





1	Linearità della risposta strumentale di un metodo analitico: definizione, allestimento curva, scelta del tipo di retta, parametri valutati.	Quali caratteristiche deve avere, in base alla normativa di riferimento, un metodo di screening per ricerca di sostanze anabolizzanti?
2	La precisione di un metodo analitico: definizione, tipi di precisione, allestimento prove, parametri valutati.	Significato del termine CCalfa, campo di applicazione, normativa di riferimento e differenze nel calcolo tra sostanze di Cat A e Cat B.
3	Standard degli analiti ricercati: caratteristiche in base allo scopo del metodo, certificazione degli standard. Preparazione delle soluzioni madri di standard e verifica della stabilità.	Quali caratteristiche deve avere, in base alla normativa di riferimento, un metodo di conferma per ricerca di farmaci non autorizzati?
4	Recupero di un metodo analitico: definizione, allestimento prove, parametri valutati.	Significato del termine CCbeta e campo di applicazione per farmaci di Cat A e Cat B.
5	Determinazione quantitativa di una micotossina in alimenti: metodi di quantificazione, criteri di identificazione e di prestazione.	Descrivere l'indice di prestazione più importante per la validazione dei metodi di screening e indicare le modalità di valutazione
6	Approcci alla stima dell'incertezza di misura associata al risultato di una misura e suo utilizzo nella valutazione della conformità di un campione per una micotossina	Caratteristiche dello standard interno nei metodi di screening e differenze nella scelta quando invece esso si utilizza in metodi quantitativi in spettrometria di massa.
7	Quali caratteristiche deve avere un materiale di riferimento e a cosa serve?	Quali caratteristiche deve avere uno standard interno utilizzato per scopi quantitativi?



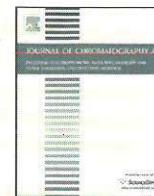
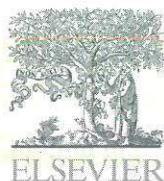
8	Strumenti per assicurare la qualità dei risultati nelle analisi di conferma per ricerca di residui di sostanze vietate o contaminanti in alimenti.	Validazione secondaria di un metodo normato.
9	Criteri di rendimento dei metodi analitici destinati alla ricerca di residui di farmaci o contaminanti in alimenti	Costruzione della curva di taratura con il metodo dello standard interno
10	Modelli di stima dell'incertezza di misura associata al risultato di una prova.	Costruzione della curva di taratura con il metodo delle aggiunte standard
11	Validazione primaria di un metodo interno sviluppato dal laboratorio: pianificazione e svolgimento	Come si assicura la qualità di un risultato nelle analisi di routine di screening?
12	Accuratezza di un metodo analitico: cosa rappresenta e come si esprime.	Come si controlla il mantenimento delle prestazioni di un metodo di analisi durante la sua applicazione in routine?
13	Come si assicura la qualità di un risultato nelle analisi di routine di conferma?	Rivelatori per HPLC: differenze nelle caratteristiche e applicazioni tra DAD, FLD E MS/MS
14	Sviluppo di un metodo interno di analisi per la ricerca di un analita nel muscolo: pianificazione e svolgimento	Criteri di espressione del risultato e del giudizio di conformità per residui di un metallo pesante nel pesce.

15	Criteri per l'identificazione di un analita in HPLC-DAD e FLD nei metodi di conferma	La norma 17025: requisiti dell'operatore addetto alle prove
16	Criteri per l'identificazione di un analita in cromatografia accoppiata alla spettrometria di massa.	La norma 17025: riferibilità delle misure e materiali di riferimento
17	Analisi di conferma per determinazione della quantità di un residuo in alimenti: metodi di quantificazione	Come si assicura la qualità del dato analitico in un laboratorio di analisi degli alimenti
18	Vantaggi dell'ICP-MS rispetto alle tecniche AAS.	Piano Nazionale Residui: indicarne le caratteristiche e le finalità
19	Il campione ufficiale legale: definizione e caratteristiche.	Come si costruisce una carta di controllo e a cosa serve.
20	Rivelatori per GC: differenze nelle caratteristiche e applicazioni tra FID, ECD, NPID E MS/MS	Indici di prestazione da verificare durante la validazione di un metodo di screening per ricerca di micotossine in alimenti
21	Proficiency test: utilità per il laboratorio, caratteristiche, normativa di riferimento, tipologia di report dei risultati	Quali caratteristiche deve avere un metodo di conferma per ricerca di micotossine in alimenti in base alla normativa di riferimento?

22	Varie tipologie di utilizzo degli standard interni aggiunti all'inizio del processo di preparazione del campione.	Approccio bottom-up alla stima dell'incertezza di misura: principali contributi da considerare e modalità di espressione dell'incertezza estesa.
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Large multiresidue analysis of pesticides in edible vegetable oils by using efficient solid-phase extraction sorbents based on quick, easy, cheap, effective, rugged and safe methodology followed by gas chromatography–tandem mass spectrometry



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ABSTRACT

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The aim of this research was to adapt the QuEChERS method for routine pesticide multiresidue analysis in edible vegetable oil samples using gas chromatography coupled to tandem mass spectrometry (GC–MS/MS). Several clean-up approaches were tested: (a) D-SPE with Enhanced Matrix Removal–Lipid (EMR–Lipid™); (b) D-SPE with PSA; (c) D-SPE with Z-Sep; (d) SPE with Z-Sep. Clean-up methods were evaluated in terms of fat removal from the extracts, recoveries and extraction precision for 213 pesticides in different matrices (soybean, sunflower and extra-virgin olive oil). The QuEChERS protocol with EMR–Lipid d-SPE provided the best reduction of co-extracted matrix compounds with the highest number of pesticides exhibiting mean recoveries in the 70–120% range, and the lowest relative standard deviations values (4% on average). A simple and rapid (only 5 min) freeze-out step with dry ice (CO₂ at –76 °C) prior to d-SPE clean-up ensured much better removal of co-extracted matrix compounds in compliance of the necessity in routine analysis. Procedural Standard Calibration was established in order to compensate for recovery losses of certain pesticides and possible matrix effects. Limits of quantification were 10 µg kg^{–1} for the majority of the pesticides. The modified methodology was applied for the analysis of different 17 oil samples. Fourteen pesticides were detected with values lower than MRLs and their concentration ranged between 10.2 and 156.0 µg kg^{–1}.

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1. Introduction

Nowadays, the olive crop as well as the soybean and the sunflower crops demand a wide range of insecticides (organophosphorus, carbamate, organochlorine, pyrethroid and other chemical classes) and fungicides (phthalimides, triazoles, imidazoles, sulfamides and others chemical classes) consumption. Herbicides (sulfonyleurea and diphenyl ethers) is another type of pesticide commonly used in these groves. Thus, pesticide residues may occur in the final vegetable oil products. According to the European Food Safety Authority (EFSA) [1], out of the 794 samples of olive oil analysed in 2012, 175 samples (22%) contained one or several pesticides in measurable concentrations. Residues above the MRL were

detected for pendimethalin (0.2%), terbutylazine (1%), endosulfan (RD) (0.2%), famoxadone (0.2%) and fenthion (0.5%). Since olive oil was not included in other EU-coordinated monitoring programs, no comparison of the 2012 results with recent years is possible. However, pesticide residues were reported by different authors between 2013 and 2016 [2–4]. The results showed that the incidence and levels of pesticides were higher in virgin olive oil than in refined olive oil. Pesticide residues were also detected in soybean [5,6] and sunflower oil [7].

Regardless of the pesticide-residue determination in edible oils, extraction and clean-up remains the main limiting step. A compilation of applications involving additional clean-up steps after solvent extraction when dealing with edible oil samples is given in several reviews [8–11], most of them related to the selective determination of pesticide residues in edible oil, but only few of these protocols were proposed for a wide-scope multiresidue analysis of pesticides in this complicated matrix [3,12–15].

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Mass spectrometric techniques (i.e. tandem mass spectrometry using triple quadrupole or ion-trap instruments) in combination with gas chromatography (GC) or liquid chromatography (LC) are the techniques of choice for pesticide residue analysis in edible oil due to their high selectivity, sensitivity and throughput. The development of multiresidue methods for the determination of pesticides in edible oil samples at low levels is yet a challenging issue to which much effort in separation of lipid material from extracts has been applied. An exhaustive clean-up of the sample extract is necessary in order to avoid high amount of fat residues in the final extract, which would decrease the column lifetime and the maintenance of the instrument in working conditions. Since lipids deposits on the source, the analyte sensitivity is highly reduced too due to ion suppression. Difficulty it is focusing on remove interfering lipids without losing certain analytes considering that many of the target pesticides are fat-soluble non-polar compounds (e.g., organochlorine, pyrethroids) and they tend to remain in the fat. Liquid partitioning between the oil matrix dissolved in petroleum ether or *n*-hexane saturated with acetonitrile was one of the most reported methods for the isolation of pesticides in edible oils [10,14,16–18]. In these procedures pesticides are partitioned into the polar acetonitrile layer while the lipids are removed in the non-polar petroleum ether or *n*-hexane phase. Usually, liquid partitioning has been used combined with GPC [10,16,17] or SPE purification using florisil cartridges [14,18]. However, these procedures are laborious, time-consuming and require large amounts of potentially hazardous solvents. MSPD [14,18] has often been combined with liquid partitioning overcoming these pitfalls with satisfactory results. Current developments involve the use of extraction methods based on modifications to the QuEChERS procedure [3,4,12,14,15,19] as originally reported by Anastassiades et al. [20]. These methods result in advantages such as low solvent consumption, simplicity, flexible approach and high workflow. They involve initial liquid-liquid partition with acetonitrile and then cleaning up by dispersive-solid-phase extraction (d-SPE) in which the extract is mixed with different sorbents combination (PSA + C18 + GCB) and anhydrous MgSO₄. Additionally, a freezing out step prior to d-SPE has been used for further clean-up of the edible oil extract. Anagnostopoulos et al. [3] proposed a method for 102 pesticides in olive oil and olives by gas and liquid chromatography coupled to tandem mass spectrometry (GC–MS/MS and LC–MS/MS) using this simple combination. Although all analytical parameters evaluated were excellent, the main drawback of this method was the significant matrix effect for most compounds.

Until now, apart from GPC, no sample preparation has been able to eliminate matrix effect which is caused by co-eluting compounds influencing ionization and, thus, signal intensity [3,14,15,18,19]. In this sense, reported multiresidue methods for pesticides in edible oils by gas and liquid chromatography/mass spectrometry show a significant or strong matrix effect for most compounds which hampers sensitivity. Recently, the use of zirconia sorbent materials (Z-Sep, Z-Sep* and Yttria-stabilized zirconium dioxide nanoparticles) for removal of lipids from fatty samples improved matrix clean-up compared to PSA, C18 and GCB sorbents [21–24], but also resulted in more analyte loss, especially for hydroxyl and carboxylic acid-containing compounds. Preliminary results with the novel sorbent material Agilent Bond Elut Enhanced Matrix Removal-Lipid (EMR-Lipid) are promising for highly selective lipid removal without unwanted analyte retention [25–29]. Application studies involving QuEChERS extraction followed by EMR-Lipid dSPE and polish salts indicate that this new product delivers fast, effective and robust sample preparation with the most complete matrix removal available for multiresidue analysis of pesticides in avocado by GC–MS/MS [27] and LC–MS/MS [28]. The performance of EMR-Lipid has also been tested for other representative high lipid content samples including bovine liver [25] and salmon [29].

Effective clean-up of EMR-Lipid and better precision results were obtained compared to alternative QuEChERS procedures.

The objective of this study was the evaluation and development of a sensitive, reliable and robust multiresidue analytical method, based on QuEChERS methodology followed by GC–MS/MS for the simultaneous analysis of an extended list of 213 pesticides in edible oils. Several clean-up methods were evaluated concentrating on efficient clean-up and the highest number of pesticides satisfying the recovery and precision criteria. The tested methods were: modified QuEChERS using d-SPE with EMR-Lipid (a), PSA (b), Z-Sep (c) as well as modified QuEChERS using SPE with Z-Sep (d) and EMR-Lipid (e). A simple and rapid freeze-out step with dry ice (CO₂ at –76 °C) for a previous removal of lipids were done before the d-SPE or the SPE clean-up.

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2. Experimental

2.1. Reagents and materials

All pesticide standards of high purity were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Riedel-de Haën (Selze, Germany) and were stored at –30 °C. Stock standard solutions of each pesticide were prepared in acetonitrile and ethyl acetate at concentrations of 1000–2000 mg L^{–1} and were stored in amber screw-capped glass vials in the dark at –20 °C. Individual standard solutions for optimisation and three standard-mix solutions for calibration were prepared from the stock standards.

Ultra-gradient HPLC-grade acetonitrile was obtained from Sigma-Aldrich (Steinheim, Germany). Trisodium citrate dihydrate was purchased from Fluka (Steinheim, Germany). Sodium chloride was purchased from J.T. Baker (Deventer, The Netherlands). Disodium hydrogencitrate sesquihydrate was obtained from Sigma-Aldrich (Steinheim, Germany). Anhydrous magnesium sulphate was supplied by Panreac (Barcelona, Spain). EMR-Lipid was purchased from Agilent Technologies (Santa Clara, CA, USA). PSA and Z-Sep were obtained from Supelco (Bellefonte, PA). A Milli-Q-Plus ultra-pure water system from Milli-pore (Milford, MA, USA) was used throughout the study to obtain the ultra-pure grade water used during the analyses. Formic acid (98% purity) was purchased from Fluka (Buchs, Switzerland). Dry ice was supplied from technical services (University of Almería).

2.2. Equipment

For GC analysis, an Agilent 7000GC (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 7693B autosampler, 7890A GC system, and an Agilent 7000 series GC–MS/MS triple quadrupole system (Agilent Technologies) were used. Data acquisition and processing were developed using Agilent MassHunter QQQ Quantitative Analysis B.05.00 software. Analyses on GC–MS/MS were performed on an Agilent Ultra Inert GC column HP-5MS UI (15 m long × 0.25 mm i.d. × 0.25 μm film thickness). The samples were injected using a multimode injector inlet in cold splitless mode through an ultra-inert inlet liner with a glass wool frit from Agilent; the injection volume was 2 μL. The injector temperature was kept at 80 °C during the solvent evaporation stage (0.1 min) and then ramped up to 300 °C at 600 °C min^{–1}. This temperature was maintained for 20 min. Helium (99.999% purity) was used as the carrier and quenching gas, and nitrogen (99.999% purity) as the collision gas. The oven temperature program was as follows: 70 °C for 1 min, up to 150 °C at 50 °C min^{–1}, then up to 200 °C at 6 °C min^{–1} and finally up to 280 °C at 16 °C min^{–1}, and then maintained for 4.07 min. The total run time was 20 min with 3 additional minutes for backflushing at 280 °C; the pressure was maintained at 60 psi. The system worked at constant pressure (14.1 psi) with

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the setting of the retention time lock employing trifluralin as the locking compound at 5.82 min. Both the transfer line and the ion source were kept at 280 °C. The ion source and quadrupole analyser temperatures were fixed at 280 °C and 150 °C, respectively. The collision gas flow was 1.5 mL min⁻¹ and the quenching gas flow was 2.25 mL min⁻¹.

For the prior screening, an Agilent 7890A gas chromatograph was used. The samples were injected using a multimode injector inlet in a splitless mode with an ultra-inert inlet liner, with a glass wool frit obtained from Agilent. The injection volume was 2 µL and was carried out at 280 °C. Helium (99.999% purity) was used as the carrier gas. The GC separation was performed using two fused silica HP-5MS UI capillary column of 15 m × 0.25 mm inner diameter and a film thickness of 0.25 µm (from Agilent) connected by a capillary flow technology (CFT) union. The oven temperature was programmed as follows: 60 °C for 1 min; 40 °C min⁻¹ to 120 °C, and finally up to 310 °C at 5 °C min⁻¹. The total run time was 40.5 min with two additional minutes for backflushing at 310 °C. The gas chromatography system was connected to a quadrupole time-of-flight (QTOF) mass spectrometer Agilent 7200 (Agilent Technologies, Santa Clara, USA), operating in electron-impact ionization (EI) mode (70 eV). The ion source and quadrupole analyser temperatures were set at 280 and 150 °C, respectively. TOF-MS was operated at 4 GHz (12 000 FWHM), with acquisition over the mass range of *m/z* 45–550.

2.3. Optimization of GC–MS/MS parameters

For the optimisation of the MS parameters, all pesticides were monitored in full scan mode in the 50–550 *m/z* range, selecting the precursor ion (PI). In the PI experiment, the PI was fragmented and the best product ions were selected. Finally, the CE was optimized for each transition in an SRM experiment. For the identification of analytes, the EU guidelines for GC–MS/MS analysis were considered (Document N° SANTE/11945/2015) [30]. The transitions obtained and collision energies chosen are shown in Table S1 (in the Supplementary material section). The most intense transition was selected as the quantifier transition and the second most intense as the qualifier transition. A thirty-nine time-segment method was created to obtain adequate sensitivity and S/N ratio. The solvent delay was 2 min.

2.4. Spiking procedure

For recovery studies, the organic extra virgin olive, sunflower and soybean oil samples obtained from a green store from Almería (South of Spain) were spiked with the standard solution at the appropriate levels. A prior analysis of the samples was performed in order to ensure that they did not contain any of the target compounds, and that samples were selected as blank for spiking, calibration curves and recovery studies. The spiked sample was stirred for 60 min to let the ethyl acetate evaporate before sample extraction and d-SPE clean-up. The final spiking concentration levels in the recovery study samples were 10 and 20 µg kg⁻¹.

2.5. Sample preparation procedure

For the extraction, 15 g of sample was weighed in a 50 mL PTFE centrifuge tube. Then, 15 mL of acetonitrile and 15 µL of a mix of surrogate standards with 10 mg L⁻¹ – triphenyl phosphate (TPP), dichlorvos-d6, malathion-d10 and carbendazim-d3- were added and the samples were shaken in an automatic axial extractor (AGYTAX®, Cirta Lab. S.L., Spain) for 4 min. Afterwards, 4 g of magnesium sulphate, 1 g of sodium chloride, 1 g of trisodium citrate dehydrate and 0.5 g of disodium hydrogencitrate sesquihydrate were added and the samples were again shaken in the auto-

matic axial extractor for 4 min. The extract was then centrifuged (2860 × *g*) for 5 min. For the freezing-out clean-up, a 10 mL aliquot of the supernatant was transferred to a 15 mL PTFE centrifuge tube and placed in a polystyrene box filled with dry ice (CO₂ at –76 °C) for 5 min. 5 mL of the extract were then separated from the precipitate using a Pasteur pipette and these were used for the following clean-up methodologies: (a) D-SPE with EMR-Lipid. 5 mL of water had been added to the EMR-Lipid d-SPE tube prior to addition of the freezing-out extract. Subsequently, the mixture was vigorously shaken by vortex for 60 s to disperse sample and then centrifuged (2860 × *g*) for 5 min. After that, a 5 mL aliquot of the above extract was transferred to a 15 mL EMR-Lipid polish tube containing 2 g salts (1:4, NaCl:MgSO₄). The contents in the tube were vortexed for 60 s and centrifuged at 2860 × *g* for 5 min; (b) D-SPE with PSA (125 mg) + MgSO₄ (750 mg). The contents in the tube were vortexed for 60 s and centrifuged at 2860 × *g* for 5 min; (c) D-SPE with Z-Sep (125 mg) + MgSO₄ (750 mg). The contents in the tube were vortexed for 60 s and centrifuged at 2860 × *g* for 5 min; (d) SPE with Z-Sep (45 mg). 1 mL aliquot of the extract was percolated through a SPE cartridge containing 45 mg of Z-Sep at a flow rate of 0.8 mL min⁻¹ in the extraction manifold system at a pressure of 1.09 psig for the vacuum system (the SPE cartridge had been preconditioned prior to percolation with 1 mL of acetonitrile). Finally, a 2 mL aliquot (1 mL for approach (d)) was transferred into a glass test tube and acidified by adding 20 µL of 5% formic acid in acetonitrile (v/v) to stabilize base-sensitive pesticides. In (b)–(d) strategies, 10 g of sample and 10 mL of acetonitrile were used for the extraction. In these cases, only 2.5 mL of the extract were obtained after using a 5 mL aliquot of acetonitrile extract in the freezing-out step. Prior to injection into the GC–MS/MS system, a 50 µL aliquot was evaporated under a gentle nitrogen stream and reconstituted with 50 µL ethyl acetate and 2 µL lindane-d6 (1.25 mg L⁻¹), which were added to each vial as the injection control standard. With this treatment, 1 mL of sample extract represents 1 g of sample. The detailed workflow for the best procedure (approach a) is shown in Fig. 1.

2.6. Method validation

A validation study was performed in terms of apparent recovery, linearity, limit of quantitation, matrix effects, as well as intra-day and inter-day precision.

The recoveries and precision of the extraction method were determined as the average of five spiked matrix blanks analyzed at concentration levels of 10 and 20 µg kg⁻¹.

The LOQ was set as the lowest spiking level that can be quantified with acceptable accuracy and precision, as described in Document No. SANTE/11945/2015 [30].

In order to demonstrate linearity, five sets of calibration curves with six concentration points (from 10 to 500 µg kg⁻¹ in the sample) were prepared by spiking the analytes before the extraction procedure to obtain a more realistic concentration in the final extracts. Procedural Standard Calibration [30] was applied to compensate analyte losses during sample preparation and variability in the matrix effects.

The repeatability of the method was estimated by determining the inter- and intra-day relative standard deviation (RSD, %) by the repeated analysis (*n* = 5) of a spiked oil sample at the 10 and 20 µg kg⁻¹ levels, from run-to-run over 1 and 5 days, respectively.

2.7. Studied pesticides

The pesticides included in this study were selected based on their relevance in terms of high probability of being found in edible oil samples. Most of the studied compounds are low- and medium-polarity pesticides (*pK_{ow}* > 3) which facilitates the contamination of edible oil matrices. Various studies revealed the presence of

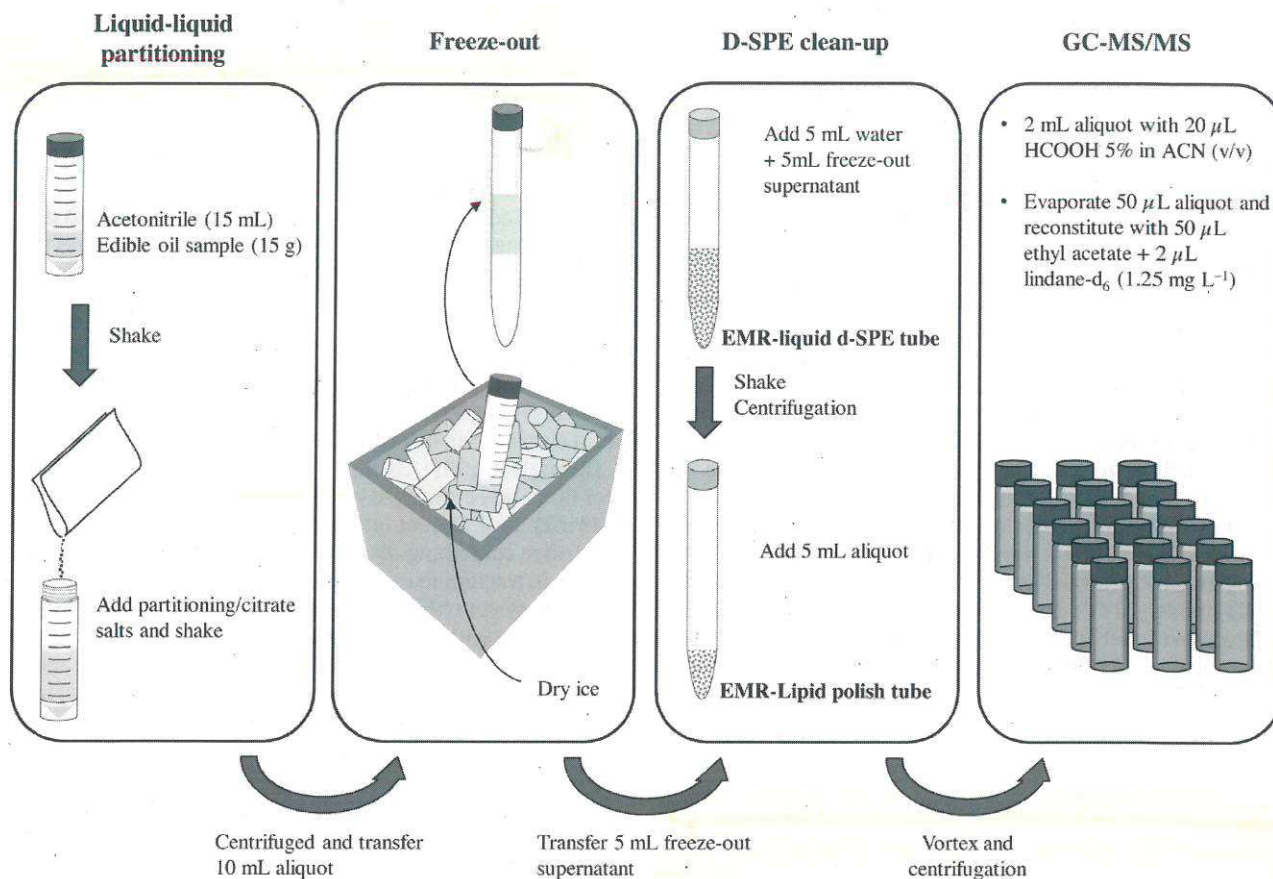


Fig. 1. Schematic diagram of the proposed sample preparation strategy based on QuEChERS method (approach a).

many of these pesticides in edible vegetable oils [1–7] at levels, in certain cases, above MRLs [1].

3. Result and discussion

3.1. Method selection

The new clean-up sorbent, Agilent Bond Elut EMR-Lipid, was applied in soybean, sunflower and extra virgin olive oil extracts and efficiency of the dispersive-solid-phase extraction (d-SPE) clean-up step as well as pesticides extraction effectiveness were evaluated by comparison with other sorbents (i.e. PSA and Z-Sep) previously used for d-SPE or SPE of this matrix [2,3,9,11,12,15,22].

The extraction/clean-up procedures were based on the QuEChERS methodology followed by analysis with a GC-EI-TOF-MS and GC-MS/MS. The extraction step involved liquid-liquid extraction of 10 g edible oil with 10 mL of acetonitrile followed by salting-out with citrate buffer, magnesium sulphate and sodium chloride. The following step was to discard in the extract an amount of matrix constituents with limited solubility in acetonitrile by precipitation at low-temperature (freezing-out clean-up). Hereafter, (a)–(d) clean-up strategies were checked.

For the freezing-out clean-up with dry ice (CO_2 at -76°C), an aliquot of the acetonitrile phase (5 mL) was transferred into a centrifuge tube and placed in a polystyrene box filled with 1.5 kg of dry ice. The freezing time study revealed that a homogenous fat precipitate in the bottom of the tube was achieved within 5 min and longer freezing times resulted in the freezing of the whole extract. Then, an aliquot of the supernatant solution (2.5 mL) was transferred into a centrifugation tube for further clean-up by d-SPE or SPE. In the case of d-SPE with EMR-Lipid (a), it was necessary to

increase the amount of edible oil sample and acetonitrile to 15 g and 15 mL, respectively, because manufacturer recommends a volume of 5 mL for the EMR-Lipid dSPE tube which could be obtained after using a 10 mL aliquot of acetonitrile extract in the freezing-out step. Highly lipophilic compounds in edible oils such as triglycerides, diglycerides, monoglycerides and free fatty acids are expected to be removed to a large extent by freezing-out. The effectiveness of freezing-out clean-up as an additional step to the d-SPE can be assessed by evaluating the amounts of matrix compounds in the final extract using a GC-EI-TOF-MS working in full-scan mode. As can be seen in Fig. 2, the Total Ion Chromatograms (TICs) obtained for the same extra virgin olive oil sample showed that freezing-out plus d-SPE with EMR-Lipid ensured better clean-up (61% cleaner) than d-SPE (EMR-Lipid) only, especially in the case of compounds eluting from 23 to 40 min due to the presence of highly non-polar compounds in this part of the chromatogram.

Regarding the evaluation of clean-up sorbents, our main criterion was to find one that gives an efficient clean-up of the oil extract with the major number of pesticides having recoveries in the 70–120% range as well as giving the lowest average RSD values. SPE with Z-Sep (d) and d-SPE with EMR-Lipid (a) were the most effective clean-up procedures (Fig. 3), obtaining 43% and 34% reduction, respectively, in the amounts of matrix compounds in the final extract compared to d-SPE with Z-Sep (c). The advantage of these two procedures over d-SPE with Z-Sep (c) and d-SPE with PSA (b) is clearly visible from 34 to 40 min (Fig. 3B). Although Agilent Technologies recommends EMR-Lipid sorbent for d-SPE procedures (a), we tried SPE with 45 mg EMR-Lipid and, in this case, it was technically impossible to pass the acetonitrile phase (5 mL) through the cartridge even when the sorbent was previously hydrated with 5 mL of ultrapure water. Since no available information from Agi-

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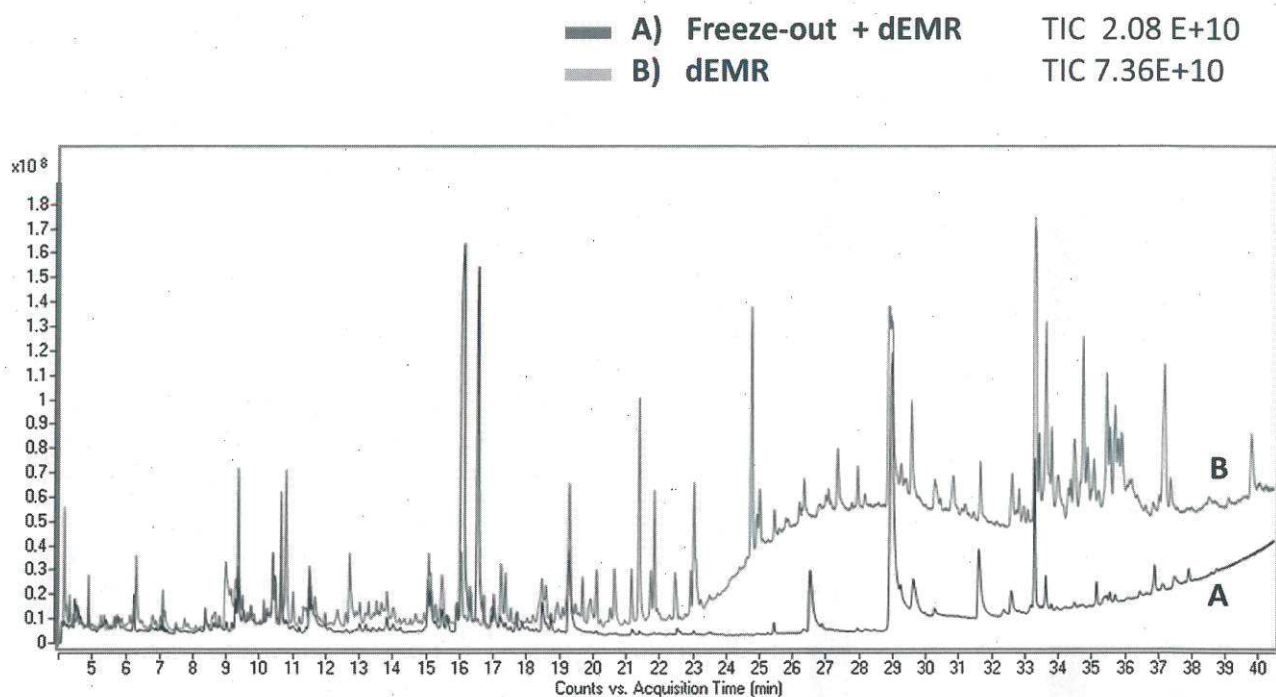


Fig. 2. GC-El-TOF-MS full-scan chromatograms of blank olive oil extracts obtained using QuEChERS methodology with freezing-out followed by d-SPE (EMR-Lipid) (A) and d-SPE (EMR-Lipid) without freezing-out (B).

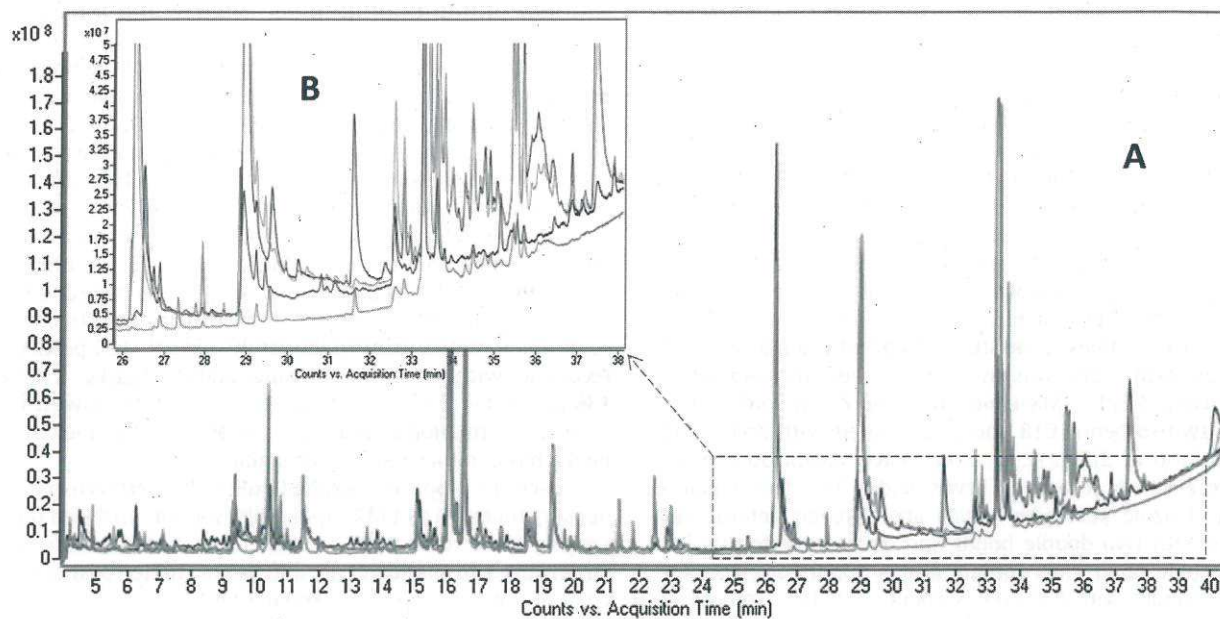


Fig. 3. GC-El-TOF-MS full-scan chromatograms of blank olive oil extract obtained using QuEChERS methodology with freezing-out followed different d-SPE or SPE clean-up procedures (a, b, c and d).

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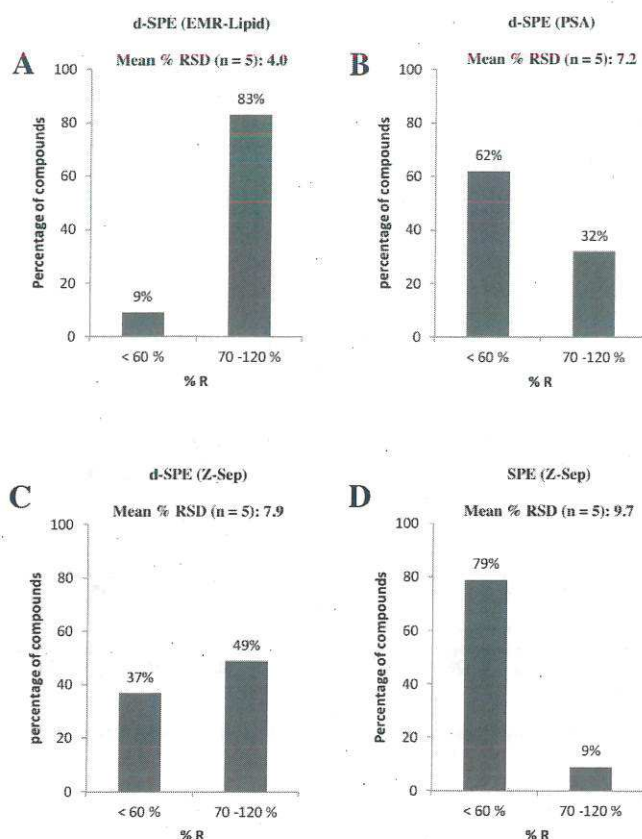


Fig. 4. Percentage of the total number of evaluated pesticides with recoveries from different ranges and mean % RSD ($n=5$) in olive oil samples. Modified QuEChERS with freezing-out followed by d-SPE (EMR-Lipid) (A), d-SPE (PSA) (B), d-SPE (Z-Sep) (C) and SPE (Z-Sep) (D).

lent about characteristics of this new material, we cannot conclude the reason for the difficulty to be used as conventional SPE.

Recovery and precision studies carried out at a concentration level of $50 \mu\text{g kg}^{-1}$ gave considerably better results for extracts cleaned with d-SPE (EMR-Lipid) than in the case of cleaning by SPE with Z-Sep (see Fig. 4). Out of the 213 pesticides, QuEChERS with d-SPE (EMR-Lipid) ensured recoveries in the 70–120% range and mean RSD equal to 4.0% for 177 pesticides whereas extracts cleaned with SPE (Z-Sep) resulted in 19 pesticides and a mean RSD equal to 9.7% (see Fig. 4A and D). A similar trend can be also observed in the case of d-SPE (Z-Sep), although extraction efficiency was better than that exhibited by the SPE (Z-Sep) alternative method tested.

It was noticed generally that azole pesticides (flusilazole, myclobutanil, penconazole, propiconazole, tetraconazole, fenbuconazole, epoxiconazole, cyproconazole, diclobutrazole, tebuconazole, paclobutrazol, metconazole, hexaconazole and flutriafol) did not show recovery values at the studied concentration level when SPE clean-up with Z-Sep was used, while they are completely recovered using d-SPE (EMR-Lipid) protocol. Z-Sep sorbent is a mixture of two sorbents, C18 and silica coated with ZrO_2 , with a $\text{ZrO}_2/\text{C18}$ ratio of 2/5 [21]. Zr atoms have vacant 3d orbitals so they are electron acceptors (Lewis acids). This fact explains adsorption of azole pesticides which are nitrogen heterocyclic compounds with two double bonds and one heteroatom is not part of a double bond, thus contain atoms donors of electrons that could interact with the ZrO_2 sorbent [21]. The strength of the interaction between the azole pesticides and this sorbent can be increased for cyproconazole, diclobutrazole, tebuconazole, paclobutrazol, metconazole, hexaconazole and flutriafol due to the presence of a hydroxyl functional group in their molecules. Ana-

lytes with hydroxyl (fenhexamid, nuarimol, orto-phenylphenol and fenarimol), hydroxyacetate (chlorobenzilate and bromopropylate), phosphate (paraoxon-methyl and dichlorvos), phosphorodithioate (disulfoton and disulfoton-sulfoxide) and methoxy (metalaxyl, terbumeton, prometon, pyrifenoxy and sebumeton) substituents could not be recovered, possibly due to their capacity to interact strongly with the ZrO_2 sorbent according to Lewis theory [21]. The same occurred for other pesticides also have Lewis base sites in their structure such as piperidine (fenpropidin), morpholine (fenpropimorph) and diamine (ametryn) groups. A similar behavior based on the electrostatic interaction between the above electron donors functional groups and the amphoteric zirconium dioxide was found by Tuzimski et al. [2] for the d-SPE clean-up with Z-Sep developed for pesticide analysis in edible oils. For other pesticides having planar structures with chloride atoms, such as chlorothalonil, a very low recovery was also obtained. Table S2 in the Supplementary data presents the recoveries and RSD values obtained in extra virgin oil with the tested clean-up procedures (experiments (a)–(d)).

Based on the results of our experiments, it was found that QuEChERS with d-SPE (EMR-Lipid) would ensure efficient and robust clean-up while maintaining quantitative recovery for most of the target pesticides (83% of pesticides). In addition, compared with SPE procedure, other advantages of d-SPE are its simplicity, lesser disposable materials and volume of organic solvent consumed and, the large number of samples that can be processed per hour. For this reason, validation experiments were carried out with d-SPE (EMR-Lipid) procedure.

3.2. Method validation

3.2.1. Recovery studies

For recovery studies, two fortification levels were selected: $10 \mu\text{g kg}^{-1}$ and $20 \mu\text{g kg}^{-1}$. All recovery experiments were performed five times at each level, as suggested by SANTE [30]. In olive oil (extra virgin quality), at the $20 \mu\text{g kg}^{-1}$ level, 171 pesticides had recoveries in the 70–120% range; 23 had recoveries below 60% and the recovery of 3 analyte (fenitrothion, fipronil, mevinphos) was above 120% (Table 1). At the $10 \mu\text{g kg}^{-1}$ level, 162 pesticides had recoveries in the 70–120% range, 23 were extracted with low recoveries or were not detected and, likewise at this level, mevinphos had an recovery higher than 120%. Low recoveries for ethoxyquin were obtained as a result of their degradation [23]. In the case of quinoxifen, 4,4-DDE, endosulfan a, endosulfan b, heptachlor, dieldrin, quintozene, 4,4-DDD, 2,4-DDE, 2,4-DDT and 4,4-DDT recoveries within the 40–60% range were obtained at both concentration levels. Different authors have also reported poor recoveries when several extraction and clean-up methods were used in high fat content matrices for these pesticides without an unambiguous reason of this fact [21,31,32]. With respect to the sunflower oil matrix, a similar situation occurred when compared with the extra virgin olive oil matrix. Results obtained in soybean oil matrix showed that at $20 \mu\text{g kg}^{-1}$, 160 pesticides had recoveries within the 70–120% range and at $10 \mu\text{g kg}^{-1}$, there were 136 pesticides (Table 1). The pesticides with the lowest recoveries were aldrin, flonicamid, HCB, paclobutrazol, paraoxon methyl, pentachloroaniline and 3-chloroaniline.

Hence, the proposed modified QuEChERS method using a freeze-out step followed by EMR-Lipid d-SPE sorbent provided recoveries ranging from 70 to 120% for 76% of studied pesticides in extra virgin olive and sunflower oil; and 64% of studied pesticides in soybean oil (at the $10 \mu\text{g kg}^{-1}$ spiking level). At this point we have to consider the large scope of pesticides presented and therefore, the difficulties to get good recoveries for all compounds by Matrix Matched Standard Calibration. To compensate this loss of analyte, we decided to use Procedural Standard Calibration providing the

Table 1
Recoveries and relative standard deviation of the proposed extraction/clean-up method in the four matrices, consisting of Freeze-out followed by d-SPE with EMR-Lipid.

Compound	Recovery (% RSD) Olive oil 1 ^a		Olive oil 2 ^b		Sunflower oil		Soybean oil	
	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹
2,4-DDE	49 (3)	42 (3)	45 (4)	44 (9)	56 (15)	40 (7)	28 (5)	18 (5)
2,4-DDT+4,4-DDD	46 (6)	51 (2)	43 (7)	50 (4)	35 (9)	43 (5)	34 (8)	25 (6)
3,5-Dichloroaniline	70 (5)	71 (3)	67 (3)	73 (3)	88 (3)	79 (2)	65 (5)	69 (5)
3-Chloroaniline	54 (20)	52 (19)	55 (5)	52 (5)	64 (5)	67 (4)	31 (5)	47 (6)
4,4-DDE	44 (14)	45 (7)	41 (5)	42 (6)	56 (7)	57 (3)	59 (9)	44 (9)
4,4-DDT	49 (8)	42 (2)	54 (8)	40 (5)	56 (5)	61 (5)	58 (7)	52 (7)
Acrinathrin	98 (5)	108 (3)	92 (1)	99 (4)	86 (5)	89 (6)	79 (6)	103 (15)
Alachlor	87 (6)	93 (3)	85 (6)	78 (5)	90 (4)	75 (4)	73 (4)	71 (8)
Aldrin	28 (10)	34 (11)	33 (3)	31 (6)	30 (5)	43 (6)	7 (35)	11 (26)
Ametryn	75 (4)	65 (4)	72 (5)	69 (4)	75 (7)	71 (6)	67 (8)	69 (6)
Anthraquinone	62 (3)	62 (2)	74 (6)	77 (5)	86 (4)	94 (7)	70 (7)	68 (8)
Atrazine	76 (7)	78 (4)	88 (7)	76 (4)	93 (6)	66 (6)	74 (3)	69 (6)
Azoxystrobin	118 (3)	118 (3)	117 (8)	105 (3)	94 (5)	98 (5)	97 (4)	119 (5)
Benalaxyl	104 (4)	107 (2)	97 (9)	100 (4)	87 (6)	103 (5)	104 (6)	87 (4)
Bifenox	74 (5)	81 (5)	76 (6)	84 (3)	89 (7)	75 (6)	89 (4)	85 (5)
Bifenthrin	65 (4)	68 (3)	65 (5)	61 (2)	73 (5)	66 (9)	67 (7)	65 (4)
Biphenyl	67 (6)	70 (6)	64 (6)	72 (6)	74 (7)	79 (4)	31 (26)	14 (23)
Bixafen	110 (2)	107 (2)	101 (5)	98 (4)	95 (3)	102 (6)	60 (18)	119 (6)
Boscalid	86 (3)	89 (2)	93 (5)	90 (7)	92 (6)	89 (5)	92 (6)	88 (7)
Bromopropylate	72 (3)	73 (3)	76 (6)	75 (5)	66 (9)	64 (5)	82 (5)	93 (8)
Bupirimate	94 (6)	97 (6)	98 (3)	99 (5)	85 (5)	101 (6)	78 (7)	78 (8)
Buprofezin	92 (2)	99 (2)	84 (5)	76 (7)	96 (5)	87 (2)	92 (2)	90 (5)
Butralin	75 (6)	78 (5)	77 (5)	91 (9)	103 (6)	87 (9)	47 (5)	57 (5)
Butylate	61 (4)	67 (4)	74 (2)	83 (1)	77 (4)	79 (7)	77 (9)	60 (5)
Cadusafos	74 (3)	71 (3)	64 (6)	70 (5)	88 (5)	77 (3)	66 (6)	68 (5)
Captan	71 (7)	72 (6)	70 (4)	75 (7)	84 (6)	73 (7)	–	63 (17)
Carbofuran	82 (3)	94 (2)	105 (5)	92 (3)	78 (5)	90 (4)	105 (5)	95 (6)
Carbophenothion	63 (3)	66 (3)	75 (2)	73 (4)	83 (7)	95 (5)	73 (15)	76 (5)
Carbosulfan	73 (3)	71 (3)	73 (4)	72 (4)	85 (5)	102 (5)	–	66 (27)
Chinomethionat	24 (3)	24 (2)	47 (4)	56 (15)	34 (15)	35 (9)	16 (6)	18 (4)
Chlorbromuron	76 (4)	77 (4)	71 (6)	77 (4)	84 (5)	81 (4)	67 (5)	73 (2)
Chlordane	61 (10)	62 (10)	64 (8)	63 (5)	64 (6)	65 (5)	–	55 (9)
Chlorfenapyr	85 (3)	90 (3)	80 (5)	89 (5)	100 (7)	85 (1)	60 (18)	71 (6)
Chlorfenvinphos	95 (5)	100 (3)	97 (3)	91 (4)	97 (5)	101 (4)	84 (4)	94 (8)
Chlorobenzilate	72 (2)	74 (2)	86 (5)	85 (3)	77 (6)	93 (5)	69 (6)	77 (7)
Chlorothalonil	77 (2)	78 (1)	79 (2)	81 (4)	90 (7)	82 (4)	82 (4)	84 (8)
Chlorpropham	67 (4)	73 (2)	74 (4)	79 (7)	81 (6)	67 (3)	63 (20)	66 (4)
Chlorpyrifos	74 (5)	72 (3)	88 (1)	79 (1)	77 (7)	70 (6)	84 (6)	88 (8)
Chlorpyrifos Methyl	74 (2)	71 (2)	74 (4)	81 (5)	65 (4)	74 (3)	62 (7)	73 (9)
Chlorthal-Dimethyl	63 (3)	73 (2)	80 (6)	81 (5)	74 (5)	90 (2)	74 (4)	68 (6)
Chlozolinate	99 (1)	109 (1)	102 (3)	111 (4)	94 (6)	96 (4)	87 (8)	102 (6)
Coumaphos	95 (2)	99 (1)	97 (5)	104 (6)	87 (5)	102 (5)	69 (18)	117 (28)
Cyfluthrin	76 (5)	80 (2)	72 (4)	75 (3)	78 (15)	102 (6)	72 (5)	78 (6)
Cypermethrin	87 (3)	87 (2)	83 (5)	75 (7)	101 (7)	91 (5)	75 (6)	75 (4)
Cyproconazole	79 (3)	75 (4)	74 (9)	68 (5)	75 (5)	76 (7)	60 (9)	79 (6)
Cyprodinil	39 (3)	43 (7)	28 (5)	40 (19)	53 (9)	47 (19)	42 (5)	36 (9)
DMST	116 (3)	119 (5)	91 (8)	85 (4)	89 (7)	76 (4)	73 (31)	105 (9)
Deltamethrin	69 (4)	76 (3)	64 (9)	79 (6)	71 (6)	87 (7)	62 (6)	86 (5)
Desmethyl-pirimicarb	–	–	45 (5)	43 (10)	25 (5)	54 (7)	48 (19)	52 (5)
Diazinon	78 (4)	80 (3)	71 (1)	82 (6)	94 (5)	102 (4)	77 (4)	73 (3)
Dichlofluanid	87 (5)	102 (3)	93 (2)	103 (6)	98 (10)	103 (5)	81 (4)	88 (6)
Dichlorvos	88 (3)	98 (4)	89 (5)	98 (4)	85 (5)	81 (4)	65 (4)	84 (8)
Diclobutrazole	71 (3)	73 (3)	97 (1)	82 (4)	86 (7)	92 (5)	74 (6)	83 (4)
Dicloran	61 (4)	71 (3)	84 (3)	82 (6)	89 (6)	100 (4)	62 (5)	76 (9)
Dicofol	33 (3)	40 (3)	21 (5)	24 (6)	38 (5)	36 (5)	30 (5)	31 (9)
Dieldrin	47 (6)	43 (4)	44 (4)	40 (23)	55 (4)	50 (7)	27 (6)	24 (8)
Diethofencarb	75 (6)	70 (3)	72 (7)	65 (5)	84 (3)	79 (4)	–	98 (5)
Dimethenamid	82 (4)	91 (1)	74 (3)	73 (5)	88 (4)	78 (2)	75 (5)	76 (4)
Dimethipin	114 (2)	119 (3)	106 (6)	84 (2)	104 (6)	103 (7)	112 (8)	104 (3)
Diphenylamine	69 (3)	79 (4)	95 (3)	74 (4)	87 (5)	95 (1)	81 (5)	88 (6)
Disulfoton	74 (5)	71 (3)	86 (8)	73 (9)	83 (5)	88 (3)	77 (6)	74 (2)
Disulfonton-sulfoxide	113 (3)	119 (3)	106 (7)	106 (6)	98 (5)	90 (4)	105 (3)	99 (4)
Dodemorph	69 (7)	71 (5)	66 (3)	70 (6)	75 (6)	67 (2)	83 (7)	76 (1)
EPN	78 (12)	86 (7)	75 (7)	80 (7)	88 (7)	95 (4)	78 (5)	76 (6)
Endosulfan Alpha	40 (4)	48 (3)	35 (6)	46 (5)	29 (9)	54 (5)	61 (6)	59 (6)
Endosulfan Beta	40 (5)	46 (3)	35 (5)	39 (8)	49 (5)	58 (15)	49 (9)	38 (4)
Endosulfan Sulfate	81 (5)	88 (5)	81 (4)	85 (4)	98 (4)	73 (5)	75 (5)	72 (9)
Endrin	41 (3)	40 (2)	37 (5)	53 (22)	45 (9)	57 (4)	32 (5)	27 (6)
Epoxiconazole	95 (8)	98 (6)	90 (5)	89 (3)	104 (7)	97 (8)	78 (8)	92 (5)
Ethion	84 (3)	93 (4)	80 (9)	92 (6)	86 (7)	96 (7)	73 (4)	71 (3)
Ethofumesate	115 (4)	114 (1)	119 (5)	103 (7)	96 (6)	75 (7)	104 (2)	98 (5)
Ethoprophos	79 (3)	85 (2)	87 (4)	85 (5)	90 (4)	81 (6)	67 (19)	70 (6)
Ethoxyquin	–	24 (9)	–	18 (24)	–	–	–	4 (15)

the VDA

Table 1 (Continued)

Compound	Recovery (% RSD) Olive oil 1 ^a		Olive oil 2 ^b		Sunflower oil		Soybean oil	
	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹
Etofenprox	57 (2)	53 (2)	46 (9)	50 (5)	57 (5)	64 (4)	33 (5)	35 (4)
Etrimfos	77 (2)	81 (3)	86 (4)	85 (7)	89 (5)	92 (3)	78 (4)	72 (8)
Fenamidone	91 (3)	102 (1)	79 (5)	94 (4)	94 (4)	101 (1)	82 (6)	93 (1)
Fenarimol	72 (2)	65 (1)	70 (6)	61 (6)	87 (5)	79 (4)	64 (7)	75 (3)
Fenazaquin	67 (3)	68 (2)	73 (4)	87 (5)	76 (2)	70 (5)	70 (6)	84 (4)
Fenbuconazole	90 (3)	94 (2)	95 (4)	97 (5)	96 (7)	88 (5)	82 (6)	91 (5)
Fenchlorphos	65 (3)	60 (4)	93 (5)	95 (3)	76 (4)	90 (8)	76 (7)	72 (6)
Fenhexamid	74 (3)	78 (1)	76 (4)	79 (5)	82 (5)	76 (6)	91 (5)	89 (6)
Fenitrothion	103 (3)	122 (5)	99 (3)	87 (6)	101 (5)	93 (7)	74 (5)	87 (8)
Fenpropathrin	79 (5)	77 (5)	76 (15)	71 (5)	84 (6)	81 (7)	66 (8)	70 (6)
Fenpropidin	91 (4)	94 (2)	90 (4)	92 (1)	83 (5)	92 (1)	95 (6)	96 (6)
Fenpropimorph	78 (2)	76 (4)	74 (3)	75 (5)	69 (2)	74 (1)	96 (9)	95 (7)
Fention	73 (7)	79 (3)	78 (2)	73 (8)	89 (5)	73 (3)	72 (5)	74 (3)
Fenvalerate/Esfenvalerate RR/SS	65 (11)	71 (4)	61 (5)	65 (9)	79 (7)	69 (5)	59 (5)	75 (15)
Fenvalerate/Esfenvalerate RS/SR	72 (5)	73 (2)	70 (7)	73 (8)	99 (4)	76 (7)	65 (6)	77 (3)
Fipronil	119 (7)	122 (5)	102 (6)	114 (4)	93 (6)	90 (8)	112 (6)	112 (4)
Fipronil Desulfinil	109 (3)	116 (2)	115 (6)	102 (5)	95 (5)	97 (5)	110 (8)	109 (4)
Fipronil Sulfone	116 (5)	119 (4)	98 (1)	97 (7)	103 (7)	105 (4)	101 (19)	106 (3)
Flamprop-Isopropyl	101 (3)	102 (2)	113 (5)	101 (6)	97 (6)	96 (5)	93 (4)	92 (6)
Flamprop-Methyl	100 (4)	107 (3)	102 (5)	104 (5)	97 (4)	95 (7)	99 (8)	97 (6)
Flonicamid	96 (3)	85 (3)	93 (4)	84 (5)	111 (9)	96 (3)	–	–
Fluacrypyrim	118 (3)	119 (2)	97 (5)	102 (9)	84 (8)	92 (4)	108 (3)	109 (5)
Fluazifop- <i>p</i> -butyl	100 (4)	106 (6)	103 (4)	99 (1)	98 (4)	102 (2)	83 (5)	82 (4)
Flucythrinate	103 (3)	101 (1)	85 (6)	94 (8)	102 (7)	98 (7)	79 (15)	110 (6)
Fludioxonil	98 (3)	101 (2)	102 (3)	96 (4)	89 (8)	76 (5)	85 (3)	91 (4)
Fluopicolide	96 (2)	100 (3)	95 (5)	103 (4)	85 (7)	77 (4)	90 (2)	92 (2)
Fluopyram	110 (2)	119 (2)	107 (2)	83 (5)	97 (4)	83 (9)	105 (7)	114 (4)
Fluquinconazole	75 (2)	81 (1)	96 (3)	88 (5)	95 (4)	85 (3)	76 (6)	77 (4)
Flusilazol	86 (4)	91 (3)	84 (6)	101 (6)	96 (3)	102 (2)	78 (5)	86 (8)
Flutolanil	108 (3)	111 (3)	100 (4)	95 (5)	102 (5)	97 (5)	100 (4)	107 (4)
Flutriafol	75 (3)	77 (2)	79 (8)	90 (7)	96 (4)	97 (2)	63 (5)	71 (4)
Fluvalinate- τ	73 (2)	78 (2)	98 (4)	76 (3)	89 (3)	85 (6)	63 (4)	89 (4)
Folpet	70 (6)	81 (3)	87 (3)	89 (6)	90 (3)	85 (5)	116 (5)	94 (7)
Fonofos	65 (7)	68 (2)	66 (22)	76 (5)	72 (9)	77 (5)	85 (5)	74 (4)
Formothion	112 (8)	111 (3)	100 (4)	106 (3)	98 (4)	91 (3)	93 (6)	120 (4)
Fosthiazate	110 (4)	119 (1)	107 (3)	95 (3)	86 (7)	106 (5)	82 (4)	119 (5)
HCB	17 (6)	17 (3)	26 (4)	22 (6)	32 (8)	20 (4)	4 (45)	5 (3)
HCH- α	66 (3)	62 (4)	78 (7)	65 (4)	79 (3)	68 (9)	63 (5)	68 (6)
HCH- β	67 (3)	71 (3)	68 (6)	73 (4)	79 (4)	89 (5)	69 (8)	57 (9)
Heptachlor	42 (8)	44 (4)	45 (3)	50 (4)	63 (3)	59 (25)	57 (9)	43 (8)
Heptachlorepoxyde-cis	70 (3)	69 (5)	62 (6)	65 (3)	74 (7)	76 (6)	71 (9)	72 (5)
Heptachlorepoxyde-trans	67 (11)	71 (4)	64 (3)	83 (5)	79 (6)	85 (7)	85 (4)	74 (6)
Heptenophos	109 (5)	115 (3)	90 (5)	109 (5)	89 (8)	97 (7)	88 (6)	88 (5)
Hexaconazole	71 (6)	75 (7)	70 (5)	73 (5)	66 (5)	95 (3)	65 (8)	63 (3)
Indoxacarb	113 (3)	107 (2)	111 (4)	95 (3)	103 (3)	96 (2)	113 (8)	114 (9)
Iprodione	88 (6)	91 (3)	96 (1)	94 (4)	93 (6)	85 (5)	76 (9)	104 (5)
Iprovalicarb	89 (6)	111 (4)	90 (2)	94 (3)	97 (6)	98 (3)	78 (6)	87 (2)
Isazofos	99 (5)	108 (3)	99 (4)	83 (6)	101 (7)	102 (4)	83 (5)	91 (7)
Isocarbophos	91 (5)	98 (5)	94 (1)	72 (5)	83 (1)	89 (2)	88 (9)	117 (3)
Isofenfos Ethyl	90 (4)	95 (3)	94 (5)	96 (3)	98 (4)	113 (3)	81 (7)	88 (4)
Isofenfos Methyl	91 (5)	99 (2)	90 (6)	96 (5)	102 (5)	97 (3)	81 (6)	74 (5)
Isoprothiolane	90 (5)	96 (3)	75 (4)	90 (3)	111 (4)	85 (3)	84 (5)	85 (7)
Isopyrazam	96 (5)	98 (3)	93 (6)	82 (4)	86 (5)	102 (3)	84 (5)	111 (3)
Kresoxim Methyl	97 (8)	114 (4)	99 (4)	103 (7)	96 (3)	87 (3)	95 (7)	97 (9)
Lambda-Cyhalothrin	88 (9)	94 (3)	89 (3)	97 (5)	101 (4)	103 (1)	75 (4)	91 (6)
Lindane	78 (3)	71 (3)	78 (4)	67 (6)	90 (3)	79 (2)	73 (7)	79 (8)
Malaoxon	112 (8)	114 (2)	111 (7)	109 (4)	99 (5)	86 (6)	29 (4)	120 (19)
Malathion	112 (6)	117 (3)	92 (3)	119 (5)	83 (3)	84 (4)	102 (7)	116 (5)
Mecarbam	104 (8)	112 (5)	91 (4)	87 (9)	88 (6)	93 (3)	111 (5)	111 (3)
Mepanpyrim	79 (8)	74 (5)	80 (3)	95 (3)	91 (5)	99 (5)	80 (3)	104 (4)
Merphos	49 (7)	37 (9)	55 (4)	45 (3)	58 (3)	61 (8)	–	57 (6)
Metalaxyl	103 (6)	100 (6)	96 (4)	98 (5)	109 (5)	112 (4)	62 (20)	79 (9)
Metazachlor	101 (5)	104 (1)	109 (6)	108 (4)	83 (7)	92 (6)	91 (4)	85 (8)
Metconazole	75 (2)	71 (3)	71 (5)	70 (7)	87 (2)	76 (3)	95 (8)	92 (7)
Methidathion	105 (8)	112 (4)	109 (3)	96 (3)	93 (3)	87 (2)	73 (6)	78 (5)
Methiocarb	78 (7)	85 (2)	83 (7)	90 (7)	88 (7)	91 (3)	84 (7)	105 (8)
Methiocarb sulfone	118 (25)	78 (2)	106 (3)	95 (4)	101 (6)	92 (7)	76 (20)	89 (3)
Methoxychlor	67 (6)	71 (3)	52 (5)	64 (7)	72 (5)	77 (6)	65 (9)	79 (5)
Metolachlor	84 (6)	88 (3)	87 (5)	100 (3)	95 (3)	90 (5)	78 (7)	71 (8)
Mevinphos	121 (9)	118 (2)	107 (2)	122 (7)	98 (6)	85 (3)	74 (7)	99 (5)
Molinate	77 (8)	73 (4)	78 (6)	75 (5)	85 (5)	93 (3)	86 (8)	63 (7)
Myclobutanil	92 (5)	98 (2)	76 (5)	95 (6)	105 (1)	99 (4)	67 (4)	79 (6)
Napropamide	85 (7)	93 (1)	86 (8)	91 (5)	87 (6)	94 (8)	80 (7)	77 (9)
Nuarimol	73 (6)	77 (3)	70 (6)	79 (4)	90 (5)	78 (1)	79 (5)	69 (9)

Table 1 (Continued)

Compound	Recovery (% RSD)		Olive oil 2 ^b		Sunflower oil		Soybean oil	
	Olive oil 1 ^a							
	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹	10 µg kg ⁻¹	20 µg kg ⁻¹
Ofurace	116 (5)	115 (2)	96 (3)	83 (2)	110 (4)	86 (3)	103 (4)	117 (3)
Ortophenylphenol	74 (4)	85 (4)	71 (3)	78 (3)	79 (6)	93 (7)	109 (4)	111 (4)
Oxadixyl	112 (3)	117 (2)	110 (6)	120 (5)	96 (4)	89 (3)	109 (7)	100 (5)
Paçlobutrazol	79 (6)	85 (5)	89 (3)	87 (3)	93 (2)	75 (2)	–	–
Paraoxon methyl	114 (4)	118 (5)	97 (3)	85 (1)	76 (5)	89 (5)	–	–
Parathion Ethyl	92 (8)	111 (5)	90 (4)	103 (3)	85 (3)	83 (7)	87 (7)	86 (1)
Parathion Methyl	105 (5)	110 (4)	115 (7)	102 (4)	86 (5)	73 (4)	83 (3)	87 (3)
Pebulate	66 (6)	61 (3)	67 (6)	75 (5)	94 (4)	57 (5)	76 (5)	81 (5)
Penconazole	78 (6)	76 (5)	70 (7)	86 (1)	84 (3)	69 (6)	73 (5)	86 (8)
Pendimethalin	69 (11)	66 (6)	70 (5)	66 (9)	76 (3)	79 (1)	65 (6)	79 (3)
Pentachloroaniline	58 (4)	51 (2)	55 (3)	64 (1)	76 (4)	60 (5)	22 (3)	17 (5)
Permethrin	32 (2)	65 (3)	32 (4)	63 (2)	65 (4)	67 (6)	56 (6)	34 (3)
Phenothrin	76 (8)	74 (2)	74 (3)	71 (5)	86 (7)	97 (3)	76 (8)	85 (5)
Phenthoate	100 (3)	102 (3)	105 (2)	97 (1)	95 (4)	79 (2)	81 (3)	90 (7)
Phorate	71 (7)	79 (4)	83 (3)	84 (2)	90 (3)	75 (4)	82 (5)	94 (3)
Phorate sulfone	106 (6)	112 (2)	95 (11)	103 (2)	102 (3)	94 (5)	98 (4)	112 (4)
Phosmet	106 (4)	101 (2)	97 (5)	95 (4)	98 (5)	90 (1)	117 (7)	120 (5)
Phthalimide	97 (2)	101 (5)	104 (3)	86 (6)	85 (4)	109 (3)	80 (4)	76 (6)
Picolinafen	71 (4)	75 (1)	70 (8)	73 (4)	92 (8)	76 (5)	75 (2)	78 (1)
Picoxystrobin	117 (8)	101 (3)	100 (3)	93 (6)	98 (6)	103 (7)	109 (2)	103 (3)
Pirimicarb	99 (4)	105 (3)	97 (5)	83 (7)	87 (3)	94 (2)	85 (7)	84 (6)
Pirimiphos-Methyl	73 (2)	75 (1)	92 (2)	71 (3)	94 (4)	96 (6)	82 (8)	79 (2)
Procimidone	82 (5)	88 (2)	66 (2)	80 (1)	88 (3)	79 (1)	74 (3)	78 (6)
Profenofos	77 (4)	73 (2)	97 (5)	79 (4)	87 (2)	90 (1)	64 (5)	86 (3)
Prometon	78 (9)	78 (4)	74 (6)	65 (2)	76 (4)	87 (5)	78 (7)	74 (8)
Prometryn	76 (3)	75 (3)	70 (7)	73 (5)	79 (3)	80 (7)	85 (3)	82 (7)
Propaphos	88 (4)	89 (3)	82 (2)	75 (1)	95 (8)	87 (6)	81 (4)	88 (3)
Propargite	71 (6)	79 (1)	72 (9)	87 (3)	89 (4)	78 (9)	72 (3)	76 (8)
Propazine	71 (5)	71 (4)	75 (6)	86 (6)	76 (3)	84 (5)	68 (5)	73 (4)
Propiconazole	72 (6)	79 (3)	72 (8)	72 (4)	87 (2)	76 (3)	78 (6)	82 (2)
Propyzamide	80 (8)	83 (3)	74 (6)	77 (4)	75 (5)	92 (6)	73 (4)	73 (2)
Prosulfocarb	66 (3)	74 (4)	65 (5)	71 (7)	69 (4)	68 (3)	55 (3)	47 (8)
Prothiophos	60 (8)	63 (4)	75 (3)	84 (8)	67 (6)	69 (3)	61 (5)	62 (4)
Pyraclostrobin	93 (5)	102 (3)	98 (5)	99 (4)	87 (6)	84 (5)	69 (5)	118 (9)
Pyrazofos	90 (5)	95 (3)	93 (6)	94 (3)	90 (6)	87 (5)	71 (19)	107 (4)
Pyridaben	61 (6)	72 (2)	44 (8)	62 (6)	79 (5)	89 (4)	69 (7)	68 (8)
Pyrifeno	70 (4)	69 (2)	65 (7)	74 (4)	75 (6)	57 (1)	64 (9)	71 (3)
Pyrimethanil	79 (3)	85 (1)	67 (4)	81 (4)	86 (7)	84 (6)	40 (4)	43 (6)
Pyriproxyfen	85 (9)	80 (2)	80 (5)	83 (2)	77 (9)	71 (7)	61 (5)	64 (1)
Quinalphos	77 (3)	83 (3)	72 (6)	77 (5)	76 (6)	70 (5)	77 (7)	79 (2)
Quinoxifen	40 (2)	46 (4)	54 (5)	55 (7)	57 (6)	51 (2)	58 (4)	24 (5)
Quintozene	47 (29)	50 (3)	41 (4)	48 (3)	58 (5)	57 (9)	47 (6)	38 (8)
Sebumeton	77 (3)	75 (4)	75 (5)	72 (6)	79 (8)	80 (6)	83 (3)	75 (8)
Spirodiclofen	87 (9)	85 (3)	88 (3)	86 (3)	86 (7)	75 (5)	83 (5)	66 (3)
Spiromesifen	78 (5)	83 (2)	85 (4)	77 (4)	64 (3)	66 (3)	76 (8)	79 (5)
Sulfotep	98 (5)	101 (2)	95 (5)	103 (6)	85 (2)	103 (1)	77 (9)	82 (7)
Sulprofos	73 (8)	77 (3)	71 (3)	76 (4)	89 (4)	73 (5)	63 (3)	67 (6)
Tebuconazole	71 (3)	71 (3)	75 (7)	94 (8)	99 (6)	65 (6)	79 (4)	85 (3)
Tebufenpyrad	70 (4)	72 (3)	75 (9)	79 (5)	83 (4)	90 (6)	67 (3)	82 (5)
Tecnazene	60 (3)	63 (2)	76 (5)	82 (6)	75 (3)	78 (7)	69 (4)	82 (5)
Tefluthrin	79 (3)	74 (4)	66 (7)	64 (6)	80 (5)	75 (2)	70 (5)	73 (5)
Terbufos	70 (5)	75 (2)	67 (9)	71 (6)	78 (4)	76 (4)	58 (6)	67 (3)
Terbumeton	73 (3)	74 (3)	62 (2)	69 (4)	70 (2)	87 (3)	79 (5)	81 (3)
Terbutryn	68 (3)	73 (6)	68 (1)	71 (5)	76 (5)	79 (3)	94 (8)	71 (5)
Tetrachlorvinphos	100 (6)	102 (2)	98 (5)	100 (9)	102 (6)	87 (4)	55 (5)	88 (7)
Tetraconazole	103 (8)	102 (2)	101 (3)	83 (3)	97 (5)	93 (5)	81 (2)	107 (4)
Tetradifon	69 (6)	73 (5)	73 (5)	91 (2)	84 (7)	76 (4)	99 (4)	100 (5)
Tetrahydrophthalimide	99 (6)	110 (4)	102 (1)	101 (2)	95 (6)	92 (3)	88 (5)	85 (5)
Tetramethrin	80 (4)	90 (3)	85 (4)	84 (4)	96 (4)	88 (7)	70 (9)	82 (7)
Tolclofos Methyl	74 (5)	72 (1)	93 (5)	87 (1)	90 (4)	81 (8)	61 (4)	80 (6)
Tolylfluamide	97 (11)	96 (3)	102 (2)	103 (4)	88 (5)	79 (5)	86 (1)	85 (6)
Triadimefon	90 (7)	95 (2)	94 (1)	99 (2)	102 (3)	111 (4)	82 (2)	85 (5)
Triazophos	106 (8)	111 (1)	103 (3)	97 (4)	98 (6)	84 (7)	83 (3)	85 (3)
Trifloxystrobin	108 (7)	114 (3)	112 (7)	109 (2)	90 (7)	92 (3)	94 (19)	113 (6)
Trifluralin	99 (6)	85 (3)	102 (4)	82 (4)	97 (5)	79 (3)	91 (3)	107 (6)
Vinclozolin	84 (7)	91 (4)	76 (3)	78 (1)	75 (7)	83 (8)	73 (6)	79 (4)
Average	81 (5)	84 (3)	80 (5)	81 (5)	83 (5)	82 (5)	75 (7)	79 (6)

^a Organic extra virgin olive oil (Type 1).^b Organic extra virgin olive oil (Type 2).

quantitative determination of pesticides with extraction recoveries below 70%. Procedural standards were prepared by spiking a series of blank samples with different amounts of analytes, prior to extraction and these procedural standards were then analysed in exactly the same way as the samples. Thus, Procedural Standard Calibration enables a correction of recoveries for all pesticides. A number of requirements were fulfilled for a successful application of this approach: i) Good linear relation in the 10–500 $\mu\text{g kg}^{-1}$ concentration range ($r^2 > 0.99$ in all instances for all target analytes); ii) Precise quantification ($\text{RSD} \leq 5\%$ for approximately 90% of the targeted compounds) of pesticides in oil samples were obtained; and iii) At 10 $\mu\text{g kg}^{-1}$ level, even in cases with low recoveries, detector signals of analyte were within the linearity range and the signal-to-noise of the quantitative transition was equal to, or higher than, 10.

Taking into account the good precision, the proposed method can be regarded as a strong alternative method to perform the determination of a large number of pesticides in routine analysis.

3.2.2. Limits of quantitation

Document N° SANTE/11945/2015 [30] describes the LOQ as the minimum concentration which meets the criteria of a mean recovery within the 70–120% range and an $\text{RSD} \leq 20\%$. Due the spiking levels evaluated by the authors, the LOQs have values of 10 or 20 $\mu\text{g kg}^{-1}$. In olive oil, 162 pesticides had limits of quantitation equal to 10 $\mu\text{g kg}^{-1}$; whilst 50 pesticides had recoveries outside the 70–120% range. In sunflower oil, 184 pesticides had LOQs at 10 $\mu\text{g kg}^{-1}$ and 3 pesticides had LOQs at 20 $\mu\text{g kg}^{-1}$. The remaining 26 pesticides had recoveries that were low or were not detected. In soybean oil, 136 pesticides had LOQs of 10 $\mu\text{g kg}^{-1}$ and 30 had 20 $\mu\text{g kg}^{-1}$. The remaining 47 pesticides were not detected or their recoveries were below 70%. Detailed limits of quantitation values are shown in Table S3 (in the Supplementary data).

However, the use of Procedural Standard Calibration and the good repeatability of the proposed method ensure the applicability of the proposed method for analytes with mean recoveries outside the above mentioned 70–120% range. Thus, in cases where a LOQ value of 20 $\mu\text{g kg}^{-1}$ were obtained, it may be practicable to include these compounds as a LOQ of 10 $\mu\text{g kg}^{-1}$.

3.2.3. Linearity

Linear calibration was checked in the range from 10 $\mu\text{g kg}^{-1}$ up to 500 $\mu\text{g kg}^{-1}$. Oil samples were spiked at different analyte concentrations in quintuplicate then, extractions were performed and weighted linear regressions ($1/x$) were calculated for each pesticide. Residuals were calculated and showed a deviation by less than $\pm 20\%$ from the calibration curve for each calibration level. Good linearity was achieved in all cases with correlation coefficients better than 0.990 (Table S3). The lowest calibrated level always had a qualifying transition with $S/N \geq 6$. In all studied matrices, detector saturation was not a problem due to the very effective cleaning procedure proposed.

3.2.4. Inter- and intra-day precision

Repeatability (intra-day precision) and reproducibility (inter-day precision) were evaluated through recovery studies using spiked blank oil samples at two concentration levels (10 and 20 $\mu\text{g kg}^{-1}$). Intra-day precision was assessed by five determinations at each spiking level in the same day. Inter-day precision was assessed by one determination at each spiking level for five days. RSD values were generally below 10% (5% on average) and never exceeded 15% for the intra-day precision, except for aldrin, biphenyl and carbosulfan in soybean oil. RSD values were also generally below 10% (6% on average) and never exceed 19% for the

inter-day precision, except for biphenyl and carbosulfan in soybean oil (Table S3).

3.2.5. Matrix effects

As consequence of coeluting sample components, the analyte signal may be enhanced or suppressed compared to the signal of the same analyte when injected in solvent. In most multiresidue procedures employing different clean-up techniques and MS detection methods, matrix effect has been described for the determination of pesticide residues in edible oils [3,14,15,18,19]. These methods suffered medium (suppression or enhancement of 20–50%) [3,18,19] or strong (suppression or enhancement >50%) [14,15] matrix effects for 27–60% of compounds. This drawback, however, may be overcome by applying Procedural Standard Calibration since this procedure of quantification corrects for matrix effects.

The assessment of ME was carried out by comparing the slopes of analyte calibration plots in the oils studied, including two organic extra virgin olive oils samples from different trademarks (Type 1 and Type 2), one organic sunflower oil and one organic soybean oil. Thus, the variability of the response when calibrating olive oils from different sources was checked since it is likely that for example signal value may be affected by differences in the variety of olives.

To calculate, the matrix effect value equation was used considering Type 1 olive oil as reference oil:

$$\text{ME}(\%) = \left(\frac{\text{Slope of calibration curve in studied matrix}}{\text{Slope of calibration curve in Type 1 olive oil}} - 1 \right) \times 100$$

In sunflower and Type 2 olive oils, calculated ME was negligible indicating similar behavior of analytes in these matrices with respect to Type 1 olive oil. However, differences in the analytical signals were observed for soybean oil. Medium signal suppression was found for most compounds. Since mean recoveries and RSD values of pesticides in soybean oil were quite similar compared to the other studied oils, the proposed explanation for this was supported by the endogenous matrix compounds of soybean oil that could suppress the signals. This signal suppression for soybean oil can be compensated by using Procedural Standard Calibration.

3.3. Analysis of real oil samples

The validated method was applied for the analysis of 17 oil samples collected locally from Almería markets. The samples (seven extra virgin olive oil, two virgin olive oil, two refined olive oil, three sunflower oil and three soybean oil samples) were analyzed the same as the modified validated method for the analysis of 213 pesticides. The results of the detected pesticides are shown in Table 2.

Chlorpyrifos, chlorpyrifos methyl, deltamethrin, endosulfan sulfate, phosmet, tetraconazole and fluopyram pesticides were detected with values higher than the LOQ. Chlorpyrifos was detected in three extra virgin oil sample (Type 1, 3 and 4) with a concentration of 26.6, 23.0 and 32.4 $\mu\text{g kg}^{-1}$, respectively. Chlorpyrifos methyl was detected in one extra virgin olive oil sample (Type 5) with a concentration of 21.2 $\mu\text{g kg}^{-1}$. Deltamethrin was detected in one refined olive oil sample (Type 2) with a concentration of 17.7 $\mu\text{g kg}^{-1}$. Endosulfan sulfate was detected in one extra virgin olive oil samples (Type 4) with a concentration of 10.2 $\mu\text{g kg}^{-1}$. Phosmet was found in two extra virgin olive oil (Type 3 and 7) with a concentration of 156.0 and 26.7 $\mu\text{g kg}^{-1}$, respectively. Tetraconazole was found in one extra virgin olive oil sample (Type 3) with a concentration of 31.0 $\mu\text{g kg}^{-1}$. Fluopyram was determined in one extra virgin olive oil sample (Type 3) with a concentration of 21.0 $\mu\text{g kg}^{-1}$. All the detected pesticides in the oil samples were with values lower than the MRLs ones (chlorpyrifos 50 $\mu\text{g kg}^{-1}$,

Table 2

Mean concentrations of pesticides found in the edible vegetable oils.

Sample	Pesticides detected ($\mu\text{g kg}^{-1}$)
Olive oil	
Extra virgin1	Chlorpyrifos (26.6)
Extra virgin2	–
Extra virgin3	Chlorpyrifos (23.0), Phosmet (156.0), Tetraconazole (31.0), Fluopyram (21.0), Tebuconazole (2.2 ^a), Lambda cyhalothrin (1.5 ^a), Chlorpropham (4.3 ^a)
Extra virgin4	Chlorpropham (6.3 ^a), Chlorpyrifos (32.4), Chlorpyrifos methyl (3.4 ^a), Cypermethrin (6 ^a), Endosulfan sulfate (10.2)
Extra virgin5	Chlorpyrifos (5.0 ^a), Chlorpyrifos methyl (21.2), Endosulfan sulfate (2.8 ^a)
Extra virgin6	–
Extra virgin7	Chlorpyrifos (7.4 ^a), Lambda cyhalothrin (3.9 ^a), Phosmet (26.7)
Virgin1	–
Virgin2	–
Refined1	Cypermethrin (4.0 ^a)
Refined2	Cypermethrin (1.5 ^a), Deltamethrin (17.7)
Sunflower oil	
Sunflower1	–
Sunflower2	Pyridaben (4.7 ^a)
Sunflower3	Pyridaben (4.7 ^a)
Soybean oil	
Soybean1	Fenthion (1.3 ^a)
Soybean2 (Soya + Walnut)	–
Soybean3 (Soya + Omega3)	–

^a This value is lower than the validated LOQ.

chlorpyrifos methyl $50 \mu\text{g kg}^{-1}$, deltamethrin $1000 \mu\text{g kg}^{-1}$, endosulfan sulfate $50 \mu\text{g kg}^{-1}$, phosmet $3000 \mu\text{g kg}^{-1}$, tetraconazole $20 \mu\text{g kg}^{-1}$ and fluopyram $10 \mu\text{g kg}^{-1}$). These MRLs values were established for the olives used for oil production, where, the exact MRLs values for oil processing should be 5 times of magnitude of these values assigned above except for deltamethrin (1.5 factor).

It was noticed that the detected pesticides were only in olive oil samples with no detection of any in soybean or sunflower ones. It was noticed also that the higher concentrations of chlorpyrifos and chlorpyrifos methyl were found in two of extra virgin olive oil samples. On the other hand, phosmet was found with higher value in extra virgin olive oil (Type 3) than value found in extra virgin olive oil one (Type 7). Regarding the different analyzed matrices, clearly results show a major occurrence of pesticide residues in extra virgin olive oil comparing with the “few” residues found in virgin and refined oil samples. The proposed explanation for this was supported by the pesticide degradation during the manufacturing process (only cold pressure for extra virgin olive oil and hot pressure for refined oil).

4. Conclusions

The combination of freeze-out with dry ice (CO_2 at -76°C) and d-SPE with EMR-Lipid for the clean-up of edible oil samples led to a development of a simple, efficient, selective, robust and sensitive method for the determination of 213 pesticides by GC-MS/MS. In general, better recoveries were achieved using QuEChERS with d-SPE (EMR-Lipid) method for the target pesticides. Using this extraction protocol, 83% of the analytes were recovered in the range 70–120%, while using the d-SPE (PSA), d-SPE (Z-Sep) and SPE (Z-Sep) extraction procedures, the percentage of analytes was 32%, 49% and 9%, respectively (see Table S2 in Material Supplementary for detailed data). An advantage is the low RSD achieved (between 1 and 15%) that allows the application of Procedural Standard approach to compensate the low recoveries obtained in some cases. As a result of selective extraction and effective removal of coextractives, negligible matrix effect was observed in extra virgin olive and sunflower oils. Soybean oil is the more complex matrix; medium matrix effect resulting in suppression of the response was found in this matrix.

The analysis of real oil samples showed that values of pesticides chlorpyrifos, chlorpyrifos methyl, deltamethrin, endosulfan sulfate, phosmet, tetraconazole and fluopyram were detected with values higher than the LOQ but still below the MRLs guidelines values established by EU.

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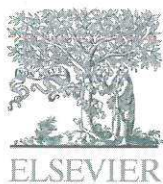
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.chroma.2016.08.008>.

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Determination of pesticides in edible oils by liquid chromatography-tandem mass spectrometry employing new generation materials for dispersive solid phase extraction clean-up



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ABSTRACT

The goal of this work was to evaluate the efficiency of several sorbents on removal fats from edible oils (olive, soya and sunflower) during the clean-up step for posterior determination of 165 pesticides by UHPLC-QqQ-MS/MS system. The extraction procedure employed in this work was the citrate version of QuEChERS method followed by a step of freezing out with dry ice and clean-up evaluation using i) PSA with magnesium sulfate (d-SPE); ii) magnesium sulfate and Z-sep sorbent (d-SPE); iii) Z-sep (column SPE) and iv) Agilent Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR-Lipid). After evaluation of the recovery results at 10, 20 and 50 $\mu\text{g kg}^{-1}$, the EMR-Lipid showed important advantages comparing to the other sorbents evaluated, such as better recovery rates and RSD%. The method was validated at the three concentrations described above. Analytical curves linearity was evaluated by spiking blank oil samples at 10, 20, 50, 100 and 500 $\mu\text{g kg}^{-1}$. The method demonstrated good recoveries values between the acceptable range of 70–120% and RSD% < 20 for most of evaluated pesticides. In order to evaluate the performance of the method, this same procedure was employed to other oils such as soya and sunflower with very good results.

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1. Introduction

Olive oil is the principal source of lipids in the Mediterranean diet, and its consumption in the world is increasing due to related potential health benefits, such as a lower incidence of cardiovascular diseases, neurological disorders, breast and colon cancers, as well as its hypolipidemic and antioxidant properties [1]. According to the data published in November 2015 by International Olive Oil Council, Spain is the main producer of olive oil in Europe with about 840 thousand tons during 2014/2015 production. Related to consumption in Europe, Italy is the main consumer with circa 520 thousand tons in 2014/2015 and in second place Spain with approximately 490 thousand tons [2].

Pesticides are chemical substances applied to crops at various stages of cultivation and post-harvest storage of crops. The use of

pesticides is intended to prevent the destruction of food crops by controlling agricultural pests or unwanted plants and to improve plant quality. The widespread use of pesticides for improving agricultural productivity has raised public concern about the possible presence of residues in crops and its byproducts. In agricultural practice for olive groves, the use of insecticides and herbicides provides an unquestionable benefit for crop protection. However, these pesticides can persist up to the harvest and processing stage, making the contamination of olives, and consequently of olive oil, possible [3,4].

The large number of pesticides to be monitored associated with low concentration of the maximum residue limits (MRL) established and non-registered residues in food require sensitive and selective methods for their identification and quantification. However, olive oil contains high level of lipid substances which can cause problems during pesticide residue analysis because they are soluble in many organic solvents used for extraction. The lipids must be removed from the extracts prior to analysis or the chromatographic and detection system can be damaged [5].

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In the last few years many studies were published aiming the development of sensitive and accurate methods for pesticide residues determination in high fat content matrices. The most common sorbent employed in these works during clean-up step was PSA [6–10] which was also evaluated in combination with other sorbents such as C18 [6,7] and GCB [8,9], due to the well known power of PSA in removing lipid content. Some methods employing Oasis Hydrophilic-Lipophilic Balance (HLB) were also reported [11]. Most recently, a new sorbent based on zirconium dioxide has been employed instead of PSA due to its higher ability on removing fat content from olive oil [12,13].

These clean-up methods were applied, in most of the cases, in combination with QuEChERS methodologies and its variations [6,7,10,13,14]. Mini-Luke was also evaluated in combination with UPLC–MS/MS in order to determine residues of 169 pesticides in soya grain [15].

Taking all these points into account and considering the importance of olive oil in Europe, the goal of this study was to develop and validate an analytical method for pesticides residue determination in olive oil by UHPLC–QqQ–MS/MS employing new sorbents for clean-up step. Four different methods using different sorbents were employed: i) PSA with magnesium sulfate (d-SPE); ii) Z-Sep sorbent with magnesium sulfate (d-SPE); iii) Z-Sep (cartridge SPE) and iv) Agilent Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR-Lipid). Furthermore, a step of low temperature precipitation (freezing-out) was evaluated before SPE clean-up. The method was fully validated in olive oil and applied for sunflower oil and soya oil in order to compare the results and check the possibility of employing only one kind of oil to quantify all of them. The method was applied in oil real samples of olive, sunflower and soya collected in local supermarkets of Almería city, in the southeastern of Spain.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, HPLC grade (99.9%), formic acid, analytical grade (>96%) and magnesium sulfate (98%) were purchased from Sigma Aldrich (Steinheim, Germany). Water, Optima®, HPLC grade was supplied by Fisher Scientific (New Jersey, USA). Sodium chloride was obtained from J. T. Baker (Deventer, Netherlands). Sodium citrate tribasic dihydrate (≥99%) and disodium hydrogen citrate sesquihydrate (99%) were obtained from Fluka (Steinheim, Germany). PSA and Z-Sep were purchased from Supelco (Bellefonte, USA). Bond Elut Enhanced Matrix Removal d-SPE and Bond Elut Final Polish from Agilent Technologies (Santa Clara, USA). Pesticides standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany), from Riedel-de-Haën (Seelze, Germany) and from Sigma Aldrich (Steinheim, Germany).

2.2. Pesticides standards solutions

Individual pesticide standard stock solutions were prepared in acetonitrile and stored in amber screw-capped glass vials at -20°C . A standard mixture solution of the pesticides was prepared in acetonitrile at 10 mg L^{-1} . This solution was used as spike solution for recovery experiments and also to prepare the analytical curves solution for linearity studies.

2.3. Final extraction procedure

The final extraction procedure employed was the citrate version of QuEChERS method [16] using the EMR-Lipid from Agilent Technologies. An amount of 15 g of olive oil was weighed in a 50 mL PTFE centrifuge tube and 15 mL of acetonitrile was added plus 15 μL of procedure internal standard solution at 10 mg L^{-1} in

acetonitrile containing triphenyl phosphate (TPP), dichlorvos-d6, malathion-d10 and carbendazim-d3. The tubes were shaken in an automatic axial extractor (AGYTAX®, Cirta Lab. S.L., Spain) during 4 min. Thereafter, 6 g of magnesium sulfate, 1.5 g of sodium chloride, 1.5 g of sodium citrate tribasic dihydrate and 0.75 g of disodium hydrogen citrate sesquihydrate were added and the samples were again shaken during 4 min in the automatic axial extractor. The extracts were centrifuged at 3500 rpm for 5 min and 8 mL were transferred to a 15 mL PTFE centrifuge tube. The tubes containing the extract were allowed to stand in dry ice during approximately 6 min in order to precipitate the fat content. The upper acetonitrile extract (5 mL) was collected and transferred to an EMR-Lipid d-SPE 15 mL tube already containing the adsorbent for clean-up step (1 g) and 5 mL of water. The mixture were homogenized in vortex during 1 min, centrifuged (3500 rpm, 5 min) and 5 mL of extract was transferred to an EMR-Lipid polish tube containing 2 g of a mixture of sodium chloride and magnesium sulfate (1:4, w/w). The mixture was homogenized during 1 min in vortex and centrifuged. Hereafter, 2 mL of extract were transferred to a vial and acidified with 20 μL of formic acid (5% in acetonitrile). Before UHPLC–MS/MS analysis, the extracts (100 μL) were diluted 5-fold with water HPLC grade and 10 μL of injection internal standard solution at 2.5 mg L^{-1} containing dimethoate-d6 was added to the vials.

2.4. Instrumentation

An Agilent UHPLC 1290 Series (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent Technologies 6490 TripleQuad LC/MS was used for this study. Data acquisition and processing were developed by using Agilent MassHunter QQQ Acquisition and Quantitative Analysis B.07.00 software using Dynamic MRM software features with a retention time window of 0.8 min. The injection volume was 5 μL , and the chromatographic separation was carried out with a Zorbax Eclipse Plus C8 column (Agilent), $1.8\text{ }\mu\text{m} \times 2.1\text{ mm} \times 100\text{ mm}$, maintained at 35°C . The mobile phases employed was a solution of formic acid 0.1% in milliQ water (mobile phase A) and 0.1% formic acid and 5% water in acetonitrile (mobile phase B) at a constant flow rate of 0.3 mL min^{-1} , with the following gradient: 20% of B for 2 min, a linear gradient up to 100% of B in 13 min and finally an isocratic mode at 100% of B for 2 min. Afterwards, an equilibration step coming back to 20% of B (2.5 min) was performed. The system was provided with a Jet-Stream electrospray ion source, employing nitrogen as nebulizer gas. This ion source was configured as follows: 120°C for drying gas temperature, 13 L min^{-1} for drying gas flow, 45 psi for pressure of the nebulizer, 375°C for sheath gas temperature and 10 L min^{-1} for the sheath gas flow. The MS used nitrogen as collision gas (99.999% purity), 380 V for the fragmentor and 3000 V for the capillary voltage both in positive and negative mode.

For the optimization of the MS parameters, all pesticides at $100\text{ }\mu\text{g L}^{-1}$ (acetonitrile:water, 1:1, v/v) were injected directly in the MS system in full scan mode with a mass range of 50–800 m/z . From this injection the precursor ion was selected and one more injection in product ion mode was needed to choose two fragment ions and the optimum collision energy (CE) for each transition. Retention times, transitions and CEs for each compound are collected in Table 1. The most intense transition was selected as the quantifier transition (SRM1), while the second most intense was chosen as the qualifier transition (SRM2).

2.5. Validation of the analytical procedure

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Validation study was performed in order to evaluate accuracy (recovery), precision, linearity, limit of quantification, matrix effects and repeatability in accordance with the Document No. SANTE/11945/2015 [17]. Recovery and precision were determined

Table 1

Acquisition conditions for mass spectrometer: retention time, precursor ion, quantifier and qualifier transition and polarity of acquisition.

Compound	Retention time (min)	Precursor ion	1st transition		2nd transition		Polarity
			Product ion	CE ^a	Product ion	CE ^a	
2,4-D	7.8	218.96/220.96	160.96	15	162.95	15	Negative
Acephate	1.1	184.00	143.00	5	125.00	15	Positive
Acetamiprid	4.4	223.00	126.00	20	56.00	15	Positive
Aldicarb	5.7	213.00	116.00	10	89.00	15	Positive
Aldicarb-sulfone	1.7	223.00	148.00	5	86.00	10	Positive
Aldicarb-sulfoxide	1.2	207.00	132.00	5	89.00	10	Positive
Azinphos-methyl	9.1	318.00	261.00	0	132.10	8	Positive
Azoxystrobin	9.6	404.00	372.00	10	344.00	20	Positive
Bifenazate	10.2	301.10	198.20	10	169.90	20	Positive
Bitertanol	10.2	338.20	269.20	5	99.10	10	Positive
Boscalid	9.7	343.00	307.10	16	272.10	32	Positive
Bromuconazole	9.5	378.00	159.00	20	70.00	20	Positive
Bupirimate	8.6	317.00	272.00	20	166.00	20	Positive
Buprofezin	10.1	306.00	201.00	10	116.00	15	Positive
Carbaryl	7.4	202.00	145.00	10	127.00	20	Positive
Carbendazim	1.2	192.00	160.00	15	132.00	20	Positive
Carbendazim-d3 (LS)	1.2	195.10	159.80	20	131.90	20	Positive
Chlorantraniliprol	8.6	483.90	452.90	16	285.90	8	Positive
Chlorfenvinphos	10.8	358.90	155.00	8	99.20	28	Positive
Chlorpyrifos-methyl	11.7	321.90	289.90	14	125.00	16	Positive
Chlorpyrifos	12.9	352/349.93	200.00	20	198.00	20	Positive
Clofentezin	11.4	303.00	138.00	12	102.00	40	Positive
Clomazone	8.6	240.10	127.80	10	124.90	20	Positive
Coumaphos	11.4	363.00	307.00	20	227.00	28	Positive
Cyazofamid	11.1	325.00	261.20	10	108.10	15	Positive
Cymoxanil	5.0	199.10	128.00	4	110.90	12	Positive
Cyproconazole	9.1	292.10	125.00	32	70.00	16	Positive
Cyprodinil	8.0	226.20	92.90	40	76.90	40	Positive
Cyromazine	0.9	167.00	125.00	15	59.90	20	Positive
Demeton-S-methylsulfone	2.3	263.02	169.00	12	109.00	24	Positive
Demeton-S-methylsulfoxide	1.4	247.00	169.00	8	109.00	24	Positive
Diazinon	11.4	305.00	169.00	15	153.00	20	Positive
Diclorvos	6.5	220.80	108.80	15	78.90	30	Positive
Diclorvos-d6 (LS)	7.8	226.90	132.90	20	115.00	20	Positive
Dicrotophos	2.0	238.09	112.10	8	72.10	28	Positive
Diethofencarb	9.2	268.00	226.00	5	180.00	15	Positive
Difenoconazole	10.9	406.00	337.00	15	251.00	20	Positive
Diffubenzuron	10.1	311.00	158.00	8	141.00	32	Positive
Dimethoate	4.2	230.00	199.00	5	171.00	10	Positive
Dimethoate-d6 (LS)	4.2	236.00	205.00	4	131.00	16	Positive
Dimethomorph	8.7	388.00	301.00	20	165.00	20	Positive
Diniconazole	10.5	326.10	159.00	28	70.00	28	Positive
Dodine	8.6	228.20	60.10	20	57.20	20	Positive
Emamectin B1a	9.6	886.50	158.10	40	81.80	50	Positive
EPN	12.0	324.05	296.01	10	156.99	20	Positive
Epoxiconazole	9.6	330.10	121.00	16	101.20	52	Positive
Ethion	13.3	385.10	199.00	5	171.00	10	Positive
Ethirimol	2.5	210.16	140.10	20	43.10	52	Positive
Ethoprophos	9.8	243.10	130.90	15	97.00	30	Positive
Fenamidone	9.7	312.00	92.20	28	65.10	56	Positive
Fenamiphos	9.5	304.10	234.00	12	217.10	20	Positive
Fenamiphos-sulfone	6.9	336.10	266.00	16	188.00	24	Positive
Fenamiphos-sulfoxide	5.8	320.11	292.10	8	108.10	44	Positive
Fenarimol	9.3	331.00	268.00	20	259.00	20	Positive
Fenazaquin	12.5	307.30	161.30	15	147.20	15	Positive
Fenbuconazole	10.1	337.10	125.10	40	70.00	33	Positive
Fenhexamid	9.7	302.00	97.00	25	55.00	30	Positive
Fenoxycarb	10.3	302.20	116.20	5	88.20	20	Positive
Fenpropimorph	7.6	304.30	147.10	30	130.00	25	Positive
Fenpyrazamine	10.0	332.20	272.10	10	230.20	20	Positive
Fenpyroximate	13.1	422.21	366.20	12	107.00	64	Positive
Fenthion	11.2	279.00	247.10	8	169.10	12	Positive
Fenthion-sulfoxide	7.1	295.02	280.00	16	109.00	32	Positive
Fipronil	11.2	434.90	329.90	12	249.90	28	Negative
Fluazifop	9.2	328.20	282.20	15	254.20	20	Positive
Flubendiamide	11.1	680.90	273.90	15	254.00	20	Negative
Fludioxonil	9.3	247.00	169.00	32	152.00	32	Negative
Flufenacet	10.6	364.10	194.10	15	152.00	15	Positive
Flufenoxuron	12.9	489.10	158.00	20	140.90	56	Positive
Fluopyram	10.1	397.10	208.00	20	173.10	20	Positive
Fluquinconazole	9.7	376.00	307.10	24	108.00	56	Positive
Flusilazol	10.1	316.10	247.10	12	165.00	24	Positive
Flutriafol	7.6	302.10	95.00	56	70.10	16	Positive
Formetanate	1.0	222.13	165.10	8	65.10	52	Positive

Table 1 (Continued)

Compound	Retention time (min)	Precursor ion	1st transition		2nd transition		Polarity
			Product ion	CE ^a	Product ion	CE ^a	
Fosthiazate	7.6	284.00	227.80	10	103.80	20	Positive
Haloxypop	10.3	362.10	316.20	12	288.10	24	Positive
Hexaconazole	10.2	314.10	159.00	30	70.10	20	Positive
Hexythiazox	13.0	353.10	228.20	10	168.20	20	Positive
Imazalil	6.4	297.00	255.00	15	159.00	20	Positive
Imidacloprid	3.8	256.00	209.00	15	175.00	15	Positive
Indoxacarb	12.2	528.10	218.00	20	203.00	45	Positive
Ioxonil	8.4	369.80	214.80	30	126.80	30	Negative
Iprovalicarb	9.5	321.20	202.90	0	119.00	16	Positive
Isofenfos-methyl	11.6	231.00	199.00	15	121.00	15	Positive
Isoprocarb	8.2	194.10	152.00	5	95.10	15	Positive
Isoxaflutole	9.7	360.00	250.90	15	219.70	50	Positive
Kresoxim-methyl	11.0	314.10	267.00	0	222.10	10	Positive
Linuron	9.1	249.02	160.10	20	133.00	36	Positive
Lufenuron	12.6	508.90	339.00	10	325.90	10	Negative
Malathion	10.4	331.00	127.00	10	99.00	20	Positive
Malathion-d10 (I.S)	10.3	341.11	132.00	12	100.00	24	Positive
Mandipropamid	9.8	412.13	356.10	4	328.10	8	Positive
MCPA	7.9	199.00	154.60	5	140.70	10	Negative
Mepanpyrim	9.7	224.10	206.80	10	190.60	20	Positive
Meptyldinocap	13.8	295.10	193.00	42	163.00	50	Negative
Metalaxyl	7.8	280.30	220.00	5	192.40	10	Positive
Metconazole	10.3	320.10	125.00	48	70.10	24	Positive
Methamidophos	1.1	142.10	125.00	10	94.10	10	Positive
Methidathion	9.1	302.90	145.00	0	85.10	15	Positive
Methiocarb	9.0	226.10	121.10	12	169.00	5	Positive
Methiocarb-sulfoxide	2.8	242.00	185.00	10	170.00	20	Positive
Methomyl	2.0	163.10	106.00	4	88.00	0	Positive
Methoxyfenozide	10.2	369.30	149.00	15	133.00	20	Positive
Metobromuron	8.1	259.00	170.00	15	148.00	10	Positive
Monocrotophos	1.7	224.20	193.10	5	127.00	10	Positive
Myclobutanil	9.6	289.20	125.10	20	70.20	15	Positive
Nitempyram	1.5	271.00	225.00	10	99.00	10	Positive
Omethoate	1.2	214.10	183.00	5	125.00	20	Positive
Oxadixyl	6.2	279.10	219.20	5	132.30	32	Positive
Oxamyl	1.7	237.00	90.00	5	72.00	10	Positive
Paclobutrazol	8.9	294.10	125.20	36	70.10	16	Positive
Paraoxon-methyl	6.3	247.80	201.90	15	108.70	30	Positive
Penconazole	10.2	284.00	159.00	20	70.00	15	Positive
Pencycuron	11.7	329.10	125.10	24	89.10	60	Positive
Pendimethalin	13.0	282.10	212.10	4	194.10	16	Positive
Phenthoate	11.4	321.00	247.10	4	79.10	44	Positive
Phosalone	11.8	368.00	182.00	8	110.90	44	Positive
Phosmet	9.4	317.99	160.00	8	133.00	36	Positive
Phoxim	11.9	299.00	129.10	4	77.10	24	Positive
Pirimicarb	2.5	239.20	182.10	15	72.20	20	Positive
Pirimicarb-desmethyl	1.4	225.10	168.10	8	72.10	20	Positive
Pirimiphos-methyl	11.2	306.20	164.20	20	108.20	20	Positive
Prochloraz	8.8	376.00	308.00	10	266.00	15	Positive
Profenofos	12.0	374.90	347.00	5	304.90	15	Positive
Propamocarb	1.1	189.20	144.10	10	102.10	15	Positive
Propaquizafop	12.2	444.10	371.00	15	99.90	20	Positive
Propargite	13.5	368.10	231.20	0	175.20	8	Positive
Propiconazole	10.4	342.10	159.00	32	69.10	16	Positive
Propoxur	7.0	210.11	168.10	5	111.10	10	Positive
Propyzamide	9.8	256.00	190.00	10	173.00	20	Positive
Proquinazid	13.2	373.00	331.00	20	289.10	20	Positive
Prothioconazole	10.4	341.90	306.10	15	99.80	20	Negative
Prothiofos	14.2	345.00	241.00	20	161.00	40	Positive
Pymetrozine	0.9	218.11	105.00	20	51.00	60	Positive
Pyraclostrobin	11.4	388.11	193.80	8	163.10	20	Positive
Pyrethrin	13.6	329.21	161.00	5	143.00	20	Positive
Pyridaben	13.8	365.20	309.20	10	147.30	20	Positive
Pyridate	14.6	379.10	351.10	5	206.80	10	Positive
Pyrimethanil	6.3	200.00	183.00	20	107.00	20	Positive
Pyriproxyfen	12.6	322.00	185.00	20	96.00	10	Positive
Quinoclamine	6.1	208.00	105.10	25	77.00	40	Positive
Quinoxifen	11.7	308.10	271.90	25	196.90	35	Positive
Quizalofop-ethyl	12.1	373.09	271.20	24	255.10	36	Positive
Rotenone	10.4	395.00	213.10	20	192.10	20	Positive
Spinosyn A	8.7	732.50	142.00	20	98.00	20	Positive
Spinosyn D	9.1	746.50	142.00	20	98.00	20	Positive
Spirodiclofen	14.2	411.10	313.00	5	71.20	15	Positive
Spiromesifen	14.1	371.00	273.00	5	255.00	20	Positive

Table 1 (Continued)

Compound	Retention time (min)	Precursor Ion	1st transition		2nd transition		Polarity
			Product ion	CE ^a	Product ion	CE ^a	
Spirotetramat	9.3	374.20	330.30	15	270.10	20	Positive
Spiroxamine	7.6	298.00	144.00	20	100.00	20	Positive
Tebuconazole	9.9	308.00	125.00	20	70.00	20	Positive
Tebuconazole	10.9	353.20	296.90	5	133.10	15	Positive
Tebuconazole	12.1	334.20	145.10	20	117.00	47	Positive
Tebuconazole	11.7	379.00	359.00	0	339.00	4	Negative
Terbutylazine	9.0	230.00	174.00	15	146.00	20	Positive
Tetraconazole	10.0	372.00	159.00	36	70.00	20	Positive
Thiabendazol	1.3	202.00	175.00	30	131.00	40	Positive
Thiacloprid	5.5	253.00	186.00	10	126.00	20	Positive
Thiamethoxam	2.4	292.00	211.00	10	181.00	20	Positive
Thiobencarb	11.5	258.00	124.70	15	99.90	10	Positive
Thiodicarb	6.9	355.06	108.10	8	88.10	8	Positive
Tolclofos-methyl	11.7	300.90	269.00	10	125.00	15	Positive
Triadimenol	9.0	296.00	227.00	5	70.00	10	Positive
Triazophos	10.4	314.10	286.20	10	162.20	20	Positive
Trichlorfon	3.0	256.90	221.00	4	109.00	12	Positive
Trifloxystrobin	12.2	409.20	206.20	10	186.20	20	Positive
Triflumuron	11.1	359.00	156.00	8	139.00	32	Positive
Triticonazole	9.0	318.10	125.20	41	70.20	33	Positive
Zoxamide	11.3	336.00	187.00	16	159.00	44	Positive

^a CE: collision energy (V).

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as average peak areas of five replicates of spiked blank olive oil samples at 10, 20 and 50 $\mu\text{g kg}^{-1}$. The mixture solution containing all the pesticides was added to the blank olive oil sample and shaken during 30 min in order to obtain a homogeneous sample due to insolubility of the acetonitrile in the oil. The linearity was evaluated by assessing signal responses of the target analytes from procedural standard calibration by spiking blank samples of olive oil at five concentration levels from 10 to 500 $\mu\text{g kg}^{-1}$. Matrix effect was calculated by comparison of the slopes obtained from analytical curves prepared in acetonitrile and in blank olive oil using the following equation:

$$\text{Matrix effect(\%)} = \left[\left(\frac{\text{slope analytical curve in matrix}}{\text{slope analytical curve in matrix acetonitrile}} \right) - 1 \right] \times 100$$

The linearity was accessed also for other two oils, sunflower and soya, and compared with the results obtained from olive oil. This procedure was done to verify the behavior of analytes in different types of oil in order to evaluate if it is possible to quantify residues of different oils with the same calibration curve.

The limit of quantification (LOQ) for each pesticide was settled as the lowest fortified concentration in blank olive oil samples that could be quantified with acceptable accuracy and precision as preconized by Document No. SANTE/11945/2015 [17].

3. Results and discussion

3.1. Clean-up evaluation

In this study a procedure of low temperature precipitation (freezing-out) was evaluated before clean-up employing various sorbents and combination of sorbents.

Primary-secondary amine (PSA) is a well know clean-up sorbent employed when removal of fat content is necessary. The chemical structure of PSA provides high retention of free fatty acids and other polar matrix compounds [18]. Z-sep is a mixture of two sorbents, C18 and silica, coated with zirconium dioxide. Distinct classes of active sites make the lipid removal efficient when this sorbents is used [19]. Agilent Bond Elut QuEChERS Enhanced Matrix Removal Lipid (EMR-Lipid) is the new generation of sample preparation products for dispersive solid phase extraction (d-SPE) employed for highly selective matrix removal without impacting analyte recovery, especially for high-fat samples [20].

PDBRUA

Blank samples of olive oil were spiked with the mixture of analytes at 50 $\mu\text{g kg}^{-1}$, extracted, allowed to freezing out procedure and submitted to clean-up step. For clean-up using PSA, 2 mL of the extract was purified using 300 mg of magnesium sulfate and 50 mg of PSA; for clean-up employing Z-sep (d-SPE), 2 mL of extract were mixed with 500 mg of magnesium sulfate and 50 mg of Z-sep sorbent; for clean-up using Z-sep (cartridge SPE), 1 mL of extract was passed through a cartridge containing 40 mg of Z-sep solid and the collected fraction in acetonitrile was analyzed; for clean-up employing EMR-Lipid, the purification was done as described in Section 2.3. Fig. 1 shows the recoveries and mean relative standard deviation obtained for all clean-up methods evaluated at 50 $\mu\text{g kg}^{-1}$. In terms of recovery Z-sep using cartridges SPE showed the worst results with almost 40% of the pesticides no recovered properly followed by Z-sep using dispersive clean-up. When PSA and EMR were employed the best results were obtained, but PSA showed a mean RSD of 15%. For the EMR method none of the pesticides was completely lost and more compounds showed recovery percentage between 70 and 120% and mean RSD of 7%.

To evaluate the effectiveness of clean-up procedure the blank sample of olive oil was extracted employing EMR-Lipid clean-up and analyzed in a LC-QTOF system (6530 Accurate Mass QTOF-MS, Agilent Technologies, Santa Clara, USA) in full scan mode. To obtain the number of co-extractives present in the matrix the data were processed employing MassHunter software. The results can be accessed by visual comparison of full scan chromatograms in Fig. 2, where is possible to see the differences between the full scan chromatograms for each clean-up procedure. Fig. 3 shows the matrix components present in olive oil extract related to its retention times when evaluated the different clean-up procedures employed. As can be seen in Fig. 3, the EMR is the clean-up procedure where more co-extractives are present, but with the best recoveries results as well as acceptable levels for routine analysis. Taking these results into account, the EMR method was chosen as clean-up procedure for pesticide determination in olive oil.

3.2. Validation

3.2.1. Accuracy (recovery), precision and repeatability

In order to assess accuracy and precision, blank samples of olive oil were spiked with the pesticide mixture at 10, 20 and 50 $\mu\text{g kg}^{-1}$ with five replicates of each concentration. Table 2 shows the

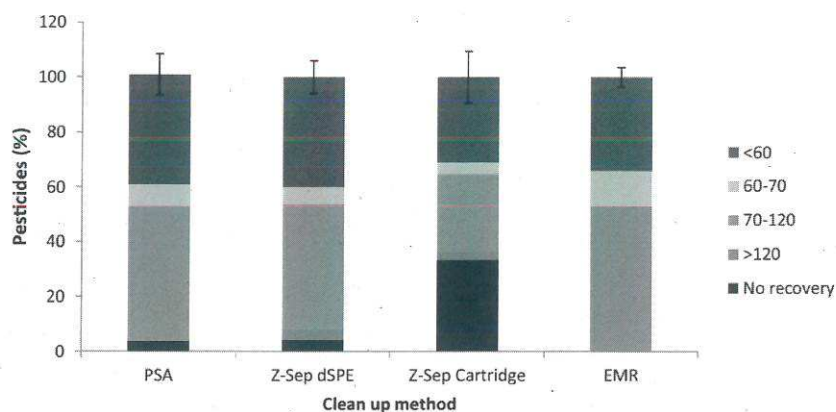


Fig. 1. Recovery (%) and RSD (%) for the pesticides determined by LC-QqQ-MS/MS employing four different clean-up methods.

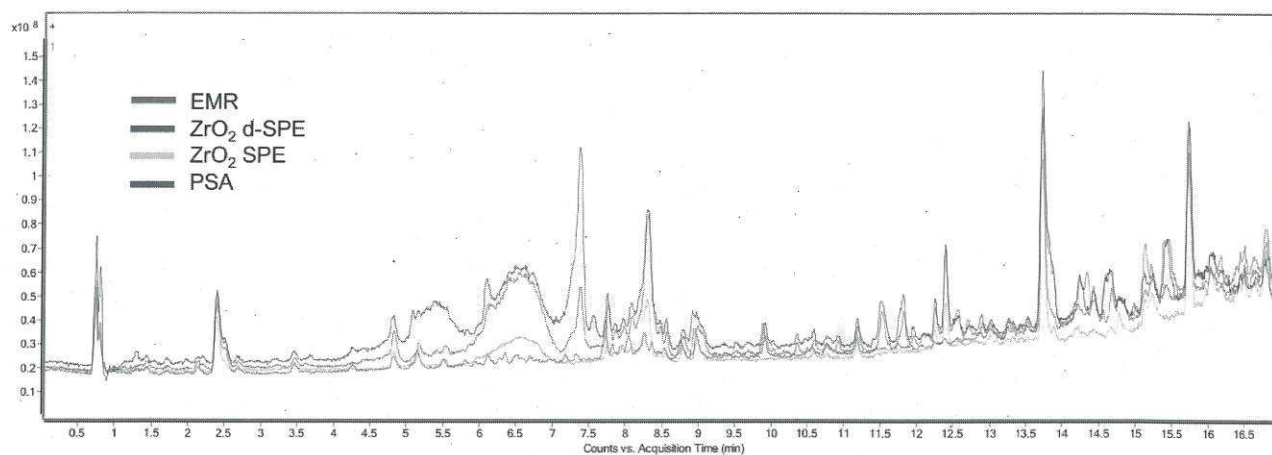


Fig. 2. LC-QTOF full scan chromatograms from olive oil extracts employing different clean-up procedures.

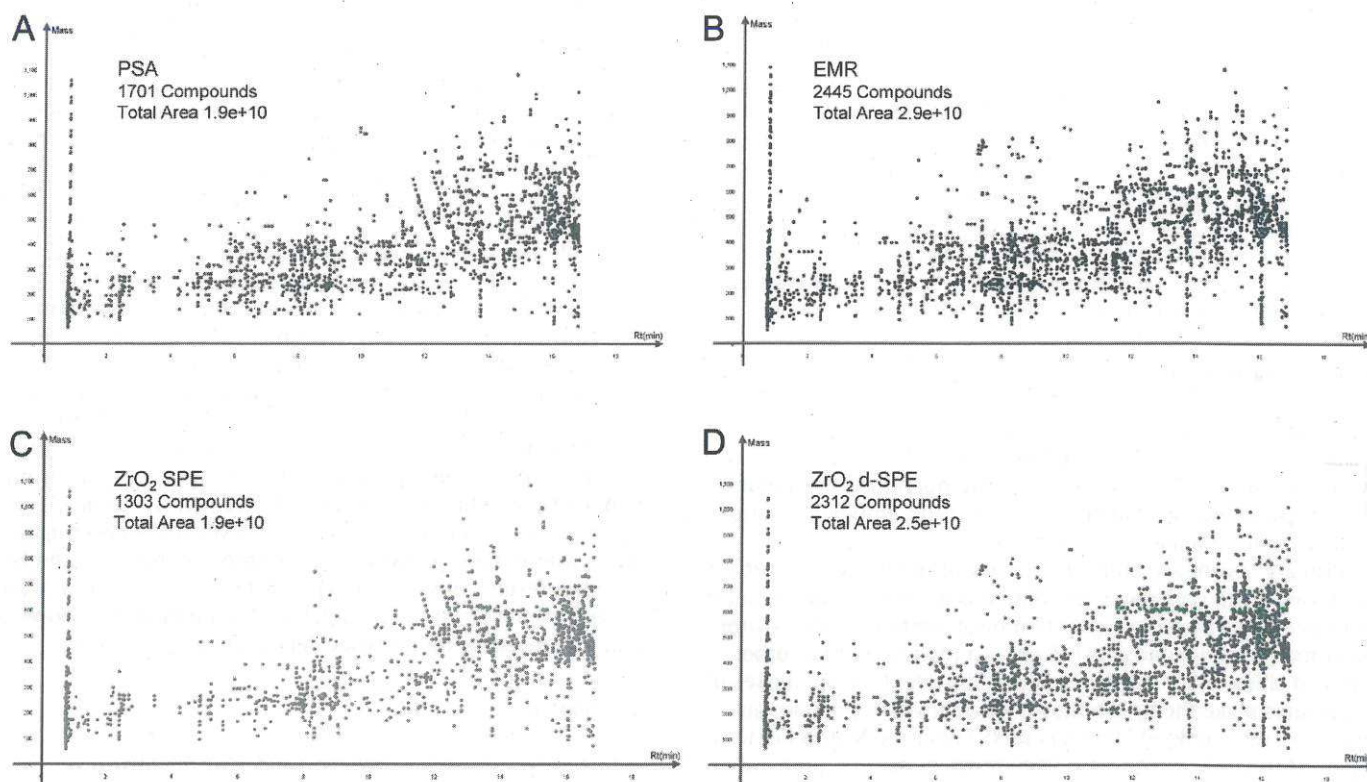


Fig. 3. Matrix components of olive oil when extracted employing PSA (A), EMR-Lipid (B), ZrO₂ cartridges SPE (C) and ZrO₂ dispersive SPE. Each point corresponds to one matrix component.

Table 2Recoveries, relative standard deviation at 10, 20 and 50 $\mu\text{g kg}^{-1}$ ($n = 5$) and LOQ for the pesticides spiked in blank olive oil sample.

Compound	Spike level (µg kg ⁻¹)						LOQ (µg kg ⁻¹)
	10		20		50		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
2,4-D	19	11	18	18	14	15	10
Acephate	48	47	46	7	69	6	20
Acetamiprid	116	2	94	8	91	2	10
Aldicarb	97	2	102	4	102	6	10
Aldicarb sulfone	144	7	100	13	90	5	10
Aldicarb sulfoxide	91	6	73	9	74	8	10
Azinphos-methyl	95	12	87	12	79	5	10
Azoxystrobin	100	8	103	8	110	3	10
Bifenazate	40	23	53	9	63	11	20
Bitertanol	59	11	61	7	61	8	10
Boscalid	89	4	74	5	85	3	10
Bromuconazole	60	10	60	7	57	7	10
Bupirimate	76	8	73	6	73	5	10
Buprofezin	51	6	50	9	48	4	10
Carbaryl	97	11	88	6	80	4	10
Carbendazim	37	11	38	6	41	6	10
Chlorantraniliprol	95	8	85	6	84	3	10
Chlorfenvinphos	92	6	82	10	83	7	10
Chlorpyrifos-methyl	76	13	58	11	57	4	10
Chlorpyrifos	57	13	48	6	51	2	10
Clofentezin	61	10	54	5	57	4	10
Clomazone	82	5	83	7	71	4	10
Coumaphos	87	8	87	8	80	7	10
Cyazofamid	108	10	94	5	119	3	10
Cymoxanil	101	3	104	6	101	3	10
Cyproconazole	56	4	53	7	58	6	10
Cyprodinil	34	7	37	5	37	4	10
Cyromazine	0	17	1	56	1	33	50
Demeton-S-methylsulfone	105	6	100	6	113	3	10
Demeton-S-methylsulfoxide	17	19	16	14	24	15	10
Diazinon	68	7	65	7	67	5	10
Diclorvos	84	7	104	6	93	5	10
Dicrotophos	70	10	75	5	80	7	10
Diethofencarb	92	8	89	5	87	4	10
Difenoconazole	70	7	69	5	66	5	10
Diffubenzuron	96	11	72	15	82	6	10
Dimethoate	115	5	105	8	95	3	10
Dimethomorph	107	6	88	7	86	6	10
Diniconazole	43	9	49	7	49	7	10
Dodine	2	17	2	74	1	54	50
Emamectin B1a	2	16	3	46	7	31	10
EPN	56	20	70	11	62	14	10
Epoxiconazole	89	5	85	8	78	8	10
Ethion	67	21	73	4	69	4	10
Ethirimol	7	11	10	38	17	24	50
Ethoprophos	74	3	72	9	68	4	10
Fenamidone	86	6	88	5	91	4	10
Fenamiphos	87	8	87	6	85	6	10
Fenamiphos – sulfone	93	3	89	7	95	5	10
Fenamiphos – sulfoxide	15	13	13	12	18	17	10
Fenarimol	58	9	51	6	51	6	10
Fenazaquin	20	23	22	7	22	3	10
Fenbuconazole	90	8	78	12	85	8	10
Fenhexamid	65	5	63	12	56	7	10
Fenoxycarb	88	6	76	8	79	3	10
Fenpropimorph	51	13	50	19	72	8	10
Fenpyrazamine	102	7	96	6	92	4	10
Fenpyroximate	48	27	50	6	49	4	10
Fenthion	65	15	75	11	70	10	10
Fenthion sulfoxide	117	4	101	5	93	4	10
Fipronil	102	5	103	8	100	2	10
Fluazifop	80	10	72	10	77	3	10
Flubendiamide	102	4	100	3	101	5	10
Fludioxonil	90	3	104	8	90	8	10
Flufenacet	97	8	91	5	92	2	10
Flufenoxuron	83	8	84	9	79	7	10
Fluopyram	107	6	100	9	93	5	10
Fluquinconazole	85	13	74	9	76	9	10
Flusilazol	84	8	71	7	76	4	10
Flutriafol	63	7	66	4	72	7	10
Formetanate	18	10	19	23	40	13	10
Fosthiazate	101	6	94	9	93	5	10

Table 2 (Continued)

Compound	Spike level (µg kg ⁻¹)						LOQ (µg kg ⁻¹)
	10		20		50		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
Haloxypop	83	8	76	9	87	11	10
Hexaconazole	36	16	41	5	45	9	10
Hexythiazox	35	18	38	7	36	3	10
Imazalil	23	12	27	25	46	15	10
Imidacloprid	116	5	94	7	96	3	10
Indoxacarb	100	6	100	8	97	9	10
Ioxonil	68	4	63	6	60	4	10
Iprovalicarb	92	8	85	7	88	7	10
Isofenfos methyl	93	7	86	5	79	3	10
Isoprocarb	101	4	86	3	75	2	10
Isoxaflutole	118	4	117	8	104	6	10
Kresoxim methyl	100	4	92	9	85	5	10
Linuron	78	5	82	8	68	7	10
Lufenuron	72	26	86	9	84	3	10
Malathion	108	5	94	7	101	4	10
Mandipropamid	125	10	115	6	104	3	10
MCPA	25	14	0	30	21	18	50
Mepanypirim	65	15	62	7	58	3	10
Meptyldinocap	16	42	18	15	38	9	20
Metalaxyl	91	8	86	8	95	5	10
Metconazole	52	9	49	5	51	6	10
Methamidophos	57	8	56	9	48	4	10
Methidathion	100	6	93	5	72	7	10
Methiocarb	82	4	79	7	72	7	10
Methiocarb sulfoxide	94	4	88	7	90	4	10
Methomyl	106	5	98	7	100	3	10
Methoxyfenozide	109	6	92	12	95	5	10
Metobromuron	83	1	78	5	71	2	10
Monocrotophos	120	6	96	6	77	5	10
Myclobutanil	85	7	82	7	82	3	10
Nitempyram	29	5	24	13	34	7	10
Omethoate	64	14	55	7	63	5	10
Oxadixyl	106	3	100	5	108	2	10
Oxamyl	134	6	102	10	89	5	10
Paclobutrazol	75	6	71	6	73	6	10
Paraoxon methyl	112	10	104	9	100	6	10
Penconazole	59	7	56	10	62	5	10
Pencycuron	65	11	61	5	59	4	10
Pendimethalin	42	18	46	7	47	5	10
Phenthoate	93	10	80	9	79	7	10
Phosalone	74	16	70	3	80	11	10
Phosmet	123	2	106	3	91	5	10
Phoxim	89	11	83	11	88	6	10
Pirimicarb	80	5	76	6	81	3	10
Pirimicarb, desmethyl-	50	10	48	5	57	5	10
Pirimiphos-methyl	67	6	64	7	63	4	10
Prochloraz	53	7	54	6	57	4	10
Profenofos	58	9	54	4	49	6	10
Propamocarb	2	20	3	52	6	26	50
Propaquizafop	69	21	71	7	73	3	10
Propargite	64	36	79	7	66	11	20
Propiconazole	66	15	66	6	65	4	10
Propoxur	110	3	94	9	92	3	10
Propyzamide	82	5	82	8	72	4	10
Proquinazid	17	20	18	6	17	5	10
Prothioconazole	2	65	4	48	4	23	50
Prothiofos	31	34	38	35	30	11	50
Pymetrozine	0	0	0	0	3	17	50
Pyraclostrobin	87	7	80	9	75	5	10
Pyrethrin	56	27	64	7	56	4	20
Pyridaben	33	34	36	6	38	5	20
Pyridate	31	43	29	25	20	16	50
Pyrimethanil	53	4	41	8	47	6	10
Pyriproxyfen	41	18	44	7	43	3	10
Quinoclamine	147	16	90	22	68	7	10
Quinoxifen	23	16	24	6	22	5	10
Quizalofop-ethyl	65	11	64	7	63	3	10
Rotenone	84	5	90	9	77	5	10
Spinosyn A	23	12	26	29	44	17	10
Spinosyn D	24	16	26	28	45	17	10
Spirodiclofen	59	5	60	6	56	6	10
Spiromesifen	75	25	77	10	69	5	10
Spirotetramat	98	7	86	8	90	6	10

Table 2 (Continued)

Compound	Spike level (μg kg ⁻¹)						LOQ (μg kg ⁻¹)
	10		20		50		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
Spiroxamine	50	9	46	16	68	10	10
Tebuconazole	51	10	57	6	56	7	10
Tebuconazole	103	5	93	6	92	4	10
Tebuconazole	46	13	50	4	44	4	10
Tebuconazole	60	11	65	7	68	4	10
Terbutylazine	60	4	62	4	58	4	10
Tetraconazole	93	5	92	5	94	5	10
Thiabendazol	32	7	33	9	44	9	10
Thiacloprid	109	2	98	3	101	2	10
Thiamethoxam	112	7	94	8	96	4	10
Thiobencarb	49	6	51	9	44	5	10
Thiodicarb	111	5	103	5	104	4	10
Tolclofos-methyl	63	7	58	17	56	11	10
Triadimenol	67	6	69	7	69	7	10
Triazophos	105	5	87	5	88	7	10
Trichlorfon	102	4	94	5	93	5	10
Trifloxystrobin	100	8	95	4	93	5	10
Triflumuron	77	7	77	8	75	5	10
Triticonazole	48	5	48	7	56	10	10
Zoxamide	84	6	78	3	70	3	10

recovery, RSD% and method LOQ results for the pesticides quantified by matrix matched calibration. As can be seen in the Table 2, the mean recovery for all spike levels was about 70% with mean RSD% of 11, 10 and 7 for the levels 10, 20 and $50 \mu\text{g kg}^{-1}$, respectively. The majority of compounds that did not fulfill the requirements for recovery demonstrated good RSD values, most of them lower than 10%. Taking these results into account, the linearity studies as well as evaluation of real samples were done applying procedural standard calibration approach (PSC), an alternative type of calibration that can compensate for low extraction recoveries and matrix effects [17].

Repeatability was evaluated by preparing standards solutions in olive oil blank extract at 10, 20 and $50 \mu\text{g kg}^{-1}$, and injected 5 times. As can be observed in Table S1 (see in the online version at DOI: <http://dx.doi.org/10.1016/j.chroma.2016.07.072>), all pesticides showed a RSD $\leq 20\%$ for the three evaluated concentrations and excellent reproducibility making the use of procedural standard calibration feasible.

3.2.2. Calibration curves, linearity and matrix effect

Linearity of analytical curves was evaluated via procedural standard calibration (PSC) by spiking blank olive oil portions at 10, 20, 50, 100 and $500 \mu\text{g kg}^{-1}$. From the 165 evaluated pesticides, 154 (93%) showed correlation coefficient (r^2) ≥ 0.99 for analytical curves evaluated using procedural standard calibration approach. However, the other 11 compounds showed $r^2 \geq 0.98$ demonstrating the good linearity of the method in the range from 10 to $500 \mu\text{g kg}^{-1}$. For analytical curves solutions in acetonitrile, just prothioconazole and pymetrozine showed $r^2 < 0.99$, both had $r^2 = 0.98$.

The same procedure (PSC) was applied for sunflower and soya oil in order to check the behavior of the analytes in different oils. For soya oil only six compounds showed $r^2 < 0.99$ but higher than 0.98. For sunflower oil, six compounds showed r^2 between 0.97 and 0.99 and all other compound demonstrated correlation coefficient > 0.99 . For all analytical curves evaluated in all oils the residuals were $< 20\%$.

For matrix effect evaluation, results obtained for analytical curves prepared in matrix extract by PSC for all oils studied were compared. As expected, the matrix effect was very similar among all oils studied as can be seen in Fig. 4 and in Table S1 (see in the online version at DOI: <http://dx.doi.org/10.1016/j.chroma.2016.07.072>). In the Table S1 (see in the online version at DOI: <http://dx.doi.org/10.1016/j.chroma.2016.07.072>).

org/10.1016/j.chroma.2016.07.072), the pesticides were grouped according to their chemical group in order to demonstrate the behavior of the different pesticides classes when evaluated in different oils. Taking the results obtained is possible to see the similarities of signals among all different oils and, with this, the quantification of pesticides is possible to be done with the same analytical curve for any oil.

3.2.3. Analysis of real samples

In order to apply the developed method in real samples, 15 samples (9 of olive oil, 3 of soya oil and 3 of sunflower oil) were purchased from local markets in Almería city, southeastern of Spain. Two samples of olive oil came from farmers that produce their own oil. From 9 olive oil samples from supermarket (extra virgin oil), four showed no contamination by any evaluated pesticide. In the other five at least one pesticide was found being dimethoate found in five samples. With exception of one sample that was contaminated with chlorpyrifos at $11 \mu\text{g kg}^{-1}$ all the other five olive oil samples showed contamination below the method limit of quantification ($10 \mu\text{g kg}^{-1}$). From the domestic olive oil samples, one of them showed contamination by phosmet at $194 \mu\text{g kg}^{-1}$, tetraconazole at $31 \mu\text{g kg}^{-1}$, chlorpyrifos at $23 \mu\text{g kg}^{-1}$ and fluopyran at $22 \mu\text{g kg}^{-1}$, besides tebuconazole where the residue was found below the limit of quantification of $10 \mu\text{g kg}^{-1}$. The other domestic olive oil was contaminated with chlorpyrifos methyl at $25 \mu\text{g kg}^{-1}$.

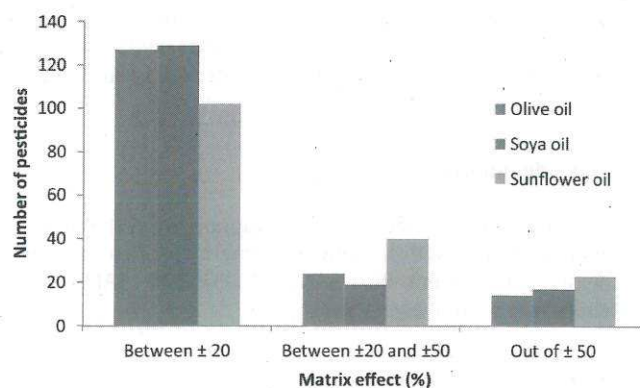


Fig. 4. Matrix effect evaluated by comparing slopes obtained from analytical curves prepared in acetonitrile and in matrix extract by procedural standard calibration.

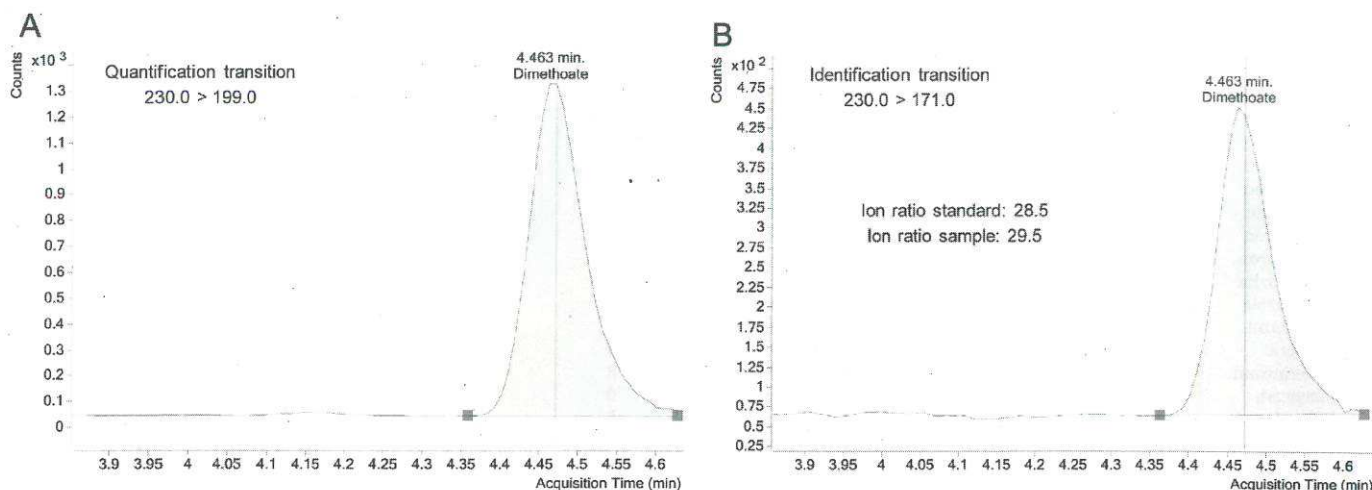


Fig. 5. SRM transitions peaks for dimethoate found in samples showing (A) quantification transition and (B) identification transition as well as ion ratio.

besides diazinon, dimethoate and pyriproxyfen below the LOQ. Dimethoate was the most found compound in olive oil samples but below the LOQ. Despite of that, in Fig. 5 is possible to see that despite the signal was below the LOQ the peak shape, quantification and confirmation transitions, retention time and ion ratio matched the criteria for quantification, demonstrating the good performance of the method.

For sunflower oil (three samples) no contamination was registered by any of the evaluated pesticides. On the other hand for soya oil, from three evaluated samples two of them showed contamination by azoxystrobin below the LOQ. For the other sample, no contamination was observed.

4. Conclusions

A multiresidue method for the determination of 165 pesticides in edible oils (olive oil, sunflower oil and soya oil) employing low temperature precipitation procedure and clean-up using Agilent Bond Elut QuEChERS Enhanced Matrix Removal-Lipid (EMR-Lipid) was developed and validated allowing quantification levels of $10 \mu\text{g kg}^{-1}$ for 91% of studied compounds. The use of EMR-Lipid in combination with freezing-out showed important advantages such as more pesticides with recovery between 70 and 120% range and no pesticide losses when compared with other clean-up procedures evaluated in this study such as PSA and Z-sep sorbent. The EMR-Lipid showed as the only drawback the use of more extract (5 mL) for clean-up procedure when compared to the other approaches. Validation was done using procedural standard calibration approach (PSC), an alternative type of calibration that compensate for recovery losses, showing good results. The method was employed to analyze 15 samples of oils sold in supermarkets as well as two domestic olive oil samples.

Acknowledgements

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GENERICI

1 Una cartella può contenere?

- ☐ File o cartelle
- ☐ Solo i file
- ☐ File e menù
- ☐ File e CD

2 Cos'è la RAM ?

- ☐ Un software
- ☐ Un file
- ☐ Una cartella
- ☐ Una memoria

WORD PROCESSOR

3 Quali comandi devo usare per spostare del testo?

- ☐ Sposta e Copia
- ☐ Copia e Incolla
- ☐ Copia e Sposta
- ☐ Taglia e Incolla

4 Formattare un testo significa ...

- ☐ Copiare il formato del testo
- ☐ Cambiare forma al testo
- ☐ Cancellare il testo
- ☐ Copiare il testo

5 Per NON salvare le modifiche fatte ad un documento?

- ☐ Dalla barra dei menu, clicco su File - Esci
- ☐ Chiudo il documento e dalla finestra che appare clicco su ANNULLA
- ☐ Dalla barra dei menu, clicco su File - Salva
- ☐ Chiudo il documento e dalla finestra che appare clicco su NO

Cognome Nome

Firma

6 Se applico l'elenco puntato, cosa succede ?

- ☐ Viene inserita una tabella con punti e simboli
- ☐ All'inizio di ogni paragrafo viene inserito un punto o un simbolo
- ☐ Viene inserito un elenco di punti e simboli
- ☐ All'inizio di ogni riga viene inserito un punto o un simbolo

FOGLIO DI CALCOLO

7 Come viene identificata una cella in Excel?

- ☐ Mediante la barra di stato
- ☐ Mediante la barra della formula
- ☐ Mediante la selezione della cella
- ☐ Mediante una riga e una colonna

8 Se in una cella digito: $=5+3*2$ e premo invio, il risultato è ?

- ☐ 11
- ☐ 0
- ☐ 16
- ☐ errore

9 Quale tipo di dati puoi inserire in singola cella?

- ☐ Solo numeri
- ☐ Solo numeri e formule
- ☐ Solo testo e numeri
- ☐ Testo, numeri e formule

10 Se in una cella digito ,35 (virgola tre cinque) e premo invio, cosa verrà visualizzato ?

- ☐ ,35
- ☐ #nome
- ☐ 0,35
- ☐ errore