 3.3 and Table 9S. Results were obtained from three replicates that were injected in triplicate, and %RSD were lower than 20% in all cases.

SPE cartridge	**Extraction efficiency (%recovery) (minimum-maximum)**	**Matrix effect (minimum-maximum)**
Florisil®	13 to 51	-10 to 31
Strata® C18	35 to 127	-14 to 24
Strata® X	52 to 99	-40 to 54
Oasis™ HLB	51 to 89	-28 to 62

Table 8S.- Procedures employed for determining the validation parameters.

Validation parameter	Experimental procedure
Selectivity	This was evaluated by comparing the chromatograms and mass spectra of standards in solvents, standard in honey extracts and blank honeys of the three different botanical origins (n = 6).
Limits of detection and quantification	The limits of detection (LODs) and quantification (LOQs) were determined by the injection of several blank samples measurement noise at the elution times for the studied acaricides and comparing this response (mean values) with the signal (peak heights) of compounds at low concentration levels. The LODs and LOQs were estimated to be three and ten times the S/N ratio, respectively.
Matrix effect	To ascertain how the matrix influenced ESI ionization for the acaricides a comparison was made of the detector responses (analyte peak area/IS area) of standard in solvent and standard in matrix extracts (AF samples) of the different botanical origins spiked at three different concentrations (QC levels). It was calculated as recommended in SANTE (2021). $ME\ (in\%) = \left(\left(\frac{Rmatrix}{Rsolvent}\right) - 1\right) \times 100$ R_{matrix}: detector response from standard in matrix extract. $R_{solvent}$: detector response from standard in solvent. In addition, the confidence intervals of the slopes in both types of standards were also compared.
Linearity/Working range	Calibration curves (n = 6) were constructed by plotting the signal on the *y*-axis (analyte peak area/IS area) against the analyte concentration on the *x*-axis. Linearity was evaluated by visual analysis of the plots, a calculation being made of the determination coefficients (R^2), and by our back calculation of the concentrations of the individual calibration standards.

Precision	Precision, which was expressed as relative standard deviation (% RSD), experiments were performed concurrently by repeated sample analysis using BF samples, either on the same day (intra-day precision, SANTE, 2021; repeatability, EURACHEM, 2014), or over three consecutive days (inter-day precision, SANTE 2021; partial reproducibility, EURACHEM, 2014).
Trueness	It was evaluated by means of recovery experiments (as a measure of trueness), by comparing the results (analyte peak area/IS area) obtained from blank honey samples spiked at three different concentrations (low, medium and high QC levels), either prior to (BF samples) or following (AF samples) sample treatment.

Table 9S.- Summary of precision studies (minimum and maximum %RSD values) for the determination of acaricides in spiked blank honey samples.

	Spiking level	Multifloral		Rosemary		Heather	
		Min	Max	Min	Max	Min	Max
Intraday precision (repeatability)	**Low**	3	8	3	12	2	14
	Medium	2	9	2	11	1	12
	High	2	10	3	10	3	10
Interday precision (partial reproducibility)	**Low**	4	10	3	10	2	11
	Medium	2	11	2	9	1	10
	High	1	12	1	11	1	12

Low- LOQ (see Table 1); **Medium-** 100 μg kg^{-1}; **High-** 800 μg kg^{-1} for multifloral and rosemary; 700 μg kg^{-1} for heather**.**

Figure 1S.- Examples of the analyzed honeys from each botanical origin.

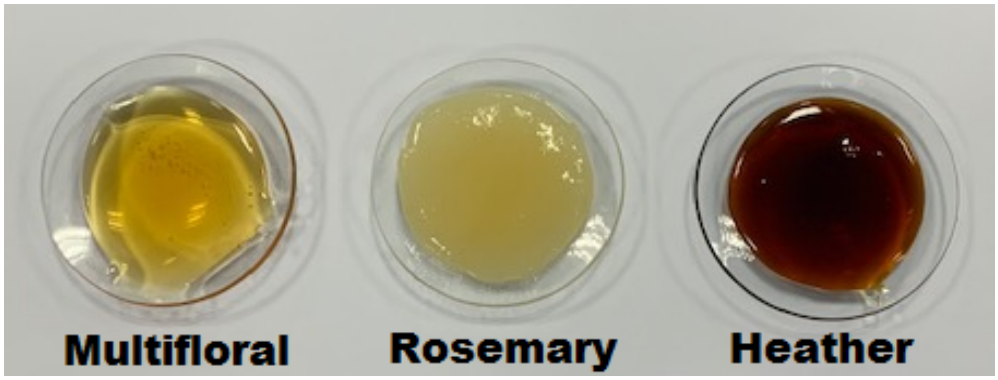


Figure 2S.- Coumaphos (0.5 mg L^{-1}) MS spectra (SIM mode of the selected ions) of (**A**) standard in solvent, (**B**) spiked heather honey, (**C**) spiked multifloral honey, and (**D**) spiked rosemary honey. GC-MS conditions are summarized in Subsection 2.4 and Table 1.

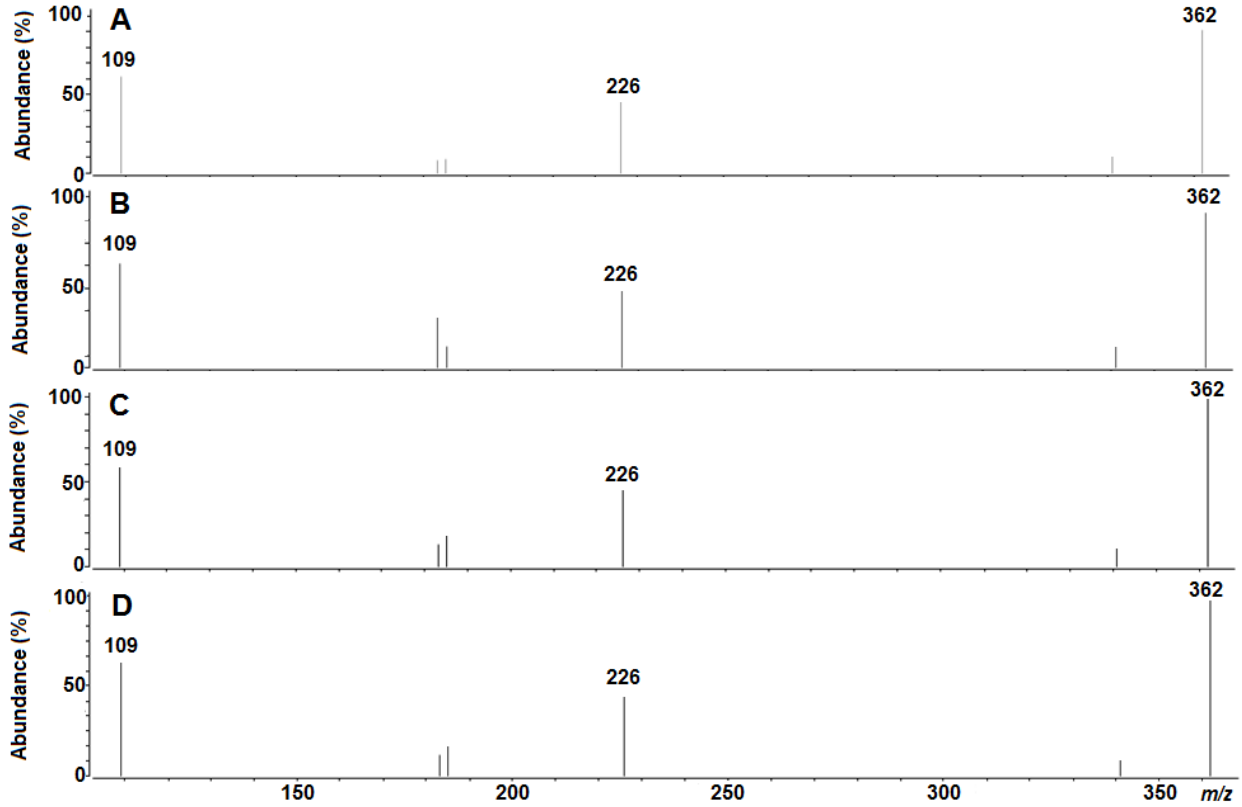


Figure 3S.- Representative GC-MS chromatograms (SIM mode using the quantification/target ions; see Table 2) obtained from multifloral (M5; 23 µg kg^{-1}) and rosemary (R4; 6 µg kg^{-1}) honey samples with endogenous τ-fluvalinate content over LOQ. GC-MS conditions are summarized in Subsection 2.4 and Table 1.