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Determination of Pesticide Residues in Olive Oil Using QuEChERS Extraction and Liquid Chromatography–Orbitrap High-Resolution Mass Spectrometry: Comparison of Different Clean-Up Sorbents and Validation Study

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Abstract: The aim of this study was the optimization of the clean–up step in the widely applied QuEChERS method for the determination of 39 representative multiclass pesticides in olive oil with Ultra-High-Performance Chromatography–Orbitrap Mass Spectrometry (UHPLC-Orbitrap-MS). The analytical methodology combines the original version of QuEChERS extraction with two different clean-up-step approaches, using firstly a combination of Z-Sep$^+$, PSA and $MgSO_4$ and secondly EMR-lipid. The methods were compared for their efficiency in the removal of fats and co-extractives and their effect on the analytical performance characteristics. Both methods were evaluated in terms of linearity, matrix effects (ME), recovery, precision, limits of detection (LOD) and quantification (LOQ) and expanded uncertainty in three spiking levels of 30, 100 and 300 μg/kg. The recoveries ranged between 70–113% for 95% of analytes (RSD_r < 14%) when EMR-lipid was used as a sorbent, while in the case of Z-Sep$^+$/PSA/$MgSO_4$ recoveries ranged between 72–107% for 92% of analytes (RSD_r < 18%). ME showed low signal suppression for 77% of analytes in the case of Z-Sep$^+$/PSA/$MgSO_4$ and for 85% of analytes in the case of EMR-lipid. According to the results, both methodologies provided good analytical performances fulfilling validation criteria; however, the EMR-lipid sorbent showed better clean-up capacity (i.e., less matrix effects and lower variability in extraction recoveries) and validation parameter values for more analytes. The validated method was successfully applied to 30 olive oil samples from different regions of Greece. No residues have been identified in the analyzed samples.

Keywords: Z-Sep$^+$; EMR-lipid; QuEChERS; multiresidue pesticide analysis; olive oil; UHPLC-Orbitrap-MS

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1. Introduction

Olive oil is a product of great importance in Mediterranean Basin and especially in Greece; it comprises the principal source of lipids in the Mediterranean diet. Its demand has increased worldwide, due to related nutritional benefits in human health. The quality of olive oil depends on the quality of olive crops. Olive trees are prone to several diseases which are caused by insects and weeds. Pesticides are applied to crops at various stages of cultivation to prevent the deterioration or the destruction of product and trees by controlling agricultural pests and to improve tree quality [1–3].

The overuse or illegal use of pesticides in agricultural production and the possible presence of their residues in crops and their byproducts have raised public concern regarding the negative effects on the environment and potential human health risks. Within this framework, the European Union (EU) and Codex Alimentarius Commission have established maximum residue levels (MRLs) for pesticides in olives and olive oil using processing factors. These MRLs are usually in the low μg/kg range [2,4].

The monitoring of pesticide residues in olive oil is challenging due to the complex matrix with high triglyceride content and the possible presence of other lipophilic analytes. Consequently, the development of sensitive and reliable multi-residue methods is therefore essential [2,5]. Over the years, different methodologies have been reported for the extraction of pesticide residues from oil samples. These include liquid–liquid extraction (LLE), solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD), solid-phase microextraction (SPME) and gel permeation chromatography (GPC). However, these procedures present one or more significant disadvantages such as the laborious and/or time-consuming procedures, the requirements of large amounts of potentially hazardous solvents and low recovery for some analytes. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) sample preparation approach was adapted initially for the analysis of fruits and vegetables but later applied also to the analysis of pesticide residues in fatty matrices. It is usually conducted in two distinct steps, the extraction step and the clean-up step carried out by dispersive solid phase extraction (d-SPE). Several sorbents have been reported in the literature for the removal of co-extracted lipids prior to pesticide analysis of fatty products. PSA, C18 and GCB have been the most commonly used clean-up sorbents in the d-SPE step. However, alternative sorbents (e.g., Z-Sep$^+$, EMR-lipid and MWCNTs) have been also studied more recently in different fatty food commodities [2–4]. Z-Sep$^+$ is a zirconia-based C18 sorbent which enables Lewis acid/base interactions, while the Enhanced Matrix Removal (EMR-lipid) sorbent combines size-exclusion and hydrophobic interactions. Finally, the determination of pesticide residues is usually carrying out by hyphenated chromatographic–mass spectrometric techniques using GC-MS/MS, LC-MS/MS and, in some cases, microflow-LC-MS, depending on the target analytes [6–10]. More rarely, high-resolution (HR) instruments are used. LC-HRMS has gained popularity in recent years, enabling the identification and characterization of chemical structures. LC-HRMS can be applied with various screening strategies depending on the goal of each analysis. These strategies can be divided into targeted, non-targeted and retrospective approaches [11]. Targeted analysis is based on the determination of specific analytes of interest with the use of standard solutions, while suspect screening includes a list of possible contaminants considered from literature prediction model data. Finally, non-targeted screening comprises the identification of novel contaminants performed with post-acquisition data tools and can be carried also retrospectively.

The aim of this study was the optimization of the clean-up step in the widely applied QuEChERS method and the application of UHPLC-Orbitrap-MS for the determination of 39 representative pesticides from different chemical families in olive oil. The analytical procedure employed in this work was a combination of the original version of QuEChERS method with two different approaches for the clean-up step: (a) a combination of Z-Sep$^+$, PSA and $MgSO_4$ in different amounts and (b) EMR-lipid in different amounts were compared for their efficiency on analytical method performance. Methods were fully validated by determining linearity, ME, recovery, precision, LOD, LOQ and expanded uncertainty. According to the best of our knowledge, the comparison of different QuEChERS methodologies in the clean-up step and the use of HR-Orbitrap instrumentation along with full validation including uncertainty measurements has not been reported so far.

2. Materials and Methods

Chemicals and reagents: LC-MS-grade (purity $\geq$ 99.9%) acetonitrile (MeCN) with methanol (MeOH) and water were obtained from Fisher Scientific (Loughborough, UK). Ammonium formate, sodium chloride (NaCl) and acetic acid (HOAc) were obtained from Merck kGaA (Darmstadt, Germany) while anhydrous magnesium sulfate ($MgSO_4$) was obtained from Alfa Aesar ThermoFisher (Kandel, Germany). Z-Sep$^+$ Supel^{TM} Que and Bond Elut EMR-lipid were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Agilent Technologies (Waldbronn, Germany), respectively, whereas PSA was supplied by Chromatific (Heidenrod, Germany).

The selection of pesticides was performed according to their authorized use in olives and according to literature sources [12]. Pesticide analytical-quality standards (>99% purity) were obtained from Sigma-Aldrich (Steinheim, Germany). Individual stock standard solutions of each pesticide were prepared in LC-MS-grade acetonitrile at concentrations of 1000–2000 mg/L. Working standard solutions were prepared by proper dilution of the stock standard solutions with LC-MS-grade acetonitrile. All the solutions were stored in screw-capped glass vials at −20 °C.

Sample preparation-extraction procedure: An organic extra virgin olive oil supplied from the local market and stored at room temperature away from bright light was used for spiking pesticide concentration levels used on method validation experiments. The final extraction procedure employed was the original version of the QuEChERS method [13] using EMR-lipid and Z-Sep$^+$ as d-SPE clean-up sorbents. For the extraction procedure, 3 g of olive oil, 7 mL of H_2O and 10 mL MeCN (1% HOAc) was weighed into a 50 mL centrifuge tube and stirred by vortex for 1 min. Then, 4 g of $MgSO_4$ and 1 g NaCl were added and stirred by vortex for 1 min. The extract was centrifuged at 4000 rpm for 5 min and the supernatant was preserved for the clean-up step. The extraction procedure described above was common in both protocols which are compared. The recovery rates of pesticide residues in the middle spiking level (100 μg/kg) were used to evaluate the extraction efficiency.

Protocol I—Clean-up step using Z-Sep$^+$ as d-SPE sorbent: For Protocol I, which uses Z-Sep$^+$ as d-SPE sorbent, five versions which differed in the proportion of absorbent materials used were evaluated. More specifically, the following combinations were studied: (a) 50 mg Z-Sep$^+$, 50 mg PSA and 150 mg $MgSO_4$ for 1 mL of supernatant; (b) 50 mg Z-Sep$^+$ and 150 mg $MgSO_4$ for 1 mL of supernatant; (c) 25 mg Z-Sep$^+$ and 150 mg $MgSO_4$ for 1 mL of supernatant; (d) 50 mg Z-Sep$^+$, 25 mg PSA and 150 mg $MgSO_4$ for 1 mL of supernatant; and (e) 25 mg Z-Sep$^+$, 25 mg PSA and 100 mg $MgSO_4$ for 1 mL of supernatant. From the above protocols, the one which used 50 mg Z-Sep$^+$, 50 mg PSA and 150 mg $MgSO_4$ for 1 mL of supernatant provided for an efficient clean-up of the oil extract with the major number of pesticides having recoveries in the 70–120% range. For the clean-up step, 2 mL of the upper layer was transferred into a 15 mL centrifuge tube which contained 100 mg Z-Sep$^+$, 100 mg PSA and 300 mg $MgSO_4$. The extract was stirred by vortex for 1 min and centrifuged at 4000 rpm for 5 min. Afterwards, 1 mL of the upper phase was filtrated through a PTFE syringe filter (0.22 μm), transferred to a vial tube and submitted to UHPLC-Orbitrap-MS analysis.

Protocol II—Clean-up step using EMR-lipid as d-SPE sorbent: For Protocol II, which used EMR-lipid as d-SPE sorbent, two strategies which differed in the proportions of the absorbent material used were evaluated. These were as follows: (a) 1 g EMR-lipid for 5 mL of supernatant and (b) 0.5 g EMR-lipid for 5 mL of supernatant. The second protocol (0.5 g EMR-lipid for 5 mL of supernatant) was assumed to be more effective according to the recovery rates. For the clean-up step, an amount of 0.5 g EMR-lipid was added in a 15 mL centrifuge tube, activated with 2.5 mL H_2O and stirred by vortex for 1 min. Then, 5 mL of the acetonitrile phase of the extract was added, shaken for 1 min and centrifuged at 4000 rpm for 5 min. Afterwards, 5 mL of the supernatant were transferred in another 15 mL centrifuge tube which contained 1.6 g of $MgSO_4$ and 0.4 g of NaCl. The extract was shaken for 1 min and centrifuged at 4000 rpm for 5 min. Finally, 1 mL of the upper phase was filtrated through a PTFE syringe filter (0.22 μm), transferred to a vial tube and submitted to UHPLC-Orbitrap-MS analysis. The studied parameters were selected according to previously published approaches for the determination of pesticides in oil matrices [14–16].

UHPLC-Orbitrap-MS analysis: A UHPLC Accela LC system coupled with a hybrid LTQ-FT Orbitrap mass spectrometer was used for the analysis of pesticide residues in olive oil extracts (Thermo Fischer Scientific, Inc., GmbH, Bremen, Germany). The ionization of target analytes was performed by an electrospray ionization source (ESI) in positive mode. Full-scan ionization was applied with mass resolving power of 60,000 FWHM

and mass range of 120–1000 Da while a data-dependent acquisition based on Collision-Induced Dissociation (CID) was performed. Detailed operational parameters of the LTQ-FT Orbitrap instrument are reported in Table S1. Chromatographic separation was performed in a Hypersil Gold analytical column with dimensions 100 × 2.1 mm and 1.9 μm particle size. The mobile phases A and B consisted of water with 0.1% ammonium formate and methanol with 0.1% ammonium formate, respectively. The elution gradient started at 70% A (initial conditions), remained at these conditions for 1 min, progressed to 30% in 2 min, then decreased to 0% in 5 min, remained at 0% for 2 min and returned to the initial conditions at 7.1 min. Total run time was 10 min, flow rate was 250 μL/min and injection volume was 10 μL (Table S2). For the qualitative and quantitative analysis Thermo Xcalibur 2.1 was used.

Method validation: The validation of the studied methods was performed according to the EU guidelines (SANTE/12682/2019, SANTE/11312/2021) [17,18]. Analytical parameters evaluated were linearity, average recovery (as a measure of trueness), repeatability and reproducibility (as a measure of precision), ME, LOQs, LODs, expanded uncertainty in three spiking levels—30, 100 and 300 μg/kg—and the Horwitz ratio (HorRat). Linearity was estimated by calculating the correlation coefficient factor (r^2). For this scope, a calibration curve of seven concentration points (5 to 500 μg/kg) was prepared by spiking the analytes to blank matrix-matched samples. The average recovery values were determined by performing the above-described protocols (Protocols I, II) in five replicates using the calibration curve. The repeatability (intra-day precision) and reproducibility (inter-day precision) of each method were estimated by determining the intra- and inter-day relative standard deviation (% RSD_r and % RSD_R, respectively) through recovery studies using spiked oil samples at three spiking levels (30,100 and 300 μg/kg). Intra-day precision was assessed by five determinations at each spiking level in the same day, while inter-day precision was assessed by one determination at each spiking level for five days. LOQs and LODs used the equations LOQ = 10 × SD and LOD = 3.3 × SD, where SD is the standard deviation of spiked sample (n = 6) concentration at low levels (10 μg/Kg). Moreover, matrix effect (ME) was determined by comparing the slope of the calibration curves obtained from matrix-matched extracts and acetonitrile solvent. ME values were calculated by the followed formula:

$$\%\mathrm{ME} = \left[\left(\frac{slope\ of\ curve\ in\ matrix}{slope\ of\ curve\ in\ solvent} \right) - 1 \right] \times 100, \quad (1)$$

Expanded uncertainty (%U) was estimated in all three spiking levels using the following formulas:

$$\mathrm{U} = \mathrm{k} \times \mathrm{U}', \quad (2)$$

$$\mathrm{U}' = \sqrt{\mathrm{U}'(\mathrm{bias})^2 + \mathrm{U}'(\mathrm{precision})^2}, \quad (3)$$

where U is the expanded uncertainty, k is the coverage factor (k = 2), U′ is the combined standard uncertainty, U′(bias) is the uncertainty of the bias and U′(precision) is the uncertainty of reproducibility within-laboratory component. Finally, the Horwitz ratio (HorRat) was calculated as a performance parameter that reflects the acceptability of a chemical method of analysis with respect to a precision according to the followed formula [19,20]:

$$\mathrm{HorRat} = \mathrm{RSD_R}/\mathrm{PRSD_R}, \quad (4)$$

where RSD_R is the among-laboratory relative standard deviation and $PRSD_R$ is the predicted inter-laboratory relative standard deviation.

3. Results and Discussion

3.1. Identification and Quantification of the Target Pesticides by UHPLC-Orbitrap-MS

The identification of pesticide residues in virgin olive oil extracts was carried out using the retention time and accurate mass spectrum of each compound. The extracted ion chromatograms of selected pesticides are shown in Figure 1, while in Figure S1 are shown representative MS/MS spectrum of several pesticide analytes. The extracted ion chromatograms were obtained using a mass window of ±5 ppm in order to obtain appropriate selectivity. For quantification purposes, peak areas of the extracted ion chromatograms of the protonated molecules ([M + H^+]) were used for all of analytes (Table S3).

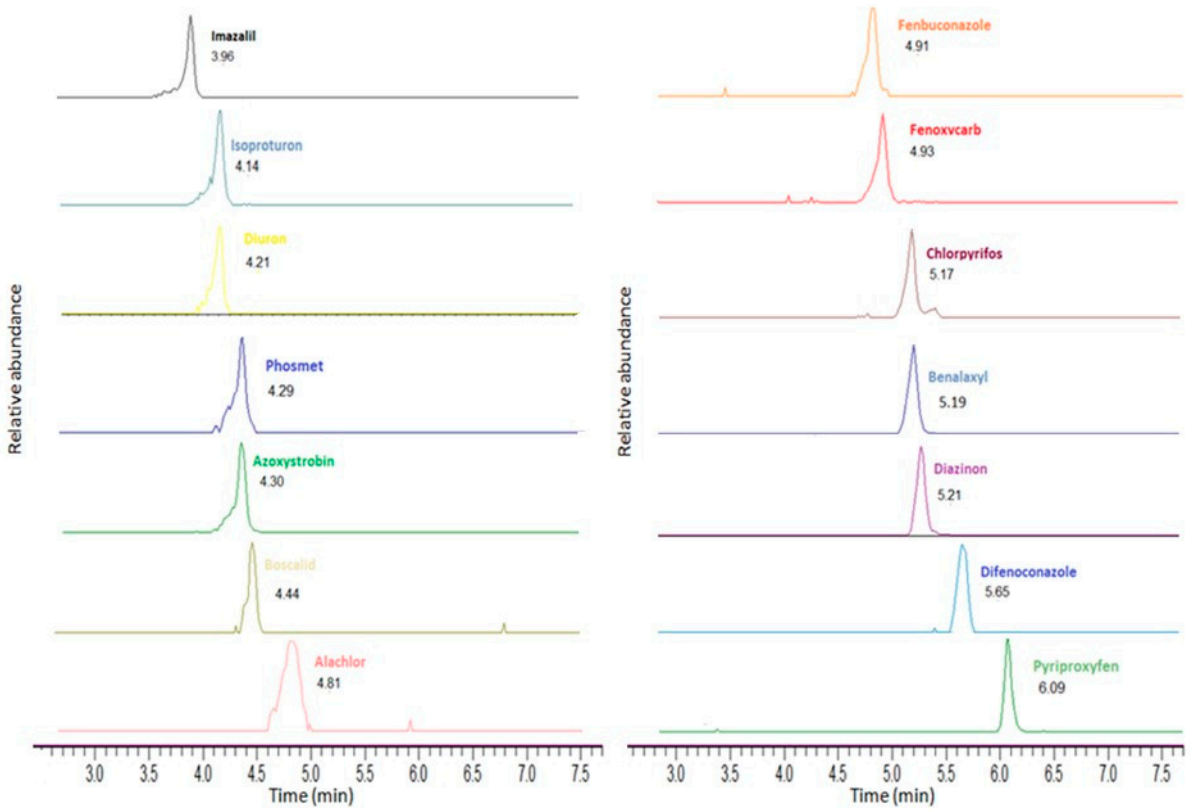

Figure 1. Extracted ion chromatograms of selected pesticides in olive oil at 10µg/kg fortification level in olive oil extract using EMR-lipid as a clean-up sorbent.

3.2. Clean-Up Optimization

For Protocol I, which used Z-Sep^+ as d-SPE sorbent, five versions which differed in the proportion of absorbent materials used were evaluated. The one which used 50 mg Z-Sep^+, 50 mg PSA and 150 mg $MgSO_4$ for 1 mL of supernatant provided for the efficient clean-up of the oil extract, with the major number of pesticides having recoveries in the 70–120% range. Figure 2 shows the percentages of pesticide recoveries for each version studied. Similarly, in Figure 3, the corresponding results for the two versions studied in the case of EMR-lipid are presented.

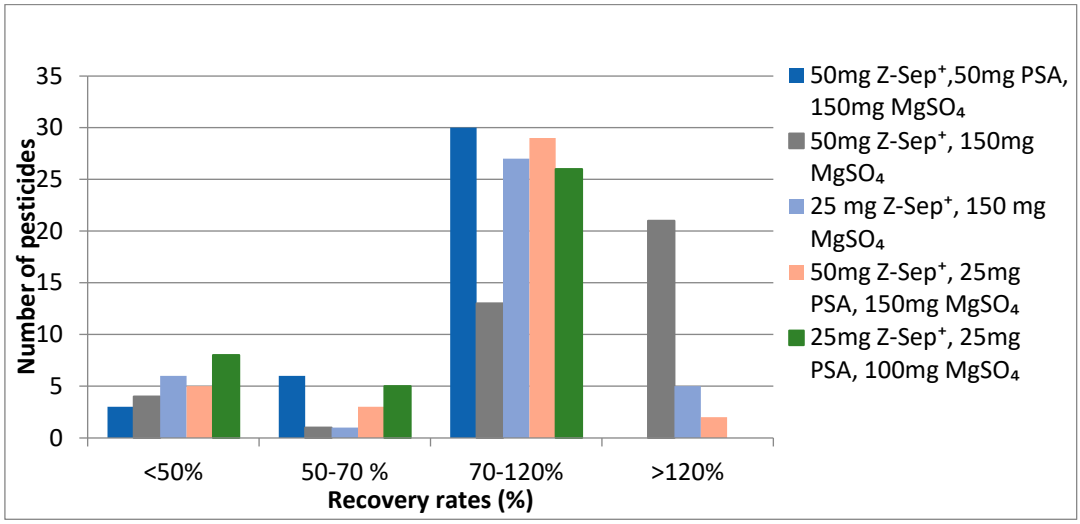

Figure 2. Recovery percentages of studied pesticides by applying the different clean-up versions of the QuEChERS method to olive oil samples using Z-Sep$^+$ as sorbent.

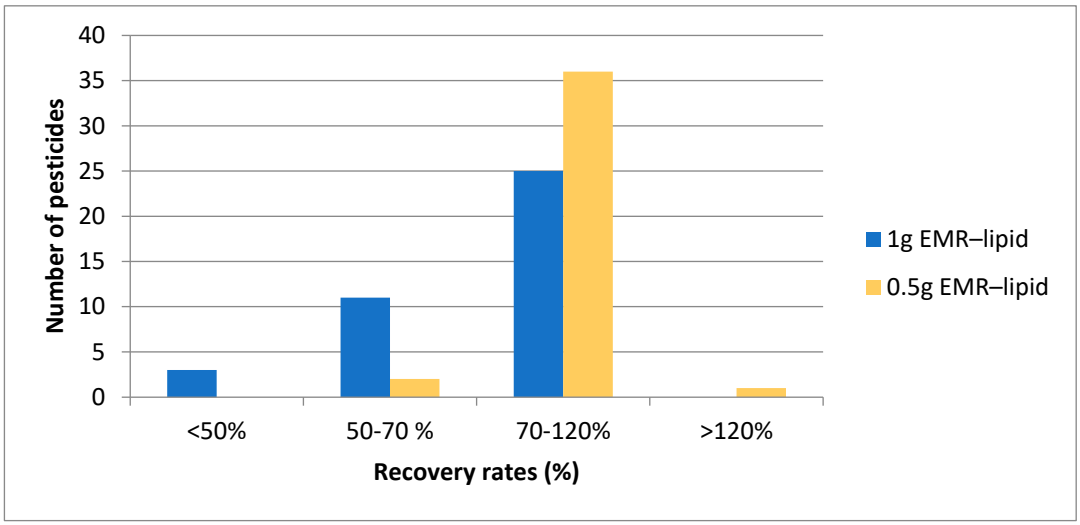

Figure 3. Recovery percentages of studied pesticides by applying the different clean-up versions of the QuEChERS method to olive oil samples using EMR-lipid as sorbent.

3.3. Method Validation

3.3.1. Accuracy, Precision and Repeatability

In order to assess accuracy and precision, blank samples of olive oil were spiked with the pesticide mixture at 30, 100 and 300 μg/kg with five replicates of each concentration. Table 1 shows the recovery and RSD% for Protocol I, while Table 2 shows the same results for Protocol II. As can be seen in Table 1 the mean recovery ranged between 72–107% for 92% of analytes, with an RSD_r lower than 18% and an RSD_R lower than 24%. The majority of compounds that did not fulfill the requirements for recovery demonstrated good RSD values, lower than 21%. For the case of EMR-lipid, as can be seen in Table 2, the mean recovery ranged between 70–113% for 95% of pesticide residues, with an RSD_r lower than 14% and an RSD_R lower than 17%. Only two pesticide residues (pyriproxyfen, λ-cyhalothrin) did not fulfilling the requirements. Protocol II provided better repeatability and reproducibility values than Protocol I. As it can be seen in Figure 4, where recovery rates are plotted against the octanol/water partition coefficient (K_{ow}) for the different sorbents tested, the behavior of analytes depended on the actual interaction with the clean-up sorbent and with the subsequent analyte retention. For example, in the case of imazalil

which has a $logK_{ow}$ equal to 3.82, the recoveries rate was 40.2% when Z-Sep$^+$ was used as d-SPE sorbent, while in the case of EMR-lipid, the recovery rate was 103.0%. In general, EMR-lipid showed less variation of recovery percentages in relation to the $logK_{ow}$ values.

Table 1. Recoveries (%) and RSD values at 30, 100 and 300 μg kg^{-1} (n = 5) for Protocol I (Z-Sep$^+$ as d-SPE sorbent).

	Intra-Day						**Inter-Day**					
	30 μg/kg		**100 μg/kg**		**300 μg/kg**		**30 μg/kg**		**100 μg/kg**		**300 μg/kg**	
	%Rec	**RSD_r**	**%Rec**	**RSD_r**	**%Rec**	**RSD_r**	**%Rec**	**RSD_R**	**%Rec**	**RSD_R**	**%Rec**	**RSD_R**
Alachlor	83.11	8.01	83.88	3.53	92.41	5.11	70.46	14.02	70.72	6.15	89.61	5.01
Azoxystrobin	90.51	3.71	98.87	2.47	101.70	3.59	77.44	16.54	80.59	11.61	105.45	4.10
Benalaxyl	72.14	4.86	92.03	2.00	99.86	4.73	72.12	2.91	75.76	13.52	103.66	3.70
Boscalid	79.09	6.53	83.56	1.83	91.47	2.82	72.07	1.61	73.10	9.93	94.28	3.32
Chlorfenviphos	101.49	5.50	109.13	2.22	107.11	5.61	84.29	17.53	91.39	12.38	110.95	5.21
Chlorpyrifos	79.81	3.68	58.99	2.82	73.72	5.72	74.60	10.05	53.33	10.90	73.81	8.16
Diazinon	107.22	5.15	122.74	1.27	115.75	5.30	99.80	16.66	102.70	10.95	119.16	4.26
Difenoconazole	75.56	4.89	70.31	2.35	81.07	5.65	89.59	23.10	70.08	11.97	83.13	3.71
Dimethoate	92.68	2.42	99.24	2.04	105.62	3.07	81.98	14.48	80.44	12.41	108.71	4.25
Diuron	77.83	3.50	78.29	2.62	89.63	2.91	73.13	6.30	74.73	11.39	73.13	6.30
Fenbuconazole	78.53	9.16	84.21	2.20	95.50	3.93	72.42	10.83	72.24	12.86	95.06	4.89
Fenoxycarb	74.56	7.35	82.59	2.19	97.50	4.50	70.32	4.81	70.36	9.07	98.00	4.46
Florasulam	102.99	5.35	75.58	7.80	83.52	4.99	88.73	10.32	72.27	14.10	90.68	6.67
Fluquinconazole	86.11	8.68	81.76	1.65	86.42	3.11	75.79	14.79	76.05	12.96	88.16	7.05
Iodosulfuron methyl	23.50	4.87	23.08	7.47	71.15	6.71	22.64	21.11	22.80	8.98	85.10	5.83
Imazalil	40.25	17.17	54.07	8.88	65.27	4.86	43.94	20.45	45.54	7.46	68.32	3.33
Deltamethin	82.34	13.39	58.17	8.20	80.60	1.67	70.20	11.25	42.65	13.06	86.59	12.16
Trifloxystrobin	92.46	5.85	99.41	0.70	99.80	5.04	77.77	17.34	82.56	11.95	103.65	5.00
Isoproturon	83.99	6.29	85.13	2.93	91.55	2.30	73.00	11.53	72.65	13.33	94.00	4.20
Kresoxim-methyl	81.50	6.54	90.77	1.56	99.14	5.26	75.73	5.95	73.51	11.58	102.82	5.03
Metalaxyl	93.55	1.82	99.28	2.67	102.51	3.75	84.77	18.61	83.72	10.72	107.54	4.02
Myclobutanil	80.94	8.73	86.44	1.27	92.84	5.02	79.95	7.92	70.60	13.20	97.68	3.52
Mefenpyr-diethyl	104.08	4.13	109.31	1.86	101.72	4.49	88.23	17.98	91.86	11.57	105.36	3.76
Nicosulfuron	92.33	6.93	42.92	21.98	52.64	3.59	83.85	11.86	49.82	10.88	58.28	8.38
Pirimicarb	80.08	3.80	86.98	3.89	99.70	3.22	77.05	4.54	71.63	11.24	101.40	3.07
Penoxsulam	82.03	5.15	74.18	7.00	83.77	4.21	72.49	12.44	68.09	12.12	87.77	5.91
Phosmet	89.77	2.62	90.14	1.67	98.17	3.49	80.41	9.10	72.53	12.90	100.71	3.07
Pyriproxyfen	40.47	3.63	52.68	3.81	59.63	2.21	34.35	19.46	42.41	15.79	58.20	3.33
Pyraclostrobin	89.51	5.99	96.90	1.17	95.73	4.99	75.75	18.85	81.36	11.77	101.58	3.60
Quizalofop-p-ethyl	63.46	4.06	72.32	2.29	82.18	5.68	56.49	1.17	72.38	12.54	84.75	5.39
Spinetoram	19.35	7.16	14.16	19.94	18.47	16.88	20.64	4.14	14.62	11.24	20.96	18.53
Spirotetramat	88.85	7.48	91.18	2.60	97.84	4.76	72.52	21.39	76.87	14.63	101.57	5.05
Terbuthylazine	73.56	2.15	78.16	2.12	85.14	3.71	73.12	2.49	74.37	11.21	87.95	2.65
Thiabendazole	80.10	1.44	60.76	7.03	73.73	9.13	62.17	0.86	62.74	10.29	80.22	4.97
Thiamethoxam	103.25	2.34	89.52	2.56	96.72	3.51	94.31	10.37	71.73	13.37	100.01	4.26
Tebuconazole	80.44	3.16	68.65	5.55	83.46	4.51	63.68	1.33	66.05	12.68	84.62	4.90
Thiacloprid	94.78	4.64	98.27	5.67	101.49	2.81	80.59	12.54	77.97	11.19	101.67	3.90
Tribenuron-methyl	82.34	3.33	85.60	1.36	95.70	2.67	71.62	14.99	71.03	15.47	96.47	2.86
λ-cyhalothrin	101.33	4.07	76.11	8.45	95.92	17.41	84.10	10.57	64.29	5.35	102.69	15.97

Table 2. Recoveries (%) and RSD values at 30, 100 and 300 μg kg^{-1} (n = 5) for Protocol II (EMR-lipid as d-SPE sorbent).

	Intra-Day						**Inter-Day**					
	30 μg/kg		**100 μg/kg**		**300 μg/kg**		**30 μg/kg**		**100 μg/kg**		**300 μg/kg**	
	%Rec	**RSD_r**	**%Rec**	**RSD_r**	**%Rec**	**RSD_r**	**%Rec**	**RSD_R**	**%Rec**	**RSD_R**	**%Rec**	**RSD_R**
Alachlor	104.51	3.38	90.34	2.71	92.81	1.88	99.11	4.36	88.81	7.83	88.58	7.17
Azoxystrobin	97.43	2.72	107.69	3.74	105.28	2.68	98.92	3.92	105.78	7.82	103.42	4.52
Benalaxyl	74.00	3.49	77.98	4.43	97.29	3.63	74.26	5.15	77.61	6.01	95.74	4.24
Boscalid	111.61	2.78	90.02	3.06	94.16	2.46	109.57	5.00	92.49	6.52	90.07	4.24
Chlorfenviphos	81.23	5.56	112.66	5.00	103.93	3.78	79.59	3.37	109.97	5.81	103.63	2.93
Chlorpyrifos	83.76	4.43	65.65	4.46	69.05	3.61	81.13	7.24	62.37	8.26	69.37	6.04
Diazinon	111.00	2.20	121.36	2.87	112.24	4.72	109.34	2.92	121.48	5.48	111.80	3.10
Difenoconazole	81.32	5.57	86.74	5.03	98.78	3.69	79.82	6.71	83.16	5.06	95.13	5.70
Dimethoate	113.33	3.61	86.55	2.66	108.36	2.04	114.95	3.05	84.70	5.63	104.82	6.11
Diuron	92.50	3.95	94.03	3.92	98.66	3.05	89.66	9.06	95.48	6.49	96.70	5.16
Fenbuconazole	109.39	2.92	99.23	6.03	99.43	3.15	102.96	6.34	99.92	6.59	95.30	5.30
Fenoxycarb	77.40	5.05	97.15	4.95	94.10	3.31	77.08	4.39	94.05	6.04	90.84	5.43
Florasulam	99.41	7.49	96.36	11.51	106.93	3.03	105.11	4.41	97.91	8.49	105.60	5.14
Fluquinconazole	102.39	13.93	86.26	8.26	89.72	3.60	91.62	16.12	88.58	7.86	85.48	5.51
Iodosulfuron methyl	81.15	9.06	112.43	5.97	106.61	6.18	76.96	9.89	119.75	3.51	101.11	1.62
Imazalil	103.04	2.44	104.00	4.13	104.95	1.59	101.98	2.29	101.33	4.81	103.56	4.20
Deltamethin	70.58	8.73	69.75	1.26	67.12	9.23	71.41	10.79	70.16	6.07	66.94	18.55
Trifloxystrobin	96.37	2.66	109.33	4.18	95.16	3.98	93.84	4.35	106.72	5.80	93.84	1.44
Isoproturon	97.77	4.27	104.54	4.21	102.66	3.14	95.53	5.96	103.73	5.32	101.82	3.14
Kresoxim-methyl	96.94	4.65	100.82	5.61	95.47	2.41	100.03	3.92	100.02	4.68	92.02	3.57
Metalaxyl	99.19	2.27	104.92	4.20	103.66	3.87	99.77	4.40	104.72	5.70	104.03	3.12
Florasulam	99.41	7.49	96.36	11.51	106.93	3.03	105.11	4.41	97.91	8.49	105.60	5.14
Myclobutanil	101.55	2.12	98.71	4.58	99.49	3.42	99.17	3.34	99.23	6.65	95.49	3.95
Mefenpyr-diethyl	75.22	4.13	75.11	4.38	93.11	3.46	73.88	4.66	74.90	5.07	93.22	2.96
Nicosulfuron	107.44	3.90	101.05	6.68	106.50	2.61	96.40	5.03	97.13	14.16	103.76	6.56
Pirimicarb	93.84	3.92	92.61	4.34	98.65	4.45	93.38	3.23	89.02	4.55	98.79	5.81
Penoxsulam	104.20	3.82	83.95	5.00	90.73	2.37	102.18	6.72	83.51	5.47	88.56	5.55
Phosmet	112.37	2.32	92.74	1.78	99.66	1.54	110.68	4.01	93.05	4.13	97.04	5.05
Pyriproxyfen	49.90	6.29	60.66	4.12	58.69	2.32	48.01	4.86	59.61	8.30	58.24	3.11
Pyraclostrobin	75.95	4.30	81.66	3.25	96.44	4.31	74.34	2.04	80.38	6.91	94.67	3.48
Quizalofop-p-ethyl	70.88	6.50	79.90	5.52	80.55	6.42	74.03	6.66	79.36	7.41	75.43	4.67
Spinetoram	100.45	2.60	92.00	7.25	101.09	2.91	100.11	5.42	92.54	8.25	96.91	2.95
Spirotetramat	95.26	6.15	104.02	3.96	103.72	3.81	86.37	4.70	104.39	7.79	103.52	4.19
Terbuthylazine	93.89	4.32	88.09	3.82	86.30	2.79	91.50	3.58	88.93	6.27	83.60	4.29
Thiabendazole	108.68	1.55	82.92	5.28	92.18	2.90	106.87	2.28	79.41	5.79	89.49	4.41
Thiamethoxam	103.35	6.66	108.93	2.23	101.83	2.06	100.15	7.22	105.40	3.47	98.02	3.90
Tebuconazole	97.86	1.94	103.34	6.84	98.53	3.81	95.55	4.77	103.44	6.42	96.85	3.59
Thiacloprid	107.01	3.54	94.54	5.00	99.79	1.93	105.42	4.32	93.79	5.02	97.29	6.37
Tribenuron-methyl	106.97	4.75	89.18	4.21	96.01	2.59	106.44	5.00	88.72	5.22	92.95	3.76
λ-cyhalothrin	68.42	2.03	71.77	8.86	80.13	11.76	71.09	7.52	79.20	3.82	78.45	23.19

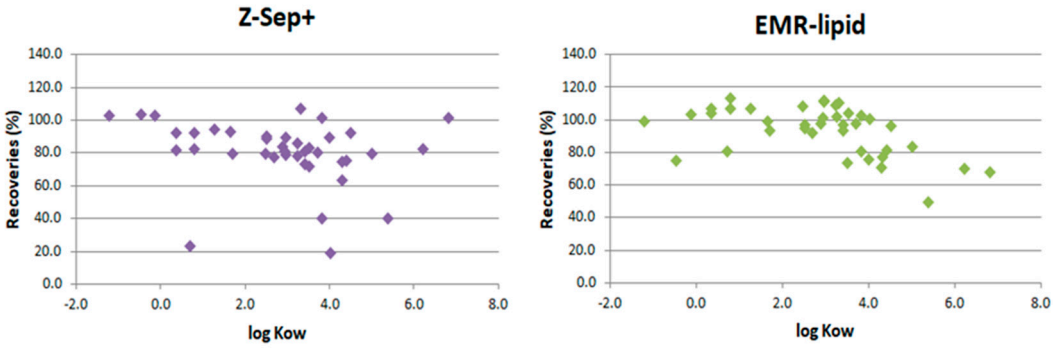

Figure 4. Plot of the recovery rates against $\log K_{ow}$ in olive oil with the different sorbents studied (Z-Sep$^+$, EMR-lipid).

3.3.2. Linearity, ME, LOQs, LODs and Method Uncertainty

Linearity was evaluated by spiking blank olive oil portions at 5, 10, 25, 50, 100, 250 and 500 µg/kg. The determination coefficients (r^2) were higher than 0.99 in all cases, showing that the linearity was adequate. For the protocol which used Z-Sep$^+$, $r^2 \geq 0.9904$, while in the case of EMR-lipid, $r^2 \geq 0.9906$ (Table S4). LODs and LOQs were assigned, taking into account signal to noise (S/N) ratio criteria (S/N of about 3 for LOD and S/N of about 10 for LOQ). LODs ranged between 0.32–3.27 µg/kg and LOQs between 0.98–9.92 µg/kg in the case of Z-Sep$^+$, while, in the case of EMR-lipid, LODs ranged between 0.44–2.58 µg/kg and LOQs between 1.32–7.83 µg/kg (Table 3). LOQ values were lower than MRLs set by the EU according to the regulation SANTE/12682/2019 in all cases.

Matrix-matched calibration curves at five different concentration levels (5, 10, 25, 50 and 100 µg/kg) were selected for method quantification to compensate ME. The results of the assessments of ME showed low signal suppression for 77% of analytes in the case of Z-Sep$^+$ and for 85% of the analytes in the case of EMR-lipid (Figure 5).

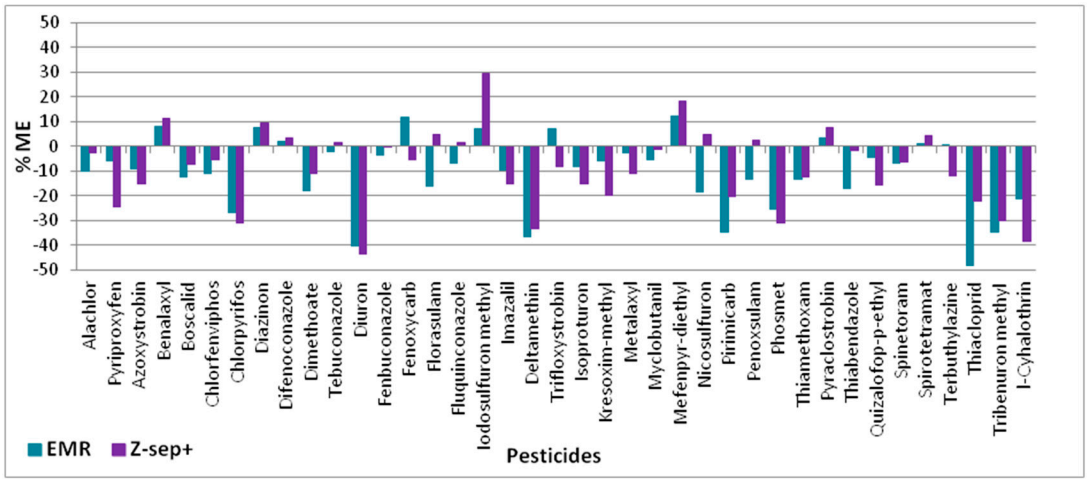

Figure 5. ME (%) of pesticides (expressed as calibration curve slope ratio) with the two compared sorbents.

U (%) was acceptable according to the SANTE regulation and ≤50% in both compared methods and in all spiking levels (30, 100, 300 µg/kg). For the Z-Sep$^+$ methodology, U% ranged between 4.1–20.5% while, in the EMR-lipid methodology, it ranged between 3.1–20.3% for the lowest spiking level (Table 4). The HorRat value was under 1, which demonstrates that the precision of both methods was better than expected.

Table 3. LOQs and LODs of the two compared protocols for each pesticide.

Pesticides	EMR-Lipid		Z-Sep$^+$	
	LOQ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	LOD (μg/kg)
Alachlor	3.17	1.05	5.99	1.98
Azoxystrobin	2.38	0.79	3.02	1.00
Benalaxyl	2.33	0.77	3.16	1.04
Boscalid	2.79	0.92	4.65	1.53
Chlorfenviphos	4.06	1.34	5.02	1.66
Chlorpyrifos	3.34	1.10	2.64	0.87
Diazinon	2.20	0.72	4.97	1.64
Difenoconazole	4.08	1.35	3.33	1.10
Dimethoate	3.68	1.21	2.02	0.66
Diuron	3.29	1.08	2.45	0.81
Fenbuconazole	2.87	0.95	6.47	2.13
Fenoxycarb	3.52	1.16	4.93	1.63
Florasulam	6.71	2.21	4.96	1.63
Fluquinconazole	7.83	2.58	6.73	2.22
Iodosulfuron methyl	6.61	2.18	3.97	1.31
Imazalil	2.26	0.75	6.22	2.05
Deltamethin	5.55	1.83	9.92	3.27
Trifloxystrobin	2.31	0.76	4.87	1.61
Isoproturon	3.76	1.24	4.75	1.57
Kresoxim-methyl	4.05	1.34	4.80	1.58
Metalaxyl	2.03	0.67	1.53	0.50
Myclobutanil	1.94	0.64	6.36	2.10
Mefenpyr-diethyl	2.79	0.92	3.87	1.28
Nicosulfuron	3.77	1.24	5.76	1.90
Pirimicarb	3.31	1.09	2.74	0.90
Penoxsulam	3.58	1.18	3.80	1.25
Phosmet	2.35	0.77	2.12	0.70
Pyriproxyfen	2.83	0.93	1.32	0.44
Pyraclostrobin	2.94	0.97	4.83	1.59
Quizalofop-p-ethyl	3.99	1.31	3.50	1.16
Spinetoram	2.35	0.77	1.25	0.41
Spirotetramat	5.27	1.74	6.00	1.97
Terbuthylazine	3.65	1.20	1.42	0.47
Thiabendazole	1.32	0.44	0.98	0.32
Thiamethoxam	6.20	2.04	2.17	0.72
Tebuconazole	1.71	0.56	1.44	0.48
Thiacloprid	3.41	1.12	3.96	1.31
Tribenuron-methyl	4.57	1.51	2.47	0.81
λ-cyhalothrin	2.82	0.93	3.67	1.21

Table 4. % U for each spiking level (30, 100 and 300 μg/kg) and HorRat for the two compared protocols.

	Z-Sep$^+$				**EMR-Lipid**			
	30 μg/kg		**100 μg/kg**	**300 μg/kg**	**30 μg/kg**		**100 μg/kg**	**300 μg/kg**
	U (%)	**HorRat**	**U (%)**	**U (%)**	**U (%)**	**HorRat**	**U (%)**	**U (%)**
Alachlor	17.98 (±1.44)	0.07	8.69 (±7.29)	5.73(±13.42)	7.69 (±2.41)	0.26	6.29 (±5.68)	2.65(±7.44)
Azoxystrobin	8.38 (±2.27)	0.25	5.78 (±5.71)	2.74 (±7.38)	6.16 (±1.80)	0.19	8.34 (±8.98)	4.20 (±12.74)
Benalaxyl	10.85 (±2.35)	0.26	4.85 (±4.46)	3.71 (±9.15)	7.80 (±1.73)	0.19	9.82 (±7.66)	3.02 (±8.00)
Boscalid	14.63 (±3.47)	0.38	5.06 (±4.23)	4.90 (±11.93)	6.37 (±2.13)	0.23	6.95 (±6.26)	3.38 (±9.46)
Chlorfenviphos	12.50 (±3.81)	0.41	5.88 (±6.42)	4.95 (±13.78)	12.46 (±3.04)	0.33	9.36 (±10.38)	3.14 (±9.45)
Chlorpyrifos	8.25 (±1.97)	0.22	8.35 (±4.86)	3.87 (±8.21)	9.96 (±2.50)	0.27	13.00 (±8.02)	5.20 (±10.61)
Diazinon	11.74 (±3.78)	0.41	4.29 (±5.27)	4.60 (±13.35)	5.03 (±1.67)	0.18	7.97 (±9.88)	3.00 (±9.40)
Difenoconazole	10.95 (±2.48)	0.27	5.60 (±3.94)	4.79 (±11.76)	12.51(±3.05)	0.33	7.28 (±6.43)	3.40 (±10.06)
Dimethoate	5.46 (±1.52)	0.16	4.66 (±4.62)	2.60 (±6.84)	8.27 (±2.81)	0.30	6.31 (±5.46)	4.12 (±13.29)
Diuron	7.84 (±1.83)	0.20	5.96 (±4.66)	4.60 (±12.16)	8.92 (±2.48)	0.27	6.13 (±5.85)	3.43 (±10.13)
Fenbuconazole	20.51 (±4.83)	0.53	6.44 (±5.42)	5.25 (±12.26)	6.67 (±2.19)	0.23	5.08 (±5.17)	3.46 (±10.17)
Fenoxycarb	16.42 (±3.67)	0.40	5.81 (±4.80)	4.49 (±10.12)	11.32 (±2.63)	0.29	7.36 (±7.29)	4.28 (±10.46)
Florasulam	12.17 (±3.76)	0.41	6.74 (±4.82)	5.48 (±13.36)	17.01 (±5.07)	0.55	17.18 (±5.27)	4.56 (±14.64)
Fluquinconazole	19.54 (±5.05)	0.55	5.73 (±4.68)	4.90 (±12.75)	13.14 (±4.11)	0.44	11.85 (±10.78)	8.50 (±11.86)
Iodosulfuron methyl	11.20 (±0.79)	0.08	14.18(±3.34)	4.40 (±9.95)	20.32 (±4.95)	0.54	11.71 (±10.41)	4.38 (±13.21)
Imazalil	7.01 (±0.78)	0.07	8.98 (±5.15)	5.68 (±10.57)	5.55 (±1.71)	0.18	9.91 (±9.53)	3.78 (±11.91)
Deltamethin	11.95 (±3.12)	0.29	10.59 (±5.67)	7.59 (±8.37)	19.49 (±4.13)	0.45	5.59 (±3.86)	4.84 (±8.99)
Trifloxystrobin	13.21 (±3.66)	0.40	3.56 (±3.54)	3.74 (±10.51)	6.02 (±1.74)	0.19	6.93 (±7.69)	4.93 (±13.66)
Isoproturon	14.13 (±3.56)	0.39	7.14 (±6.08)	5.67 (±13.31)	9.68 (±2.84)	0.31	10.0 (±10.50)	4.33 (±13.61)
Kresoxim-methyl	14.68 (±3.59)	0.39	4.66 (±4.23)	3.39 (±9.71)	10.53 (±3.06)	0.33	7.25 (±7.15)	3.39 (±9.36)
Metalaxyl	4.11 (±1.15)	0.12	5.93 (±5.89)	2.91 (±9.10)	5.16 (±1.54)	0.17	9.29 (±9.75)	1.84 (±5.88)
Myclobutanil	19.59 (±4.76)	0.52	5.04 (±4.36)	5.39 (±12.65)	4.82 (±1.47)	0.16	6.67 (±6.70)	3.46 (±10.49)
Mefenpyr-diethyl	9.41 (±2.94)	0.32	4.81 (±5.26)	4.39 (±11.77)	9.23 (±2.08)	0.23	9.78 (±7.35)	3.74 (±10.54)
Nicosulfuron	15.65 (±4.33)	0.47	15.93 (±8.15)	6.43 (±10.47)	8.90 (±2.87)	0.31	10.43 (±10.98)	3.59 (±11.50)
Pirimicarb	8.52 (±2.05)	0.22	8.72 (±7.58)	2.95 (±8.96)	8.86 (±2.49)	0.27	6.16 (±5.80)	4.63 (±14.08)
Penoxsulam	11.55 (±2.84)	0.31	7.30 (±4.97)	5.30 (±11.32)	8.69 (±2.72)	0.29	10.41 (±8.62)	4.96 (±13.60)
Phosmet	5.92 (±1.59)	0.17	3.91 (±3.52)	3.32 (±9.92)	5.32 (±1.79)	0.19	4.19 (±3.89)	3.71 (±11.10)
Pyriproxyfen	8.00 (±0.97)	0.11	5.43 (±2.82)	5.02 (±8.98)	13.91 (±2.08)	0.23	9.03 (±5.48)	5.42 (±9.54)
Pyraclostrobin	13.51 (±3.63)	0.39	4.09 (±3.96)	4.34 (±11.03)	9.62 (±2.19)	0.24	7.38 (±6.02)	4.91 (±12.54)
Quizalofop-p-ethyl	13.94 (±2.59)	0.29	5.53 (±3.99)	2.51 (±6.73)	13.94 (±2.96)	0.33	5.23 (±4.08)	2.76 (±6.55)
Spinetoram	15.71 (±0.91)	0.10	26.38 (±3.22)	23.59 (±14.0)	5.90 (±1.78)	0.19	5.90 (±5.60)	3.00 (±9.01)
Spirotetramat	16.87 (±4.50)	0.49	6.94 (±6.33)	4.29 (±12.96)	13.92 (±3.98)	0.43	9.34 (±9.72)	4.47 (±13.94)
Terbuthylazine	4.81 (±1.06)	0.12	4.73 (±3.70)	3.72 (±7.97)	9.76 (±2.75)	0.30	4.78 (±4.28)	4.33 (±11.18)
Thiabendazole	12.88 (±2.73)	0.08	5.66 (±3.27)	4.76 (±10.95)	3.09 (±1.00)	0.11	6.56 (±6.05)	5.63 (±12.14)
Thiamethoxam	5.33 (±1.65)	0.18	5.78 (±5.17)	3.04 (±7.42)	15.16 (±4.70)	0.51	6.45 (±7.02)	4.79 (±14.51)
Tebuconazole	15.91 (±3.30)	0.12	13.20 (±8.41)	3.40 (±8.69)	4.40 (±1.29)	0.14	8.39 (±8.79)	2.88 (±8.74)
Thiacloprid	10.51 (±2.99)	0.32	8.24 (±8.27)	3.52 (±9.16)	8.07 (±2.59)	0.28	10.24 (±9.82)	4.78 (±14.32)
Tribenuron-methyl	7.47 (±1.84)	0.20	3.40 (±2.91)	2.83 (±8.03)	10.84 (±3.48)	0.37	5.75 (±5.21)	3.94 (±11.15)
λ-cyhalothrin	9.25 (±2.81)	0.30	10.43 (±7.06)	6.22 (±9.32)	10.53 (±2.10)	0.23	13.16 (±8.74)	6.32 (±13.87)

The validated method, which used the EMR-lipid sorbent for the clean-up procedure, was then applied in 30 olive oil samples in order to evaluate the applicability of the QuEChERS-UHPLC-Orbitrap-MS method. The olive oil samples were collected from different regions of Greece (e.g., Peloponnesus, Crete, Athens and Central Greece). However,

the results of the analysis demonstrated the absence of pesticide residues (below LOD) in all studied samples.

4. Discussion

According to the data collected in this work, EMR-lipid provides some better analytical characteristics against Z-Sep$^+$. Briefly, the recoveries ranged between 70–113% for the 95% of analytes (RSD_r < 14%) when EMR-lipid is used as a sorbent whereas, in the case of Z-Sep$^+$, recoveries ranged between 72–107% for 92% of analytes (RSD < 17%). ME showed low signal suppression for both protocols (85% of the analytes in the case of EMR-lipid and 77% in the case of Z-Sep$^+$). LODs ranged between 0.44–2.58 μg/kg for the EMR-lipid protocol and between 0.32–3.27 μg/kg for the Z-Sep$^+$ protocol. LOQs were between 1.32–7.83 μg/kg for the EMR-lipid protocol and between 0.98–9.92 μg/kg for the Z-Sep$^+$ protocol. U (%) was acceptable according to the SANTE regulation and ranged between 3.1–20.3% for the EMR-lipid protocol compared to 4.1–20.5% for the Z-Sep$^+$ protocol for the lowest spiking level. Although LOQs, LODs and U (%) were quite similar in both methods, the EMR-lipid protocol has advantages in terms of recoveries and ME.

For comparison of the proposed method with previous studies conducted in olive oil using EMR-lipid as the clean-up sorbent, Lopez-Blanco et al. [6] determined 67 pesticide residues in different fatty matrices, including olive oil, using the QuEChERS AOAC 2007.01 method combined with UHPLC-QqQ-MS/MS. They compared three different approaches which differed in the sorbents used for the clean-up step [(a) 50 mg C18 + 50 mg PSA, 150 mg $MgSO_4$, (b) 50 mg Z-Sep$^+$ and (c) 1 g EMR-lipid]. The identification and quantification were performed in terms of recovery rates, ME and precision. According to the results, the EMR-lipid approach provided the best extraction efficiencies in the case of olive oil. The recoveries ranged between 70–120% for 82% of analytes with an RSD value lower than 10% for the majority of pesticide residues. LOQs were between 0.10 to 90 μg/kg allowing for their determination at the low concentration levels demanded by olive oil regulations in most cases. The results of the assessment of ME showed low signal suppression for 79% of analytes. On the other hand, in the case of Z-Sep$^+$, the recoveries ranged between 70–119% for 75% of analytes (RSD < 20%) while the results of the assessment of ME showed low signal suppression for the majority of analytes.

Another study performed by Dias et al. [1] determined 165 pesticides residues in different edible oils including olive oil using the EN QuEChERS method (1 g EMR-lipid) followed by a UHPLC-QqQ-MS/MS system. A validation study was performed in terms of accuracy, precision, linearity, LOQs, ME and repeatability. Recovery rates were between 70–120% for 53% of pesticide residues with an RSD value lower than 20% for 90.9% of analytes in the lowest spiking level, i.e., 10 μg/kg. LOQs were between 10 and 50 μg/kg, whereas ME showed low signal suppression for the majority of analytes. Moreno–Gonzalez et al. [2], were determined 162 multiclass pesticide residues in olive oil using QuEChERS method (1 g EMR-lipid) and nanoflow LC/ESI Q-Orbitrap-MS system. In this work recoveries ranged between 75–119% (RSD < 19%). ME were also evaluated showing a negligible effect for the majority of the analytes when a dilution factor of 50 was applied. Lowest concentration levels ranged from 0.05 to 50 μg/kg.

Moreover Moreno-Gonzalez et al. [21] determined 34 carbamates in olive oil and other edible oils using the QuEChERS method and UHPLC-MS/MS. For the clean-up procedure, they used 50 mg Z-Sep$^+$ and 50 mg $MgSO_4$ for 1 mL of supernatant. Recovery rates were between 72–110% for all the analytes with an RSD value lower than 7%. LOQs ranged from 0.13 to 2 μg/kg while LODs ranged from 0.03 to 0.60 μg/kg.

The method studied in this work provides similar or even better analytical performance characteristics when it is carried out for a full validation study including uncertainty measurements, which has not been reported from other researchers. Moreover, the use of different amounts of EMR-lipid for the clean-up step and the determination of new compounds (e.g., florasulam, penoxsulam) are also new developments in the pesticide residue analysis of olive oil. Finally, new data on the analytical performance of the QuEChERS

method combined with an HR-Orbitrap instrument—for comparison with the QqQ-MS/MS instruments that have been mostly used till now—is also presented in this study. Overall, the HR-Orbitrap-MS method has enhanced performance in terms of confirmatory and retrospective analysis capabilities compared to MS/MS methods while similar LODs and LOQs were obtained in most cases, with the HR-Orbitrap-MS method providing better performance in some cases due to the high sensitivity obtained for the diagnostic ions used for quantification.

5. Conclusions

In this work, multiresidue methods for the determination of 39 representative pesticide residues in olive oil were developed and compared. The analytical procedure empSloyed is a combination of the original version of the QuEChERS method with UHPLC-Orbitrap-MS. Regarding the QuEChERS procedure, two different d-SPE sorbents (EMR-lipid and Z-Sep$^+$) were evaluated. The analytical methods were satisfactorily validated according to EU guidelines (SANTE/12682/2019, SANTE/11312/2021) and compared in terms of linearity, average recovery (as a measure of trueness), repeatability and reproducibility (as a measure of precision), ME, LOQs, LODs, U (%) and the HorRat value in three spiking levels: 30, 100 and 300 μg/kg. The EMR-lipid methodology which provides slightly better performance than Z-Sep$^+$ was then applied in 30 real samples of olive oil and demonstrated the absence of the examined pesticides. Therefore, this method can potentially be applied for the monitoring of a wide range of pesticide residues in olive oil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su15118714/s1, Figure S1: Representative MS/MS spectrum of pesticide analytes with close retention times; Table S1: Operational parameters of LTQ-FT Orbitrap instrument; Table S2: LC—HR-MS elution program; Table S3: LC-MS analytical characteristics of the studied pesticides; Table S4: Calibration curve equation and correlation coefficient factor (r^2) of the compared protocols.

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