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Determination of pesticide residues in olives by liquid extraction surface analysis followed by liquid chromatography/tandem mass spectrometry

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SUMMARY: Nowadays, pesticides are essential in modern agriculture for crop protection, however, this use supposes a potential risk for human health and the environment. Traditional techniques of pesticide determination require the use of laborious and complex extraction methods to separate pesticides from the matrix, above all in fatty matrices like olives. For this reason, a new simple, rapid, cheap and selective method for the extraction and quantification of the most frequently used pesticides in olive growing has been developed. Pesticide determination was carried out by ultra-performance liquid chromatography (UPLC) coupled with triple-quadrupole tandem mass spectrometry (MS/MS). Mean recoveries were found in a range between 73 and 114% with relative standard deviations lower than 20% in most pesticides evaluated and the limits of detection (*LOD*s) and quantification (*LOQ*s) were lower than 4 $\mu g \cdot kg^{-1}$ and 8 $\mu g \cdot kg^{-1}$, respectively. Finally, this method was applied to the analysis of 25 olive samples where Dimethoate and Terbuthylazine were detected in some cases, but their results were lower than 15 $\mu g \cdot kg^{-1}$.

***KEYWORDS**: Food analysis; Liquid extraction; Olives; Pesticide residues; UPLC-MS/MS*

RESUMEN: ***Determinación de residuos de plaguicidas en aceitunas empleando análisis por extracción líquida de la superficie seguida por cromatografía líquida / espectrometría de masas en tándem***. Hoy en día los pesticidas son esenciales en la agricultura moderna para la protección de los cultivos pero su uso supone un riesgo para la salud y el medio ambiente. Las técnicas tradicionales de determinación de pesticidas requieren el uso de métodos de extracción complejos a fin de separar los pesticidas de la matriz, sobre todo en matrices grasas como las aceitunas. Por ello, se ha desarrollado un nuevo método simple, rápido, barato y selectivo para la extracción y cuantificación de los pesticidas más frecuentemente utilizados en el cultivo del olivo, empleando cromatografía líquida de ultra-resolución (UPLC) acoplada a espectrometría de masas (MS/MS). Las recuperaciones alcanzadas variaron entre el 73 y 114% obteniendo desviaciones estándar relativas inferiores al 20%. Los límites de detección (LD) y cuantificación (LQ) fueron inferiores a 4 y 8 $\mu g \cdot kg^{-1}$, respectivamente. Finalmente, este método fue aplicado en 25 muestras de aceitunas donde se detectaron Dimetoato y Terbutilazina en algunos casos pero con valores inferiores a 15 $\mu g \cdot kg^{-1}$.

***PALABRAS CLAVE**: Aceitunas; Análisis de alimentos; Extracción líquida; Residuos de pesticidas; UPLC-MS/MS*

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1. INTRODUCTION

Olive oil is the most important edible vegetable oil in the Mediterranean countries and is the base of the well-known Mediterranean diet. This popularity is due to its manifest sensorial characteristics and its healthy nutritional properties. In recent years, many studies have repeatedly demonstrated its role in the reduction in cardiovascular diseases, the prevention of atherosclerosis, the improvement of the nervous system and bone development, antioxidant and anti-aging properties or the prevention of tumours (Covas, 2007; Ruiz-Canela and Martínez-González, 2011).

Pesticides and crop protection products are commonly used in modern agriculture. Unfortunately, the misuse of pesticides can lead to food security issues, risking the health of consumers and the environment. Virgin olive oil is a product which, potentially, can contain pesticides if the processed olives contain this type of contaminants. Recent studies have analyzed the presence of pesticide residues in olive oil, olives and even in their washing water (Aramendía, *et al*., 2007; Cunha, *et al*., 2007a; Cunha, *et al*., 2007b; Cunha, *et al*., 2007c; García-Reyes, *et al*., 2007a; Guardia Rubio, *et al*., 2006; Guardia Rubio, *et al*., 2007a; Guardia Rubio, *et al*., 2007b; Guardia Rubio, *et al*., 2007c). The reason for the possible contamination by pesticides is mainly due to an inappropriate use of the products by farmers, who could use high doses of pesticides or also do not respect the guidelines for their usage, resulting in a contamination that could be significantly favored if flight olives are mixed with soil olives during harvesting. There are many types of pesticides used in olive cultivation, but in recent years the pesticides chiefly applied have been Diuron, Terbuthylazine, Endosulfan, Oxyfluorfen, Glyphosate, Diflufenican, Dimethoate, Phosmet and Chlorpyrifos. The use of Endosulfan, Diuron and Terbuthylazine has been restricted in certain areas but they have been occasionally found in some samples since they remain in the soil after application (Ferrer, *et al*., 2005; Guardia Rubio, *et al*., 2007c). Other herbicides such as Oxyfluorfen and Diflufenican or insecticides such as Phosmet, Chlorpyrifos and Dimethoate have high adsorption coefficients and therefore their decomposition is difficult, generating an environmental concern (Long, *et al*., 2005; Yuzhou and Xin, 2012).

Traditional methods for the determination of residues in olives or olive oil involve a lengthy analysis, especially in the separation of pesticide residues in the matrix due to its high fat content. Moreover, these traditional methods require a large amount of organic solvents, which are expensive and generate considerable wastes. The extraction methods usually applied are matrix solid phase dispersion (MSPD), liquid-liquid extraction using different solvents, gel permeation chromatography (GPC) or solid phase extraction (SPE) (Ferrer, *et al*., 2005; García-Reyes, *et al*., 2007a; Guardia Rubio, *et al*., 2006; Guardia Rubio, *et al*., 2007c). All of them use different detectors for further analysis, such as flame ionization detector (FID), nitrogen-phosphorus detector (NPD) or electron capture detector (ECD) (Amvrazi and Albanis, 2006), and in the last ten years the use of mass spectrometry (MS) or time of flight mass analyzer (TOF) have become more common (Angioni, *et al*., 2011; Cervera, *et al*., 2012; García-Reyes, *et al*., 2007b; Koesukwiwat, *et al*., 2010).

The current trend in the new methods of pesticide determination is the development of rapid, inexpensive and simple procedures. The main feature of these procedures is the use of fewer steps of analysis and the absence of interferences for a correct determination. In addition, the isolated extract must also comprehend most of the pesticides in a simple analysis. Pesticide multi-residue analysis is now a challenge due to the low levels present in the samples and the wide variety and different chemical family they belong to. Strict limits of detection are required for the quantification of pesticide residues in food samples, and therefore the use of very sensitive, selective, robust and accurate technologies is required.

Nowadays, the most commonly used extraction method for pesticide determination in food matrices is the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe), with different modifications depending on the fat content in the matrix (Lehotay, *et al*., 2010; Wilkowska and Biziuk, 2011). This method gives good results in terms of quality, speed, ease and cost. Thr method is based on a liquid-liquid extraction with MeCN, followed by a cleaning step using several absorbents. The original procedure (Anastassiades, *et al*., 2003) has been modified using various versions but the acetate buffer version has become the official method for the AOAC as the AOAC Official Method 2007.1 (Lehotay, *et al*., 2005; Lehotay, *et al*., 2007) and the version using citrate buffer has been taken as standard method EN 15662 by the European Committee for Standardization (CEN) (European Committee for Standardization, 2013).

However, to our knowledge this method has not been reported in the scientific literature to determine the following collection of pesticides: Amitrole, Chlorpyrifos, Diflufenican, Dimethoate, Diuron, Terbuthylazine, Phosmet and Oxyfluorfen on the olive surface, and it has only been described as applied to olive oil or olives treated with another set of pesticides (Cunha, *et al*., 2007a; García-Reyes, *et al*., 2007a; Gilbert-López, *et al*., 2010a; Gilbert-López, *et al*., 2010b; Gilbert-López, *et al*., 2010c). Unfortunately, in recent years these compounds have been found in real samples of olives (Guardia Rubio, *et al*., 2007c) and olive oil (Ballesteros, *et al*.,

2006; Sánchez, *et al*., 2006), either above or below their Maximum Residue Levels (MRL) and their rapid determination is necessary.

If the olives are contaminated with pesticides, it is expected that they are found on the surface of them because of an incorrect use of pesticide or, more than likely, owing to the contact of the fruit with the ground previously treated with pesticides. For these reasons, in this paper we propose a new method for pesticide determination based on liquid extraction surface analysis of the olive surface.

Liquid extraction surface analysis coupled with infusion nano–electrospray high–resolution mass spectrometry and tandem mass spectrometry (MS/MS), has previously been applied to the qualitative determination of surface chemical residues resulting from the artificial spraying of selected fresh fruits and vegetables with a sample of representative pesticides (Eikel and Henion, 2011). The surface sample is automatically extracted by a robotic micropipette using a small volume (1–3 μL) of solvent (80:20 methanol-water with 0.1% vol. acetic acid as a modifier) which was directly injected by infusion into the MS detector without chromatographic separation.

The aim of this paper was to develop a new simple, rapid, cheap and selective method for the extraction and quantification of the most frequently used pesticides in olive growing. For this purpose, a new method based on the liquid extraction surface analysis of olives was evaluated. In this method the whole surface of olives is extracted, and this extract is analyzed by LC-MS/MS. The method is simple, rapid and cheap, and was applied to the determination of 8 of the most important pesticides in olive-growing. The pesticides studied were three insecticides (Dimethoate, Chlorpyrifos and Phosmet) and five herbicides (Amitrole, Oxyfluorfen, Terbuthylazine, Diflufenican and Diuron). The levels of MRLs are shown in Table 1. The new method was evaluated using different solvents, times and speeds of extraction in order to choose the best possible analytical conditions for the multi-residue determination. The validation studies were applied and gave good results in spiked and real samples.

2. MATERIALS AND METHODS

2.1. Reagents and materials

All pesticide standards (purity higher than 99%) were obtained from Sigma-Aldrich (Madrid, Spain). All solvents (viz.) acetonitrile (99.8%), methanol (99.8%), formic acid (puriss.~98%) were LC/MS-grade and supplied by Sigma-Aldrich (Madrid, Spain). UV-Vis-grade acetone was purchased from Sigma-Aldrich (Madrid, Spain). Ultrapure water was produced by a Milli-Q Reference water purification system (Millipore, Bedford, MA, USA).

Stock standard solutions of individual compounds were prepared by exact weighing of 25 mg of the compound followed by dissolution in 50 mL of MeCN and then stored at −18 °C in the dark.

A multi-compound working standard solution (1 $mg \cdot L^{-1}$ concentration of each compound) was prepared weekly with appropriate dilutions of the stock solutions with MeCN and stored at 4 °C. This solution was used to prepare the calibration curves.

A multi-pesticide spiking solution was prepared by mixing appropriate dilutions of the stock solutions with MeCN:acetone (45:55) to obtain two concentration levels at 2 and 10 $mg \cdot L^{-1}$. Then, 5 mL of each one of them were used to fortify two samples of 500 g of olives contained in a tray with two concentration levels of 20 and 100 $\mu g \cdot kg^{-1}$ each.

2.2. Apparatus

An automatic shaker of oscillating movement Vibromatic, Selecta (Barcelona, Spain) was used to extract the pesticides from the olive samples.

Table 1. Pesticide residues at maximum residue levels ($mg \cdot kg^{-1}$) allowed in the olive: EU-MRLs Regulation (EC) No 396/2005. *MRL*s updated on 18/04/14 (DG SANCO, 2014)

Pesticides	*CAS Registry Number*	*Molecular Formula*	*Log K_{ow} (pH 7, 20 °C)*[1*]	*MRLs of* Table Olives ($mg \cdot kg^{-1}$)	MRLs of Olives for Oil Production ($mg \cdot kg^{-1}$)
Diflufenican	83164-33-4	C19H11F5N2O2	4.20	0.05[2**]	0.20
Chlorpyrifos	2921-88-2	C9H11Cl3NO3PS	4.70	0.05[3**]	0.05[4**]
Phosmet	732-11-6	C11H12NO4PS2	2.96	3.00	3.00
Diuron	10290-37-6	C9H10Cl2N2O	2.87	0.01[5**]	0.02[6**]
Terbuthylazine	5915-41-3	C9H16ClN5	3.40	0.05[7**]	0.05[8**]
Dimethoate	60-51-5	C5H12NO3PS2	0.704	2.00	2.00
Amitrole	61-82-5	C2H4N4	−0.97	0.05	0.05
Oxyfluorfen	42874-03-3	C15H11ClF3NO4	4.86	1.00	1.00

[*]The PPDB: Pesticide Properties Database. AERU. University of Hertfordshire. http://sitem.herts.ac.uk/aeru/footprint/index2.htm.
[**]Indicates lower limit of analytical determination.

A high speed vortex mixer Heidolph Reax Top (Nuremberg, Germany) was used to vortex-mix the vials prior to the chromatographic analysis.

2.3. Instruments

2.3.1. Liquid Chromatography

Chromatographic analyses were performed in a WATERS ACQUITY UPLC system (Waters, Manchester, UK) consisting of an Acquity UPLC binary solvent manager, an Acquity UPLC sample manager and an Acquity UPLC column heater. Chromatographic separation was performed using a Waters Acquity UPLC BEH C18 column (2.1 mm×100 mm) with 1.7 μm particle size (Milford, MA, USA). The column was maintained at 40 °C, with a mobile phase flow rate of 0.25 mL min^{-1}. The mobile phase contained water (A) and MetOH (B) both with 0.1% formic acid. A gradient elution was employed, starting at 10% B and rising linearly to 90% over 2 min. The composition was held at 90% B for 2min before being returned to the initial conditions of 10% B, followed by re-equilibration for 1.5 min and giving a 5-minute total cycle. The injection volume for each sample was 10 μL in "full loop" modus and the temperature inside the sample manager was maintained at 10 °C.

2.3.2. Mass Spectrometry

Mass detection was performed using an Acquity TQD tandem quadruple mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ionization interface (ESI) and operating in the positive ion mode. The ion source was operated at 150 °C with a capillary voltage of 1.0 kV. Nitrogen was employed for both the dissolvent and cone gases at 650 °C and 50 $L \cdot hr^{-1}$, respectively. The mode of acquisition was multiple reaction monitoring (MRM) at an argon collision gas pressure of 3.5×10^{-3} mBar. MRM conditions were optimized for each pesticide during infusion. Data acquisition and processing were performed using MassLynx 4.1 (Waters, Manchester, UK).

2.4. Samples and spiking procedure

2.4.1. Samples

Flight olives (cultivar *Picual*) were collected from the "*Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica* IFAPA Centro 'Venta del Llano'" (Jaén, Spain). Olive fruits with a maturation degree of 4–5 were directly collected from trees and they were used as a starting material for preparing working samples: blank samples, spiked samples and matrix-matched calibration standards for validation studies.

2.4.2. Spiking procedure

500 g of olive fruits were arranged on a small tray (25 cm of diameter) for the olives to be divided into, at least, two or three layers. Then, 0.5 ml of multi-compound pesticide spiking solution was sprayed onto the whole sample surface and let stand until the solvents were virtually evaporated. The olives were mixed in order to reach a random distribution and then were sprayed again. This process was repeatedly performed until all the olives were homogeneously sprayed with the pesticide spiking solution using a total volume of 5.0 mL. This process was carried out at two spiking levels. The spiking solution was prepared using a MeCN: acetone (45:55) mixture to ensure both a correct adherence and a rapid evaporation.

2.5. Liquid extraction surface analysis

30 g of olives were introduced into a Hybex stoppered borosilicate bottle (extraction flask) and then 30 mL of MeCN were added. After closing the flask, the bottle was shaken with an automatic shaker for 10 minutes at a speed of 750 oscillations per minute. A portion of the extract was collected with a syringe and filtered through a 0.2 μm PTFE-filter. The filtrate (1.0 mL) was transferred to a vial and analyzed in the UPLC-MS/MS system. The extracts were pre-filtered through a filter paper of a pore size of 0.45 μm under vacuum conditions when the samples contained a large amount of leaves, soil or sludge in order to facilitate their passage through the filter of 0.2 μm.

2.6. Method performance

The optimization study was carried out according to the univariate method. The precision and accuracy of the method were tested with spiked olive samples. Recoveries were determined for five replicates at two spiking concentrations (0.02 and 0.1 $mg \cdot kg^{-1}$). Matrix-matched multi-level calibration standards were used for the calibration.

3. RESULTS AND DISCUSSION

3.1. Optimization of LC-MS/MS conditions

The optimization of the MS conditions for the determination of selected pesticides was performed by examining the spectra in "full scan" and MS/MS obtained for each compound. Firstly, experiments were directly conducted by infusion of the MS standards in order to optimize the cone voltage conditions necessary to maximize the signal for each molecule. Then, the collision energy required for the fragmentation of the molecules was optimized.

The optimization of the precursor ion and product ions was carried out by the injection of 7 µL of the individual pesticide standard solution (4 µg·mL^{-1} in MeCN) directly into the mass spectrometer with a flow rate of 0.25 mL·min^{-1}. Different cone voltages were applied and when the optimal cone voltage was found, different collision energies were studied. The collision energy was adjusted to produce the highest intensity for the main fragment. The dwell time parameter was evaluated for each pesticide in the range of 5 to 200 ms.

Finally, the most intense transition was used as a quantifier while the other transition with less intensity was used as a qualifier peak for the confirmation analysis. The optimal conditions are summarized in Table 2.

3.2. Optimization of extraction conditions

Four variables were studied to obtain the best conditions for the liquid extraction surface analysis method: extraction solvent, sample/solvent ratio, extraction time and oscillation speed. The matrix effect and the number of necessary extraction steps were evaluated as well.

3.2.1. Matrix-matched calibration and Matrix Effect

The matrix effects in LC-MS with an electrospray ionization source must be studied in complex matrices like olives. This effect is produced when other compounds present in the matrix can interfere in the ionization of the target compound. Interferences can be caused by a decrease or enhancement of the analytical signal, which would cause difficulties concerning the correct detection and quantification of pesticides.

In all the methods previously described in the literature, olives are first crushed and then extracted with an appropriate solvent. These procedures provide a co-extraction of variable amounts of olive oil, which must be later removed as much as possible by applying of different clean up steps.

In the new proposed method, the olives are not crushed so that there should not be a co-extraction of oil. But considering that the liquid extraction surface analysis extract is directly analyzed without any cleaning steps, this potential effect has to be elucidated.

At first, it was assumed that the matrix effect in the surface analysis method would be low. However, other components present on the olive surface (waxes, triglycerides, pigments, etc.) might be co-extracted during extraction and incorporated into the final extract, affecting the correct detection of the target compounds. For this reason, the matrix effect was tested by comparing the slope of the calibration curves in matrix-matched or in the solvent. Then, the matrix/solvent slope ratio for each pesticide was calculated. Calibration curves in the matrix or in the solvent were made with the use of standards prepared at the concentrations of 0, 10, 25, 50, 75, 100, 150 and 300 ng·mL^{-1}.

The potential matrix effect can be quantified by comparing the slopes of the calibration curves prepared with or without the matrix. Taking into account the ratio between them, a classification of the matrix effect can be carried out, so that

Matrix Effect=100 * [1- (slope matrix / slope solvent)]

Based on the classification studied by B. Kmellár (Kmellár, *et al.*, 2008), and depending on the decrease /increase in the slope, different kinds of matrix effects could be considered: soft matrix effect, when the ratio is lower than 20%; moderate, from 20 to 50%; or strong when it is higher than 50% since this could mask the correct detection of the pesticides.

Chromatographic signal suppression was noted in all the pesticides. The matrix effect for some pesticides like Diflufenican, Terbuthylazine or Diuron could be considered as *soft* matrix effect, and other

TABLE 2. Pesticides analyzed by *LC-ESI-MS/MS positive mode, molar masses, MRM parameters, ion ratios and retention time data

				1st Transition (Quantification)		2nd Transition (Confirmation)				
Pesticide	**Molar Mass**	**Cone Voltage (V)**	**Precursor ion (m/z)**	**Product ion (m/z)**	**Collision Energy (eV)**	**Product ion (m/z)**	**Collision Energy (eV)**	**Ion Ratio**	**Dwell Time (ms)**	**t_R (min)**
Amitrole	84.04	45	85.06	43.30	16.00	57.30	14.00	0.51	50	0.99
Dimethoate	229.00	25	230.00	199.00	25.00	125.10	24.00	1.41	50	3.01
Phosmet	317.00	25	318.04	160.10	16.00	133.20	52.00	25.00	20	3.36
Diuron	232.02	40	233.05	72.20	31.00	160.00	33.00	28.00	15	3.36
Terbuthylazine	229.11	35	230.22	174.10	20.00	96.20	33.00	6.01	20	3.43
Diflufenican	394.07	48	395.22	266.20	44.00	246.20	44.00	1.29	20	3.65
Oxyfluorfen	361.70	33	362.00	316.00	10.00	237.50	20.00	15.85	20	3.78
Chlorpyrifos	348.93	33	350.03	198.25	23.00	107.00	56.00	0.62	20	3.94

*Liquid Chromatography coupled with mass spectrometry working in positive electrospray ionization (ESI).

compounds present in the matrix would have a small impact in the correct determination of these pesticides. However, other pesticides like Dimethoate, Phosmet and Chlorpyrifos should always be calibrated with the matrix, because its matrix effect was greater than 30%. The matrix effects for Oxyfluorfen and Amitrol were extremely strong (more than 60%) and it was very difficult to build a good calibration curve for these pesticides. As a conclusion, all the pesticides were determined from the standard matrix-matched calibrations. The results can be observed in Table 3.

3.2.2. Selection of the extraction solvent

In spite of the fact that MeCN is the most commonly used solvent for pesticide extraction in the analysis of olives, several potential solvents (or mixtures) were evaluated: MeCN, MetOH, MeCN/MetOH (80:20 v/v) and water/ MetOH (1:3 v/v). In order to select the best extraction solvent, the amount (weight) of matrix extracted by each solvent was studied as a first approach. For this purpose, the amount of solvent used for each extraction was 30 mL and the amount of olives selected was 30 g. The extraction was carried out by manual shaking for 5 minutes.

The absolute amount of extract obtained from each solvent was tested from four types of extraction solvents. MetOH extracted 13.2 mg of matrix per g of olives compared to 4.6 mg of matrix that the solvent mixtures extracted or 2.5 mg extracted by MeCN (values calculated as a solid weight after vacuum evaporation of an aliquot of the extract). With these results, MetOH initially could be discarded because the amount of matrix extracted by this solvent was much greater than that found for the rest of solvents. Nevertheless, MetOH was not discarded in order to evaluate its performance from other points of view.

The next parameter evaluated was the recovery of each solvent by extracting samples spiked at 100 $\mu g \cdot kg^{-1}$. The best recoveries were obtained when MeCN was used in the extraction. Poor recoveries were obtained using the other three extraction solvents in the study, which were not able to reach 30% and were therefore discarded.

Finally, the optimization of the other variables in the extraction method continued with the use of MeCN as extraction solvent.

3.2.3. Selection of the solvent/sample ratio

The next variable studied was the amount of solvent to extract the analytes, i.e., the solvent/sample ratio. For this purpose, 30 g of spiked samples (100 $\mu g \cdot kg^{-1}$) were extracted during 5 min by manual shaking in vessels containing 30, 90 or 150 mL of MeCN (w/v ratio 1/1, 1/3 and 1/5, respectively). A sample/solvent ratio of 1:1 was selected because increasing the solvent volume did not produce a better extraction yield.

3.2.4. Optimization of time and speed of oscillation

In order to automatize the extraction step in order to improve the repeatability of the process, the use of an automatic shaker was studied. Two experimental variables were studied: speed of oscillation and extraction time. For this purpose, samples spiked at the 100 $\mu g \cdot mL^{-1}$ level were analyzed, and the obtained recoveries were compared. Firstly, 5 speeds were tested using 5 minutes in each extraction: 100, 250, 500, 750 and 990 oscillations per minute (opm). Recoveries were shown to rise as the speed increased until a maximum speed of 750 opm. Recoveries of the tested pesticides decreased dramatically when the speed of oscillation was higher than 750 opm. Once the speed of 750 opm had been selected, the extraction time was optimized.

TABLE 3. Calibration parameters of matrix and solvent curves. The calibration range was 10–300 $ng \cdot mL^{-1}$

	Solvent Curve		Matrix Curve				
Pesticides	**Slope**	**Correlation Coefficient**	**Slope**	**Correlation Coefficient**	**Ratio Slope (Matrix/Solvent)**	**Matrix Effect**	**Type of Effect**
Diflufenican	84.20	0.9988	72.04	0.9984	0.86	14%	Soft
Chlorpyrifos	16.81	0.9971	10.28	0.9933	0.61	39%	Moderate
Phosmet	333.92	0.9980	217.81	0.9995	0.65	35%	Moderate
Diuron	321.87	0.9986	256.07	0.9986	0.80	20%	Soft
Terbuthylazine	4212.60	0.9985	3949.44	0.9959	0.94	6%	Soft
Dimethoate	302.26	0.9997	205.89	0.9996	0.68	32%	Moderate
Amitrole	30.69	0.9954	7.63	0.9991	0.45	55%	Strong
Oxyfluorfen	14.32	0.9980	0.39	0.9587	0.03	97%	Strong

Five replicates were made for the standard solutions. Matrix effect expressed as ratio between slope matrix-matched and slope solvent calibration.

For this purpose, 4 shaking times were tested: 1, 2, 5 and 10 minutes. It was noted that the recoveries increased according to an increase in time, reaching almost 100% recoveries after 10 minutes.

Finally, the effect of solvent/temperature during extraction was studied as well. Three temperatures were evaluated (25, 30 and 35 °C), while maintaining a constant speed of 750 opm for 10 min, but the respective recoveries did not show significant differences.

The final conditions of the liquid extraction surface analysis method were: MeCN as a solvent extraction in a 1:1 (w/v) ratio, and 10 min of extraction by shaking at 750 opm using a solvent temperature of 25 °C. These results are summarized in Table 4.

3.2.5. Optimization of number of extractions

In order to know if a single surface extraction was enough, or whether by applying a second extraction on the treated olives a greater amount of pesticides would be extracted, the recoveries of a successive extraction step were evaluated. The results are shown in Table 5. As can be seen, good recoveries were obtained, and reached a range from 70 to 99% for 6 of the 8 pesticides studied when a single extraction was applied. Recoveries between 73–102% were reached when a second extraction was used. It is noteworthy that the second extraction did not reach an appreciable amount of pesticides, and the remaining amount that could not be extracted with a single extraction step was 3%. However, since it is a small and constant value, it could be considered that a single extraction is enough to reach good recoveries for the whole of the pesticides.

Unfortunately, Oxyfluorfen and Amitrole determination were not successful, and this is probably because this extraction method was not effective enough to extract these pesticides from the olive surface.

3.3. Validation of the analytical method

In order to validate the proposed method, selectivity, linearity, repeatability, recovery and limits of detection (LOD) and quantification (LOQ) were tested according to the criteria set by the EU guidelines SANCO/12571/2013 (DG SANCO, 2013).

The selectivity was evaluated as to the possible presence of peaks in blank samples, free of pesticides. The absence of peaks of the target pesticides in their retention times in the chromatograms belonging to blank extracts confirmed the selectivity of the method, and therefore, there were no false positives signals in the samples.

The linearity of the method was evaluated with matrix-matched calibration standards at seven concentration levels ranging from 10 to 300 ng·mL^{-1}. The seven-point-calibration curves in solvent or in matrix were constructed and compared at the

TABLE 4. Summary of variables studied to optimize the liquid extraction surface analysis method. The variables finally selected were those that gave the highest recoveries of the pesticides studied

	Variable Studied	Conditions	mg·g^{-1} matrix	Recovery range	Optimal variable*
Extraction Solvent	MeCN	100%	2.5	>40%	**MeCN**
	MetOH	100%	13.2	<30%	
	MeCN/MetOH mixture	80:20 (v/v)	4.6	Values out of range	
	Water/MetOH mixture	1:3 (v/v)	4.6	<30%	
Solvent/Sample Ratio (weight/volume)	Ratio (w/v)	Sample Weight (g)	MeCN Volume (mL)	Recovery range (%)	Optimal variable
	1:1	30	30	44–78	**1:1 ratio**
	1:3	30	90	13–80	
	1:5	30	150	19–75	
Oscillation	Oscillation speed (opm)	Recovery range (%)	Oscillation time (min)	Recovery range**(%)	Optimal variable
	100	<10	0	<10	**750 opm**
	250	<50	1	50–83	**10 min**
	500	<70	2	57–92	
	750	70–98	5	83–97	
	990	37–50	10	89–103	
Solvent Temperature (°C)	Variables	No significant differences were found			Optimal variable
	25				**25**
	30				
	35				

*Final conditions of the liquid extraction surface analysis in bold.
**Excluding Oxyfluorfen and Amitrole.

TABLE 5. Average recovery (%), n=5; RSD (%) obtained by extraction of samples spiked at 100 μg·kg^{-1}, analyzed by *LC-ESI-MS/MS positive mode in one or two extractions

	1st Extraction (30 mL)		2nd Extraction (30 mL)		Overall (%)
Pesticide	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	
Diflufenican	99	18	3	14	102
Chlorpyrifos	79	16	3	10	82
Phosmet	93	20	2	20	95
Diuron	81	17	2	20	83
Terbuthylazine	96	13	3	15	99
Dimethoate	70	11	3	16	73
Amitrole	8	5	2	40	10

Notes: RSD, Relative standard deviation.
Liquid Chromatography coupled with mass spectrometry working in positive electrospray ionization (ESI).

10-25-50-75-100-150 and 300 ng·mL^{-1} concentration levels. Seven pesticides presented correlation coefficients higher than 0.99 and only one presented poor linearity in the range studied (Oxyfluorfen). The results are shown in Table 3.

The precision of the method was studied by performing repeatability experiments that were evaluated by applying the extraction procedure five times to the same sample. The repeatability was acceptable for all analytes so that RSDs were not higher than 20%. Diflufenican was an exception, because samples at a concentration level of 20 μg·kg^{-1} presented 25% RSDs as the most unfavorable case.

The method accuracy was evaluated through recovery studies and was determined by running five extractions from olive samples spiked with pesticides at 20 and 100 μg·kg^{-1} concentration levels as described in section 2.4. Mean recoveries lie within an acceptable range according to EU Guidelines, from 73 to 114%. Table 6 shows an average of recoveries at two concentration levels. The recoveries of Amitrole and Oxyfluorfen were discarded due to the fact that its LODs and LOQs were higher than the concentration levels evaluated.

The limits of detection and quantification were estimated as the lowest concentration level to reach a signal-to-noise ratio of three (S/N=3) and ten (S/N=10), respectively. Table 6 summarizes the values obtained during the validation of the method.

3.4. Application of the method to olive samples

To check the validity of the method, 25 samples of olives from different points of the Andalusian geography were analyzed by the proposed method. Some pesticides were found in the samples but none were above the Maximum Residue Limit (MRL) established by current European Legislation (DG SANCO, 2013). Particularly, Dimethoate and Terbuthylazine were detected in some samples, but the results were lower than 15 μg·kg^{-1}. The results are shown in Table 7.

TABLE 6. Average recovery (%), RSD (%) *LODs* and *LOQs* obtained by extraction of samples spiked at 20 and 100 μg·kg^{-1} levels (n=5)

	Spiking Levels					
	100 (μg·kg^{-1})		20 (μg·kg^{-1})			
Pesticide	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	***LODs* (μg·kg^{-1})**	***LOQs* (μg·kg^{-1})**
Diflufenican	79	10	82	14	0.40	1.00
Chlorpyrifos	83	10	80	20	4.00	8.00
Phosmet	98	4	114	18	0.20	0.80
Diuron	73	11	93	20	0.30	0.80
Terbuthylazine	96	4	94	18	0.03	0.06
Dimethoate	74	10	74	10	0.40	0.80
Amitrole	14	17	n.d.	n.d.	10.00	30.00
Oxyfluorfen	n.d.	n.d.	n.d.	n.d.	100.00	200.00

Notes: RSD, Relative standard deviation.
LODs and *LOQs* were calculated using a level of 0.1 μg·kg^{-1}.
LOD, limit of determination; LOQ, limit of quantification.

TABLE 7. Average results (average ± relative standard deviation) obtained after application of the liquid extraction surface analysis (n=3) in 25 olive samples that were collected from the soil. Samples were analyzed by LC-ESI-MS/MS in positive mode (results are expressed in $\mu g \cdot kg^{-1}$)

Sample	Pesticides	$\mu g \cdot kg^{-1}$
Sample 2	Terbuthylazine	14.59±1.35
Sample 5	Terbuthylazine	11.67±2.55
	Dimethoate	3.51±0.64
Sample 8	Terbuthylazine	8.31±1.09
Sample 22	Diflufenican	6.09±2.54
Sample 25	Dimethoate	7.00±1.33

The rest of pesticides evaluated were not detected in these samples.

4. CONCLUSIONS

A new rapid, simple and economical method, based on liquid extraction solvent analysis of the olive surface has been developed. The method employs MeCN as the extraction solvent with an automatic shaker. Pesticide quantification required matrix-matched calibration in order to avoid interference that could mask the correct determination of pesticides. To optimize the method, several variables were studied such as the type of solvent, the extraction time, the oscillation speed or the number of extractions. Optimal conditions for the liquid extraction surface analysis method were reached with MeCN as solvent extraction (1:1 w/v), 10 minutes of shaking at 750 opm and a single extraction. The results of the validation of the method in terms of linearity, repeatability, selectivity and recoveries were evaluated from samples spiked at two concentration levels, obtaining results between 73 and 114%.

The new proposed method is more rapid and simple than other pesticide extraction methods, because it uses a smaller number of analytical steps and consumes less volume of extraction solvent. Moreover, the new method is more representative because it uses a higher amount of sample (30 g).

Finally, the LODs and LOQs reached were small enough to apply this method in routine laboratories. Nevertheless, Oxyfluorfen and Amitrole could not be successfully quantified by this procedure because of their high LODs and LOQs.

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