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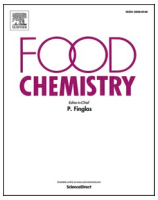

Development and validation of an analytical methodology based on solvent extraction and gas chromatography for determining pesticides in royal jelly and propolis

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ABSTRACT

We propose a new analytical methodology to determine seven pesticides (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, and τ-fluvalinate) in royal jelly and propolis products using gas chromatography-mass spectrometry. Sample treatment, with minor modifications for propolis, consisted of a solvent extraction with a hexane and isopropanol mixture, and a further clean-up step. Meanwhile, chromatographic analysis (<25 min) was performed in a DB-5MS column under programmed temperature conditions. In all cases we validated the method in terms of selectivity, limits of detection (0.1–2.8 μg kg^{-1}) and quantification (0.3–9.2 μg kg^{-1}), linearity, matrix effect (<±20 %), trueness (recoveries between 93 % and 118 %), and precision (relative standard deviation < 11 %). All royal jelly liquid dietary supplements were positive for chlorfenvinphos and, in the case of one of them, for α-endosulfan; chlorfenvinphos was determined in some fresh royal jelly samples, and no pesticide residues were detected in the propolis samples analysed.

1. Introduction

Bee products, such as honey, beeswax, pollen, propolis and royal jelly, have multiple benefits for human health due to their nutritional and medicinal properties (antioxidant, antibacterial, antiviral and anti-inflammatory). Therefore, their consumption has increased in recent years, despite having been valued since ancient times (Fuente-Ballesteros, Priovolos, Ares, Samanidou, & Bernal, 2023). Royal jelly is a prized bee product consisting of a thick milky-white or yellowish fluid, sweet and acidic in taste, secreted from the hypopharyngeal gland by nurse honeybees (Chen et al., 2023). Propolis, meanwhile, is a resinous material produced by bees collecting exudate from plants and buds mixed with saliva and beeswax. The purpose of this mixture is to protect the colony from diseases, intruders, and pathogens and to keep the temperature of the hive constant (González-Martín, Revilla, Vivar-Quintana, & Betances Salcedo, 2017). However, several food alerts have been reported in the last few years due to the detection of pesticides in bee products like honey, beeswax, propolis, royal jelly and bee pollen (Kasiotis, Zafeiraki, Manea-Karga, Anastasiadou, & Machera, 2023; Végh, Csóka, Mednyánszky, & Sipos, 2023). Pesticides can enter beehives in several ways, primarily through pesticide drift and nectar and pollen contamination from plants pollinated by bees, and they can also be found in water from which bees drink (Fuente-Ballesteros, Augé, Bernal, & Ares, 2023). Moreover, the existence of mites that cause diseases among bees, such as the *Varroa destructor* that provoked a worldwide emergency in terms of huge losses of bee populations, has led to an increase in the frequency and amount of pesticides, in this case acaricides, used by beekeepers (Fuente-Ballesteros, Augé et al., 2023). These compounds are used in the autumn to prevent winter losses by the varroa mite. Beekeepers apply them from September to December in different ways (fumigation, contact exposure, or spraying), and tiny residues could be passed on to beeswax and honey, causing them to

Abbreviations: **AF**, samples spiked after sample treatment; **BF**, samples spiked before sample treatment; **dSPE**, dispersive solid phase extraction; **EI**, electronic impact; **EMR-Lipid**, enhanced matrix removal lipid; **FRJ**, fresh royal jelly; **GCB**, graphitized carbon black; **GC-MS**, gas chromatography coupled with mass spectrometry; **IS**, internal standard; **LC-MS/MS**, liquid chromatography coupled with tandem mass spectrometry; **LOD**, limit of detection; **LOQ**, limit of quantification; ***m/z***, mass-to-charge; **MRL**, maximum residue level; **MS**, mass spectrometry; **MSPD**, matrix solid phase dispersion; **PLDS**, propolis dietary supplement; **PSA**, primary secondary amine; **QuEChERS**, quick, easy, cheap, effective, rugged, and safe; **RJLDS**, royal jelly liquid dietary supplement; **RSD**, relative standard deviation; **SE**, solvent extraction; **S/N**, signal-to-noise; **SCI**, slope confidence intervals; **SIM**, selected ion monitoring; **SPE**, solid phase extraction.

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enter the bee's body. Therefore, the risk of contamination of the honey bee colony, and subsequently of their food-derived products, generally relates to two different channels: i) pollination, mainly by pollen and nectar (Zioga, Kelly, White, & Stout, 2023; Zioga, White, & Stout, 2023), although forager bees can be directly exposed to pesticides when they are applied to crops/plants (Kadlikova et al., 2021); and ii) resistance against mites, mainly *Varroa destructor* (Wueppenhorst, Eckert, Steinert, & Erler, 2022). However, it should be noted that contamination and content of pesticides in bee colonies are greatly affected by the season in question. For example, if plants are in flower at the time of application, nectar and pollen can be directly contaminated by pesticides (Zioga et al., 2020), and the chances of these products and others generated in the hive becoming highly contaminated thereby increase. In addition, bees can be affected by pesticides by direct contact or by ingesting contaminated products (Kadlikova et al., 2021), leading not only to their potential poisoning, but, for example, to the production of royal jelly and its composition possibly becoming disrupted (Chaves, Faita, Ferreira, Poltronieri, & Nodari, 2021; Milone, Chakrabarti, Sagili, & Tarpy, 2021). Regarding the specific cases of royal jelly and propolis, the sources of contamination are not exactly the same. In both cases, if the bees are contaminated with pesticides, these can be transferred to the two products (Milone et al., 2021; Chaves et al., 2021), whilst in other cases the paths are different. Royal jelly is concerned with the process of feeding the colony, and its contamination depends on the levels of pesticides present in the other products of the hive. Propolis, meanwhile, is collected during periods of tree flowering and is unlikely to be contaminated unless the source (buds) or the bees contain pesticides, although several cases of propolis contaminated with in-hive acaricides have been reported (Végh et al., 2023).

Pesticide residues in bee matrices such as beeswax, honey and bee pollen have been the object of study in many publications, but there are few studies relating to royal jelly and propolis. In this paper we will focus attention on seven specific pesticides (herbicides: atrazine; acaricides: chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, and τ-fluvalinate). These were selected for two main reasons: i) residues have been found in beehive products all over the world, even though the use of some of them is not allowed in certain countries (Murcia-Morales, Heinzen, Parrilla-Vázquez, Gómez-Ramos, & Fernández-Alba, 2022; Nozal et al., 2021); ii) this study follows a line of investigation in collaboration with researchers from the Centro de Investigación Apícola y Agroambiental (CIAPA; Marchamalo, Guadalajara, Spain), beginning with an analysis of seven of the most commonly detected pesticides in beeswax (Nozal et al., 2021); these are the same as those of the present study. In subsequent investigations, specific methods were developed for other beehive products (honey and bee pollen), in which residues of some of these compounds were found (Fuente-Ballesteros et al., 2023; Fuente-Ballesteros, Priovolos et al., 2023). It is true that many other pesticides, such as amitraz, were not included in this study, but these were chosen by CIAPA experts based on previous work carried out both in the field and in experimental apiaries.

The determination of these pesticides in royal jelly and propolis (see Supplementary Material, Table 1S) has been mainly performed by solvent extraction (SE; Balayannis, 2001; Chen et al., 2009; Hu et al., 2019; Karazafiris, Menkissoglu-Spiroudi, & Thrasyvoulou, 2008; Karazafiris et al., 2022; Simsek, Kuzukiran, Yurdakok-Dikmen, Snoj, & Filazi, 2020; Simsek et al., 2021; Škerl, Kmecl, & Gregorc, 2010; Umsza-Guez, Silva-Beltrán, Machado, & Balderrama-Carmona, 2021; Wang et al., 2020), solid phase extraction (SPE; Chen et al., 2009; González-Martín et al., 2017; Hu et al., 2019; Karazafiris et al., 2008, 2022; Martínez-Domínguez, Romero-González, & Garrido-Frenich, 2014; Notardonato, Avino, Cinelli, & Russo, 2016; Simsek et al., 2020, 2021; Wang et al., 2020), or modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methods (Böhme, Bischoff, Zebitz, Rosenkranz, & Wallner, 2019; Gérez, Pérez-Parada, Cesio, & Heinzen, 2017; Oellig, 2016; Zheng et al., 2018), followed by gas chromatography (GC) with mass spectrometry or tandem mass spectrometry (GC–MS or GC–MS/MS), electron capture (ECD), nitrogen phosphorus (NPD) and flame ionization/photometric (FID or FPD) detectors (see Supplementary Material, Table 1S). Matrix-solid phase dispersion has been selected in several publications as sample treatment for propolis analysis (Acosta-Tejada, Medina-Peralta, Moguel-Ordóñez, & Muñoz-Rodríguez, 2011; Medina-Dzul, Muñoz-Rodríguez, Moguel-Ordoñez, & Carrera-Figueiras, 2014; Pareja et al., 2011; Pérez-Parada et al., 2011), while a dilute and shoot procedure was only once employed in royal jelly (Martínez-Domínguez, Romero-González, & Garrido Frenich, 2016). Otherwise, high performance liquid chromatography has been also employed in some cases (Oellig, 2016; Martínez-Domínguez et al., 2016; Pareja et al., 2011; Umsza-Guez et al., 2021; Zheng et al., 2018), especially for coumaphos analysis.

Therefore, the main goal of the present study is to propose a method for determining simultaneously seven pesticides (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos and τ-fluvalinate) in different types of royal jelly (fresh royal jelly-FRJ, and liquid dietary supplement-FRLDS) and propolis (liquid dietary supplement in glycerine-PLDS) by means of GC–MS. The GC–MS conditions were selected from a recent study undertaken by our group (Fuente-Ballesteros et al., 2023). Our aim is to propose an efficient, simple, cheap and fast sample treatment that can be used in royal jelly and propolis. These conditions are intended to ensure good recovery, minimizing the potential matrix effect and respecting as far as possible the principles of green analytical chemistry (a reduction in time and cost, the number of steps and the amount of reagents; Gałuszka, Migaszewski, & Namieśnik, 2013). To the best of our knowledge, this is the first study in which an analytical methodology has been proposed for determining pesticides, including those given above, in different types of royal jelly and propolis. Our study also aims to validate the proposed method for different bee foods in accordance with current European legislation (EURACHEM guide, 2014; European Commission Directorate-General for Health and Food Safety, 2021), and analyse commercial samples of both types of royal jelly samples and propolis.

2. Materials and methods

2.1. Reagents and materials

Pesticide standards (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos, τ-fluvalinate and chlorfenvinphos-d_{10}; 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, hexane, acetic acid, acetonitrile, and isopropanol) 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. Varian Bond Elut^{TM} C_{18} (500 mg of sorbent; Agilent, CA, USA), Sep-Pak® C_{18} (100 mg of sorbent; Waters, Milford, MA), Strata® C_{18}-E (500 mg of sorbent; Phenomenex, Torrance, CA) cartridges, and a 10-port Visiprep vacuum manifold (Supelco, Bellefonte, PA), were used for the SPE extractions. In addition, QuEChERS dispersive solid phase extraction (dSPE) enhanced matrix removal lipid (EMR-Lipid) sorbent was supplied by Agilent Technologies (Folsom, CA, USA); while $MgSO_4$ was obtained from Sigma-Aldrich Chemie Gbmh (Steinheim, Germany), and PSA and C_{18} were provided by Supelco (Bellefonte, PA, USA).

2.2. Standards

Standard stock (≈ 1000 mg/L) and working solutions of the studied compounds were prepared in a mixture of ethyl acetate and cyclohexane (50:50, v/v). FRJ, RJLDS, and PLDS blank samples were spiked with different amounts of the analytes before (BF samples) or after (AF samples) sample treatment (see subsection 2.3). The spiking protocol was adapted from a previous work (Fuente-Ballesteros et al., 2023; see Supplementary Material, Table 3S). The internal standard (IS; chlorfenvinphos-d_{10}) was always added at the same concentration (0.1 mg/L). These samples were used for validation (spiked samples (low, medium, and high) and calibration curves), as well as sample treatment studies. Three replicates which were injected three times, were prepared for all the studies. Each spiked sample was prepared with a blank sample spiked with three different concentrations of the acaricides within the linear range. These were as follows: low-LOQ (see Table 1); medium-156 μg kg^{-1}; high-625 μg kg^{-1}. The standard stock solutions were stored in glass containers in darkness at −20 °C; working and standard matrix solutions were stored in glass containers and kept in the dark at 4 °C.

2.3. Sample procurement and treatment

2.3.1. Samples

Three types of samples, RJLDS (n = 5), FRJ (n = 4), and PLDS (n = 6), were investigated in the present study. All of them were purchased in local markets (Valladolid, Spain) and stored at 4 °C until analysis. They were selected according to their physical state and composition since LDS of royal jelly and propolis with other excipients were available. Three replicates (sub-samples) of each sample, which were injected in triplicate, were examined to determine the pesticide concentration.

2.3.2. Sample treatment

It has been specifically developed and optimized in this work for the matrices under study. Briefly, 4.0 g of homogenized royal jelly (FRJ or RJLDS) or propolis (PLDS) sample was weighed in a 50 mL centrifuge tube, after which 3 mL of ultrapure water was added, and the tube was shaken for 30 s in a vortex device. It should be mentioned that the addition of ultrapure water was only required for royal jelly products. Next, 10 mL of a hexane and isopropanol (50:50, v/v) mixture was added to the tube and then shaken again in a vibromatic device for 5 min. After that, 1.0 g PSA was added and shaken again prior to centrifugation (10000 rpm; 5 °C) for 5 min. The supernatant was collected and evaporated to dryness at 60 °C in a rotary evaporator. Finally, the dry extract was reconstituted with 1 mL of an IS solution (0.1 mg/L), and it was passed through a 0.45 μm nylon filter prior GC–MS analysis. Fig. 1 summarizes the steps of the selected sample treatments.

2.4. GC–MS parameters

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 used. The GC–MS parameters, including the column (Agilent DB-5MS; 30 m × 0.25 mm × 0.2 μm) were selected according to previous work (see Table 2; Fuente-Ballesteros et al., 2023), and under these conditions all compounds eluted in less than 22 min (see Fig. 2). Analyses were performed in selected ion monitoring mode (SIM), with one target/quantification and two qualifier ions for each analyte (see Table 1). In the chromatographic analysis τ-fluvalinate showed two peaks according to its stereoisomerism (Frison et al., 1999; Fuente-Ballesteros, et al., 2023; Pérez-Fernández et al., 2010), and the sum of their corresponding areas was employed for quantification purposes.

3. Results and discussion

3.1. Optimization of the sample treatment

We optimised sample treatment with RJLDS by evaluating some of the procedures (SE, SPE and QuEChERS) that have been recently tested in other bee matrices such as beeswax, bee pollen or honey (Fuente-Ballesteros, Augé et al., 2023; Fuente-Ballesteros et al., 2023; Fuente-Ballesteros, Priovolos et al., 2023; Nozal et al., 2021), and which have already been proposed in the literature (see Supplementary Material, Table 1S). 2.0 g of samples (pesticide free-blank, AF and BF) were employed in these preliminary experiments. We verified the suitability of SPE by evaluating the performance of three different C_{18}-based sorbents (Varian Bond Elut^{TM} C_{18}, Sep-Pak® C_{18}, and Strata® C_{18}-E), as these were generally employed in previous publications (see Supplementary Material, Table 1S). The results obtained were unsatisfactory, due to the clogging of the cartridges, the presence of interference peaks at the same retention times as those of the analytes, and the recovery values being below 50 % for atrazine, chlorpyrifos, and bromopropylate (data not shown). Next, we performed two QuEChERS-based methodologies that proved satisfactory when determining the same pesticides in beeswax (Nozal et al., 2021) and bee pollen (Fuente-Ballesteros, Augé et al., 2023). Unfortunately, the results were not good due to the poor dissolution of RJDLS in acetonitrile acidified in acetic acid and the ineffectiveness of the cleaning salts for removing matrix interferences. Therefore, we decided on a simple SE methodology recently proposed for analysing pesticides in honey (Fuente-Ballesteros et al., 2023), consisting of an ethyl acetate and cyclohexane (50:50, v/v) mixture as the solvent extractant. For the first time, the recovery rate (60–120 %) and matrix effect values (<±35 %) were acceptable for all the analytes with the exception of the matrix effect for coumaphos, a problem already detected in previous publications (Fuente-Ballesteros et al., 2023). Consequently, we decided to continue the optimisation procedure with a SE by testing the influence of some of the most relevant parameters: i) amount of sample; ii) amount of water for sample dissolution; iii) amount of extractant; iv) shaking time; and v) amount of supernatant. The values considered for each parameter were selected as a result of some preliminary experiments. From all the tests performed

Table 1
Quantification and qualifier ions, limits of detection (LOD), quantification (LOQ), and maximum residue levels (MRLs) of the studied pesticides.

Compound	Quantification Ions (*m/z*)	Confirmation Ions (*m/z*)	RJLDS		FRJ		PLDS		MRLs (μg kg^{-1})
			LOD (μg kg^{-1})	LOQ (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	
Atrazine	200	173; 215	0.1	0.4	0.3	0.9	1.6	5.2	50
Chlorpyrifos	197	258; 314	0.4	1.2	0.4	1.2	2.7	9.2	10
Chlorfenvinphos	267	270; 329	0.3	0.9	0.6	2.1	2.8	9.2	10
α-Endosulfan	241	195; 207	0.5	1.8	1.2	4.1	2.3	7.5	10
Bromopropylate	341	183; 185	0.6	1.9	1.5	5.1	2.7	8.8	10
Coumaphos	362	109; 226	0.2	0.8	0.5	1.6	2.2	7.3	100
τ-Fluvalinate	250	181; 208	0.1	0.3	0.7	2.2	0.5	1.5	50
Chlorfenvinphos-d_{10}	333	–							

FRJ, fresh royal jelly; **PLDS**, propolis liquid dietary supplement; **RJLDS**, royal jelly liquid dietary supplement.

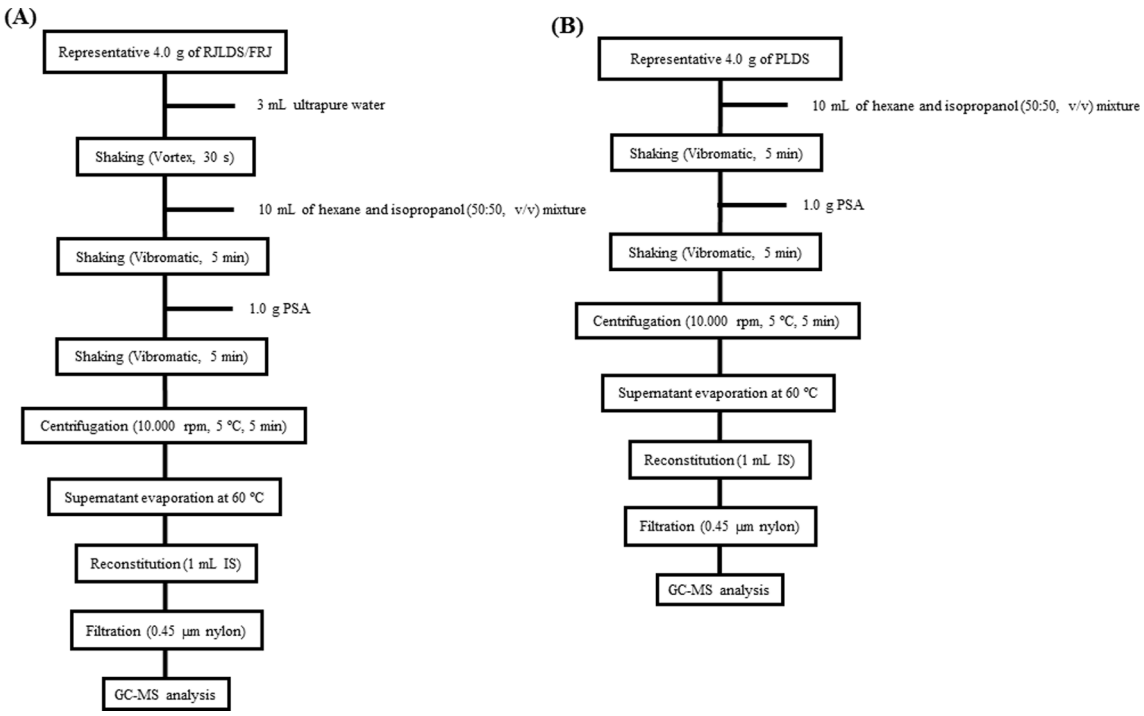

Fig. 1. Analytical procedures work-up flow charts: (A) royal liquid dietary supplements (RJLDS) and fresh royal jelly (FRJ); (B) propolis liquid dietary supplements (PLDS).

Table 2
GC-MS parameters. Adapted from Food Chemistry, 408, Adrián Fuente-Ballesteros, Patricia Brugnerotto, Ana C. O. Costa, María J. Nozal, Ana M. Ares, José Bernal, Determination of acaricides in honeys from different botanical origins by gas chromatography-mass spectrometry, 135245, Copyright (2023), with permission from Elsevier.

GC parameter	Final setting
Programmed temperature conditions	from 60 °C (1 min) to 170 °C (0 min), at 40 °C/min and then increased to 310 °C (3 min) at 8 °C/min.
Carrier gas	Helium
Flow-rate (mL/min)	1.2
Injection mode	Pulsed splitless
Injector temperature (°C)	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 (°C)	230
Quadrupole temperature (°C)	150
Nebulizer gas (N_2) pressure (psi)	40

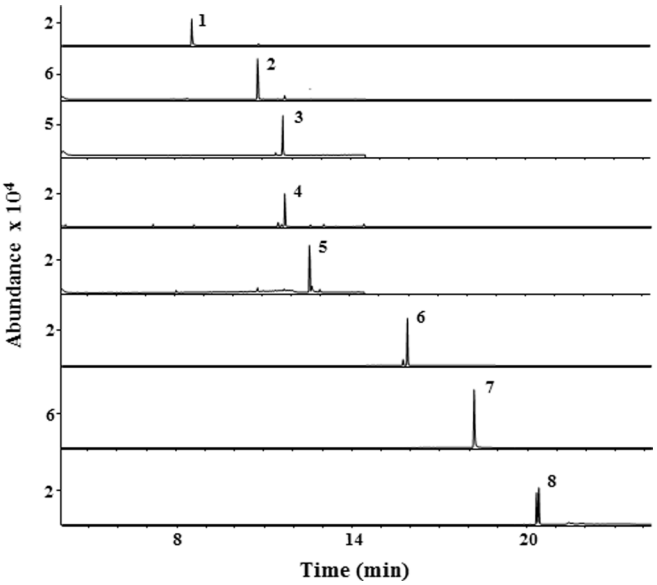

Fig. 2. Representative GC-MS chromatograms (SIM mode using the quantification/target ions; see Table 1) obtained from a standard in solvent mixture (0.25 mg/L; IS, 0.1 mg/L). GC-MS conditions are summarized in Subsection 2.4 and Tables 1 and 2. 1, atrazine; 2, chlorpyrifos; 3, chlorfenvinphos-d_{10} (IS); 4, chlorfenvinphos; 5, α-endosulfan; 6, bromopropylate; 7, coumaphos; 8, τ-fluvalinate.

(see Supplementary Material, Table 4S), the best values in terms of recovery percentages and matrix effect were obtained when using 4 g of sample, 3 mL of water, 10 mL of solvent extractant, 5 min shaking time and 4 mL of supernatant. However, the recovery and matrix effect values for all the compounds were not acceptable; consequently, we managed to verify the effectiveness of an extraction mixture (hexane and isopropanol; 50:50, v/v) that provided good results for determining pesticides like coumaphos in a bee matrix (Škerl, Kmecl, & Gregorc, 2010). With this solvent extractant and the conditions previously selected (data not shown), the recovery values of all the analytes considerably improved (between 90 and 120 % in all cases), and the matrix effect was successfully minimised, with the exception of α-endosulfan (-27 %) and coumaphos (+40 %). New alternatives were then evaluated for reducing the matrix effect. Firstly, we considered implementing a cooling stage (dry ice) in the extraction process, as this has proven useful for removing/precipitating certain matrix components like lipids of proteins; yet this was discarded as the reduction in matrix effect was not significant (see Supplementary Material, Table 5S). Next, several salts/sorbents were studied in different amounts and combinations to reduce the interferences from the matrix (EMR-Lipid, PSA, C_{18}, and $MgSO_4$).

For example, EMR-Lipid enables selective extraction from high-fat samples while minimising analyte loss; the inclusion of PSA effectively removes organic acids and polar pigments; C_{18} efficiently eliminates certain lipids; and $MgSO_4$ removes residual water content from the sample. As can be seen in Table 6S (see Supplementary Material), using 1 g of PSA provided quite acceptable results in terms of reducing matrix effect recoveries (from −2% to 4 %), without affecting the satisfactory recoveries of the analytes (from 100 % to 109 %); we therefore decided to include this sorbent in the sample treatment method. Finally, 4 mL of supernatant was transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 60 °C. No loss of pesticides was observed during this stage. Less reconstitution solution (from 1.5 mL to 0.5 mL) was used to improve method sensitivity without affecting extraction efficiency. The results showed a decrease in recovery percentages for only some of the compounds when using 0.5 mL, and similar values for 1.0 mL and 1.5 mL (data not shown). Consequently, we opted for 1.0 mL of reconstitution solution.

Once the sample treatment for RJLDS had been optimised, and we saw that the recoveries (from 96 % to 109 %) and matrix effect (from −4% to + 8 %) were acceptable for all the compounds at the different concentration levels assayed (see Table 3), we evaluated the suitability of this proposed sample treatment for FRJ. The results showed that it can be successfully employed with this matrix, due to the satisfactory recovery (from 94 % to 118 %) and matrix effect studies (from −9% to + 19 %) for all the pesticides at the three concentration levels examined (see Table 3). Once we had validated both methods for royal jelly products, we tested the same procedure for propolis samples. Following several tests, we were able to conclude that the procedure we used for royal jelly was also suitable for propolis, albeit with a minor modification; this entailed removing the initial addition of water, as it caused propolis precipitation. Suitable values for recovery (from 94 % to 118 %) and matrix effect (from −12 % to + 18 %) were obtained for the three concentration levels studied (see Table 3).

Finally, the proposed method can be considered a promising alternative to existing methods summarised in Table 1S (see Supplementary Material), owing to its being faster, simpler (few stages), involving little use of reagents (solvents, salts/sorbents), and being applicable to different bee-related matrices. Therefore, we may also conclude that the proposed method is more in keeping with the objectives of green analytical chemistry than most of those previously published. This is because the use of solvents and reagents is among the lowest (<10 mL), it does not require solvents that are incompatible with the environment (such as acetonitrile), it is the shortest of those proposed (<30 min), and it is also one of the simplest; this means that it can also be considered economical in comparison with other proposals. Moreover, recovery values were satisfactory for all the analytes studied, and, more importantly, the matrix effect was not significant for any pesticide, which is an important step forward with regard to previous studies (see Supplementary Material, Table 1S). The latter issue is of great concern when working with complex beehive products, which often goes unnoticed by many authors (Oellig, 2016). In conclusion, we have shown that the proposed procedure displays several advantages and differences in comparison with existing sample treatments. However, the main difference and substantial novelty in this regard lies in the fact that it can be applied with slight modifications to other beekeeping matrices (royal jelly and propolis) which are quite varied in their composition; this, to the best of our knowledge, is the first time that it has been proposed.

3.2. Method validation

We performed method validation according to current legislation (EURACHEM guide, 2014; European Commission Directorate-General for Health and Food Safety, 2021), and recent publications of our group (Fuente-Ballesteros et al., 2023; Fuente-Ballesteros, Priovolos et al., 2023).

3.2.1. Selectivity

This was evaluated by comparing the chromatograms and mass spectra of standards in solvents, standard extracts, and blanks of the different royal jelly and propolis products (n = 6). As can be seen when comparing Fig. 2 and Fig. 1S–3S (see Supplementary Material), we observed no matrix interferences at analyte retention times for the different bee products we studied. Moreover, we obtained similar mass spectra for the pesticides in solvents and standards in the matrix extracts (see Supplementary Material, Figure 4S). We compared the relative intensities of the selected ions for each pesticide in both types of standards, and in all cases these were within ± 20 % of the relative intensity (data not shown); this is within the established values ± 30 % (European Commission Directorate-General for Health and Food Safety, 2021).

3.2.2. Limits of detection and quantification

We determined the limits of detection (LODs) and quantification (LOQs) by injecting several blank samples measurement noise at the elution times for the acaricides studied and comparing this response (mean values) with the signal (peak heights) of the compounds at low concentration levels. We estimated the LODs and LOQs to be three and

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 Subsections 2.2, 3.3, and the results were obtained from three replicates that were injected in triplicate.

Compound	RJLDS						FRJ						PLDS					
	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	100 ± 5	101 ± 2	96 ± 4	−4 ± 3	−2 ± 4	0 ± 7	99 ± 9	94 ± 3	96 ± 4	3 ± 5	−9 ± 8	−1 ± 9	110 ± 3	103 ± 3	101 ± 5	4 ± 7	1 ± 5	−1 ± 5
Chlorpyrifos	97 ± 5	100 ± 4	100 ± 2	0 ± 5	−1 ± 2	3 ± 2	94 ± 9	117 ± 7	114 ± 7	2 ± 8	9 ± 9	0 ± 7	94 ± 3	108 ± 2	110 ± 9	4 ± 8	14 ± 3	−2 ± 5
Chlorfenvinphos	104 ± 5	107 ± 4	101 ± 2	1 ± 6	4 ± 2	7 ± 4	111 ± 5	112 ± 8	116 ± 1	14 ± 3	18 ± 8	7 ± 9	106 ± 6	114 ± 11	107 ± 4	−3 ± 6	8 ± 11	−12 ± 7
α-Endosulfan	101 ± 2	104 ± 3	99 ± 2	−1 ± 2	0 ± 2	−3 ± 3	105 ± 9	118 ± 7	111 ± 10	7 ± 8	6 ± 7	5 ± 8	102 ± 8	107 ± 8	93 ± 8	5 ± 2	−6 ± 7	7 ± 1
Bromopropylate	99 ± 4	107 ± 2	107 ± 4	7 ± 1	2 ± 3	4 ± 3	118 ± 10	117 ± 8	1117 ± 6	1 ± 7	12 ± 8	18 ± 5	118 ± 6	99 ± 4	114 ± 8	2 ± 6	11 ± 3	19 ± 3
Coumaphos	102 ± 1	109 ± 2	101 ± 7	1 ± 2	−1 ± 4	8 ± 8	102 ± 9	110 ± 7	115 ± 5	11 ± 6	10 ± 7	−1 ± 8	97 ± 1	110 ± 6	112 ± 9	16 ± 4	−8 ± 4	−4 ± 7
τ-Fluvalinate	100 ± 3	105 ± 2	100 ± 5	0 ± 5	−1 ± 4	−2 ± 5	117 ± 5	116 ± 5	108 ± 10	19 ± 9	9 ± 8	11 ± 6	117 ± 5	105 ± 2	99 ± 4	18 ± 10	5 ± 7	7 ± 6

EE, extraction efficiency; **ME**, matrix effect; **FRJ**, fresh royal jelly; **PLDS**, propolis liquid dietary supplements; **RJLDS**, royal jelly liquid dietary supplements; **LL**, low level (LOQ, see Table 1); **ML**, medium level (156 μg kg^{-1}); **HL,** high level (625 μg kg^{-1}).

ten times the S/N ratio, respectively. LOD values ranged from 0.1 to 0.6 μg kg^{-1} for RJLDS, from 0.3 to 1.5 μg kg^{-1} for FRJ, and from 0.5 to 2.8 μg kg^{-1} for PLDS propolis (see Table 1). Meanwhile, LOQ values ranged between 0.3 and 1.9 μg kg^{-1} for RJLDS, 0.9 and 5.1 μg kg^{-1} to FRJ, and 1.5 and 9.2 μg kg^{-1} for propolis (see Table 1). All the values for each matrix were below the established maximum residue levels (MRLs; see Table 1; European Union Pesticide Database, 2023), were comparable to or better than the values reported in royal jelly and were also much lower than those obtained in previous studies dealing with these compounds in propolis (see Supplementary Material, Table 1S).

3.2.3. Matrix effect

To ascertain how the matrix influenced the MS signal of the studied compounds, we compared the detector responses (analyte peak area/IS area) of standards in matrix extracts (R_{matrix}; AF samples) and standards in solvents ($R_{solvent}$) spiked at three different concentrations (low-LOQ (see Table 1); medium-156 μg kg^{-1}; and high-625 μg kg^{-1}). This was calculated in accordance with the recommendation of the European Commission Directorate-General for Health and Food Safety (2021): Matrix effect (%) = $[(R_{matrix}/R_{solvent}) -1] \times 100$. Analyte responses at the three levels assayed in each matrix ranged in all cases between −12 % of signal suppression to + 19 % of signal enhancement (see Table 3), lower than the permitted values according to current legislation (±20 %; European Commission Directorate-General for Health and Food Safety, 2021). We also compared the slope confidence intervals (SCIs) with standards in solvent and standards in matrix extracts, and we found an overlapping of SCIs in all cases (see Supplementary Material, Table 7S). Therefore, we may conclude that with the proposed sample treatment the bee-related matrices we selected did not significantly affect the MS signal of the analytes.

3.2.4. Linearity/Working range

Standard in solvent calibration curves could be used to quantify pesticides in royal jelly and propolis products due to the absence of a significant matrix effect. Calibration curves (n = 6) were constructed by plotting the signal on the *y*-axis (analyte peak area/IS area) against analyte concentration on the *x*-axis. Concentration of the analytical curves varied between LOQ and 1000 μg/L (LOQ (see Table 1), 50, 100, 250, 500, and 1000 μg/L), which corresponds to those between LOQ and 625 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 Supplementary Material, Table 7S). Moreover, the deviation of back-calculation concentration from true concentration was lower than 20 % (data not shown; European Commission Directorate-General for Health and Food Safety, 2021).

3.2.5. Precision

We carried out concurrent experiments for precision, which was expressed as relative standard deviation (% RSD), by repeated sample analysis using BF samples spiked at three different concentration levels: low-LOQ (see Table 1); medium-156 μg kg^{-1}; and high-625 μg kg^{-1}. These took place either on the same day (intra-day precision, European Commission Directorate-General for Health and Food Safety, 2021; repeatability, EURACHEM guide, 2014), or over three consecutive days (inter-day precision, European Commission Directorate-General for Health and Food Safety, 2021; partial reproducibility, EURACHEM guide, 2014). %RSD values were lower than 11 % in all cases (see Supplementary Material, Table 8S), which is consistent with current European legislation (%RSD ≤ 20 %; European Commission Directorate-General for Health and Food Safety, 2021).

3.2.6. Trueness

This was evaluated by means of recovery experiments (as a measure of trueness), by comparing the results (analyte peak area/IS area) obtained from blank samples spiked at three different concentrations (low-LOQ (see Table 1); medium-156 μg kg^{-1}; and high-625 μg kg^{-1}), either prior to (BF samples) or following (AF samples) sample treatment. Mean recoveries for the pesticides we studied ranged in all cases from 93 % to 118 %, while %RSD values were lower than 11 % in all cases (see Table 3). These values met the requirements stipulated by European legislation (recovery percentages between 70 % and 120 %; RSD ≤ 20 %; European Commission Directorate-General for Health and Food Safety, 2021), and are similar to or better than the recoveries obtained in previous studies (see Supplementary Material, Table 1S).

3.3. Application of the method

We analysed the samples in triplicate, handling them in accordance with the procedures outlined in subsection 2.3.2. All the royal jelly LDSs (n = 5; RJLDS1-RJLDS5) exhibited chlorfenvinphos residues over LOQ; α-endosulfan was also determined in one of them (RJS3; see Table 4 and Supplementary Material, Figure 5SA). All concentrations were above the established MRLs (10 μg kg^{-1}; European Union Pesticide Database, 2023), which represents a potential risk to consumers. Regarding FRJ samples (n = 4; RJF1-RJF4), we observed chlorfenvinphos residues in three of the samples but at concentrations lower than the established MRLs (see Table 4 and Supplementary Material, Figure 5SB**)**. Meanwhile, we detected no acaricide residues in the propolis products we analysed (n = 6; PLDS1-PLDS6).

The detection of pesticide residues in royal jelly is not new, as several cases of contaminated royal jelly samples have been reported in the literature (Böhme et al., 2019). This includes coumaphos (10–92 μg kg^{-1} in treated beehives, Balayannis, 2001; 5–12500 μg kg^{-1} in treated beehives, Karazafiris et al., 2022; 9-15 μg kg^{-1} in commercial and home-made samples, Notardonato et al., 2016; 170-400 μg kg^{-1} in treated beehives, Škerl et al., 2010), bromopropylate (10–36 μg kg^{-1} in commercial and home-made samples, Notardonato et al., 2016), and τ-fluvalinate (44–73 μg kg^{-1} in treated beehives, Böhme et al., 2019; 11-14 μg kg^{-1} in commercial and home-made samples, Notardonato et al., 2016). Our detecting certain pesticides in the royal jelly samples we analysed may be tentatively attributed to some samples possibly consisting of a mixture of royal jelly with pollen or with other bee products (royal jelly, nectar, or propolis); consequently, the transfer of contaminants between products is plausible. In addition, and as mentioned in the Introduction, if the bees are contaminated with pesticides, they can transfer them to the royal jelly (Chaves et al., 2021; Milone et al., 2021).

The absence of pesticide residues in the propolis samples we analysed, a finding which has been also reported in some studies (Acosta-Tejada et al., 2011; Chen et al., 2009; Medina-Dzul et al., 2014; Oellig, 2016; Simsek et al., 2020), does not mean that the applicability of the method is limited. In fact, several of the pesticides studied have been previously detected in propolis samples: atrazine (9700–17400 μg kg^{-1} in commercial samples, Umsza-Guez et al., 2021), coumaphos (4–37 μg kg^{-1} in commercial samples, Gérez et al., 2017; 600–1000 μg kg^{-1}, Pareja et al., 2011; concentration not provided, Pérez-Parada et al.,

Table 4
Results (means of triplicate analyses (μg kg^{-1}); (%RSD < 11 % in all cases) of the pesticides found in the analyzed royal jelly-based products. The other acaricides under study were below LOD in the samples.

Sample	a-Endosulfan	Chlorfenvinphos
RJLDS1	< LOD	15
RJLDS2	< LOD	16
RJLDS3	41	14
RJLDS4	< LOD	19
RJLDS5	< LOD	13
FRJ1	< LOD	6
FRJ2	< LOD	7
FRJ3	< LOD	< LOD
FRJ4	< LOD	6

LOD, limit of detection (see Table 1); **FRJ**, fresh royal jelly; **RJLDS**, royal jelly liquid dietary supplements.

2011), τ-fluvalinate (12–587 μg kg^{-1} in commercial samples, Wang et al., 2020), and chlorpyrifos (10–22 μg kg^{-1} in commercial samples, Gérez et al., 2017; 70-150 μg kg^{-1}, Pareja et al., 2011; concentration not provided, Pérez-Parada et al., 2011). The absence of pesticides in the propolis samples may be attributed to the following reasons: i) the bees collected resins from plants in an uncontaminated environment, ensuring the absence of pesticide exposure. As occurs in other European countries, in Spain - the country of origin of the samples - environmental contamination is unlikely as most of this comes from wild willows or poplars, not agricultural lands; ii) the hive had not undergone any treatment with chemicals prior to the collection of propolis samples. This is related to the fact already mentioned about the influence of the collection period on the presence of pesticides; iii) the frequent collection of propolis by beekeepers prevented the accumulation of pesticides in the hive.

4. Conclusions

In this study we develop and optimise an analytical methodology to determine seven pesticides (atrazine, chlorpyrifos, chlorfenvinphos, α-endosulfan, bromopropylate, coumaphos and τ-fluvalinate) in samples of royal jelly and propolis by means of GC–MS. We propose an efficient, simple, fast, economical and environmentally-compatible sample treatment, consisting of solvent extraction followed by a clean-up step with PSA. To the best of our knowledge, this is the first time that the same method, with minor modifications, has been developed and applied for different royal jelly and propolis products. This procedure makes it possible not only to obtain good recovery rates, but also to minimise the matrix effect in all cases; this is not usually the norm when these bee products are analysed. These are some of the advantages of developing specific methods instead of multi-residue approaches. In addition, the chromatographic conditions of a previous study can be used in these very different matrices, as our proposed sample treatment minimises both the presence of interferents and the matrix effect. We validated the method according to current legislation, and the results show that its analytical performance was in many cases similar to or better than that of previous studies. The LODs and LOQs we obtained were lower than the MRLs established for the compounds studied in these matrices and comparable to the best published values. We applied our proposed validated method to analyse several commercial samples of royal jelly and propolis. Pesticide residues (chlorfenvinphos and α-endosulfan) were detected in certain royal jelly samples, but no residues were found in propolis. Occasionally, the concentrations were higher than established MRL limits. This could represent a potential risk for consumers and justified/responded to the hypothesis included in the Introduction concerning the need to develop selective and sensitive methods for determining pesticides in these bee foods.

Finally, the detection of pesticide residues in royal jelly and propolis - and especially considering the results of this study regarding royal jelly products - emphasizes the importance of developing analytical methodologies which are both sensitive and selective to guarantee the safety of these products. It is true that the amount of royal jelly and propolis consumed by a person on a daily basis is very small, but in some cases the concentrations found in royal jelly were above established MRLs, and the harmful effects of pesticides on health can have long-term consequences. Therefore, once the advisability of our proposed method has been demonstrated, it can be used in future research to analyse more samples, including ones from different countries and different harvesting periods, and may even be tested with other families of pesticides.

CRediT authorship contribution statement

Adrián Fuente-Ballesteros: Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ana Jano:** Investigation, Methodology, Validation, Visualization, Writing – original draft. **José Bernal:** Conceptualization, Methodology, Investigation, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ana M. Ares:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137911.

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