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Analysis of Herbicides in Olive Oil by Liquid Chromatography Time-of-Flight Mass Spectrometry

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The application of liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS) for the identification and quantitation of four herbicides (simazine, atrazine, diuron, and terbuthylazine) in olive oil samples is reported here. The method includes a sample treatment step based on a preliminary liquid−liquid extraction followed by matrix solid-phase dispersion (MSPD) using aminopropyl as a sorbent material. A final cleanup step is performed with florisil using acetonitrile as an eluting solvent. The identification by LC/TOF-MS is accomplished with the accurate mass (and the subsequent generated empirical formula) of the protonated molecules $[M + H]^+$, along with the accurate mass of the main fragment ion and the characteristic chlorine isotope cluster present in all of them. Accurate mass measurements are highly useful in this type of complex sample analyses since they allow us to achieve a high degree of specificity, often needed when other interferents are present in the matrix. The mass accuracy typically obtained is routinely better than 2 ppm. The sensitivity, linearity, precision, mass accuracy, and matrix effects are studied as well, illustrating the potential of this technique for routine quantitative analyses of herbicides in olive oil. Limits of detection (LODs) range from 1 to 5 μg/kg, which are far below the required maximum residue level (MRL) of 100 μg/kg for these herbicides in olive oil.

INTRODUCTION

Olive oil is one of the most consumed food products in the Mediterranean countries. The positive effects of olive oil on health have prompted a demand for this product worldwide. "Virgin" olive oil is obtained from the fruit of the olive tree (*Olea europaea*) exclusively by mechanical and physical processes without any further treatment, which do not alter the olive-oil quality. The most extensively applied agrochemicals in olive plantations of Mediterranean countries (Greece, Spain, and Italy) are by far herbicides and insecticides. Although herbicides are mainly applied to soils, some residues can persist to the harvest stage, thus contaminating the olives picked up from the soil. This can cause the presence of trace amounts of these herbicides in olive oil. Consequently, both the European Union and the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations (FAO) have established maximum pesticide residue levels in olives and olive oil. Currently, various olive-oil pesticide residue regulatory programs are being carried out to up-date and to establish new and more stringent regulations concerning the maximum residue levels in these commodities. This fact has promoted the development of new analytical methodologies in order to provide enough sensitivity and specificity to meet these requirements in food samples such as edible oils, which have a complex matrix due to the high fat content of the extracts obtained after the sample treatment step.

Many multiresidue procedures employing different cleanup techniques and a variety of detection methods have been reported for the determination of pesticide residues in olive oil. The most commonly used methodology is based on gas chromatography (GC) (*1*−*6*) after a comprehensive cleanup step, in most cases based on liquid−liquid partitioning (*7, 8*), or gel permeation chromatography (GPC) (*9, 10*) to separate the low molecular mass herbicides from the higher molecular mass fat constituents of the oil, such as triglycerides. The preparation of oil samples for the determination of herbicides by GC requires the complete removal of the high-molecular-mass fat from the sample to maintain the chromatographic system in working order. Recently, a multiresidue method for the determination of triazines and organophosphorous pesticides using matrix solid-phase dispersion (MSPD) followed by GC/MS and ion trap MS techniques was reported (*11*). The use of HPLC has not been widely studied (*12*). In a recent work from our group (*11*), liquid chromatography coupled with ion trap mass spectrometry was used for the identification and quantitation of several herbicides in olives and olive oil samples.

In this study, we have explored the potential usefulness of liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS) for the quantitative analysis of herbicide residues in olive oil samples. Recently, LC/TOF-MS has been proven to

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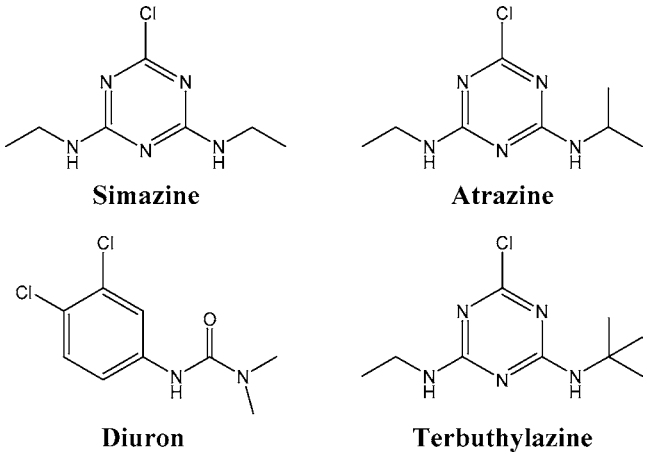

Figure 1. Structures of the selected herbicides.

Table 1. Effect of the Fragmentor Voltage on CID Fragmentation for LC/TOF-MS

		relative abundance		
compound	*m/z* ion	160 V	190 V	230 V
simazine	202 $[M + H]^+$	100	100	100
	174 $[M + H - C_2H_4]^+$		<5	10
	132 $[M + H - C_3H_6N_2]^+$		<5	17
atrazine	216 $[M + H]^+$	100	100	100
	174 $[M + H - C_3H_6]^+$		7	75
diuron	233 $[M + H]^+$	100	100	55
	255 $[M + Na]^+$	5	7	6
	72 $[M + H - C_6H_5NCl_2]^+$		12	100
terbuthylazine	230 $[M + H]^+$	100	100	20
	174 $[M + H - C_4H_8]^+$	5	30	100

be a sensitive and selective method for the determination and confirmation of pesticide residues in vegetables and fruits (*13*−*16*). The proposed methodology reported here consists of a preliminary liquid−liquid extraction step followed by matrix solid-phase dispersion (MSPD) using aminopropyl as a sorbent material. Finally, mass spectrometric identification and quantitation of the selected herbicides is achieved using LC/TOF-MS in positive ionization mode. The analytical methodology developed for the identification and quantitation of simazine, atrazine, terbuthylazine, and diuron is applied to olive oil samples. The identification by time-of-flight was accomplished with the determination of the accurate mass of the protonated molecule ($[M + H]^+$), along with the accurate mass of a characteristic fragment ion and the characteristic chlorine isotope cluster present in all the studied compounds. Several analytical parameters, such as sensitivity, linearity, precision, mass accuracy, matrix effects, and limits of detection (LODs) are evaluated. In general, this methodology compares well with the values generally achievable with triple quadrupole instruments in multiple reaction monitoring (MRM) mode, illustrating thus the potential of LC/TOF-MS for routine quantitative analyses of herbicides in olive oil.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Pesticide analytical standards were purchased from Dr. Ehrenstorfer (Ausburg, Germany). Individual pesticide stock solutions (200−300 μg/mL) were prepared in pure methanol and stored at −18 °C. HPLC grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). Petroleum ether was purchased from Panreac (Barcelona, Spain). Petroleum ether saturated with acetonitrile was prepared by adding 100 mL of acetonitrile to 500 mL of petroleum ether. Acetonitrile saturated with petroleum ether was prepared by adding 100 mL of petroleum ether to 500 mL of acetonitrile. A Milli-Q-Plus ultrapure water system from Millipore (Milford, MA) was used throughout the study to obtain the HPLC-grade water used during the analyses. The chemical structures of the herbicides studied in this work are shown in Figure 1.

Olive Oil Extraction. A methodology based on matrix solid-phase dispersion (MSPD), which has been recently developed and validated

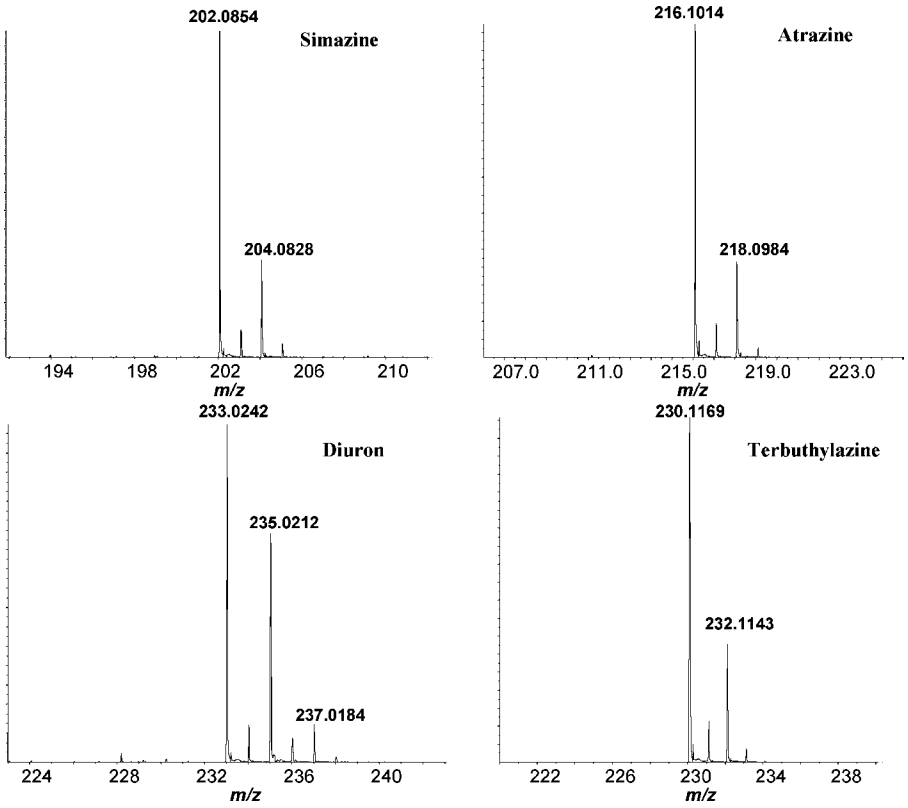

Figure 2. LC/TOF-MS accurate mass spectrum of the protonated molecules for the selected herbicides.

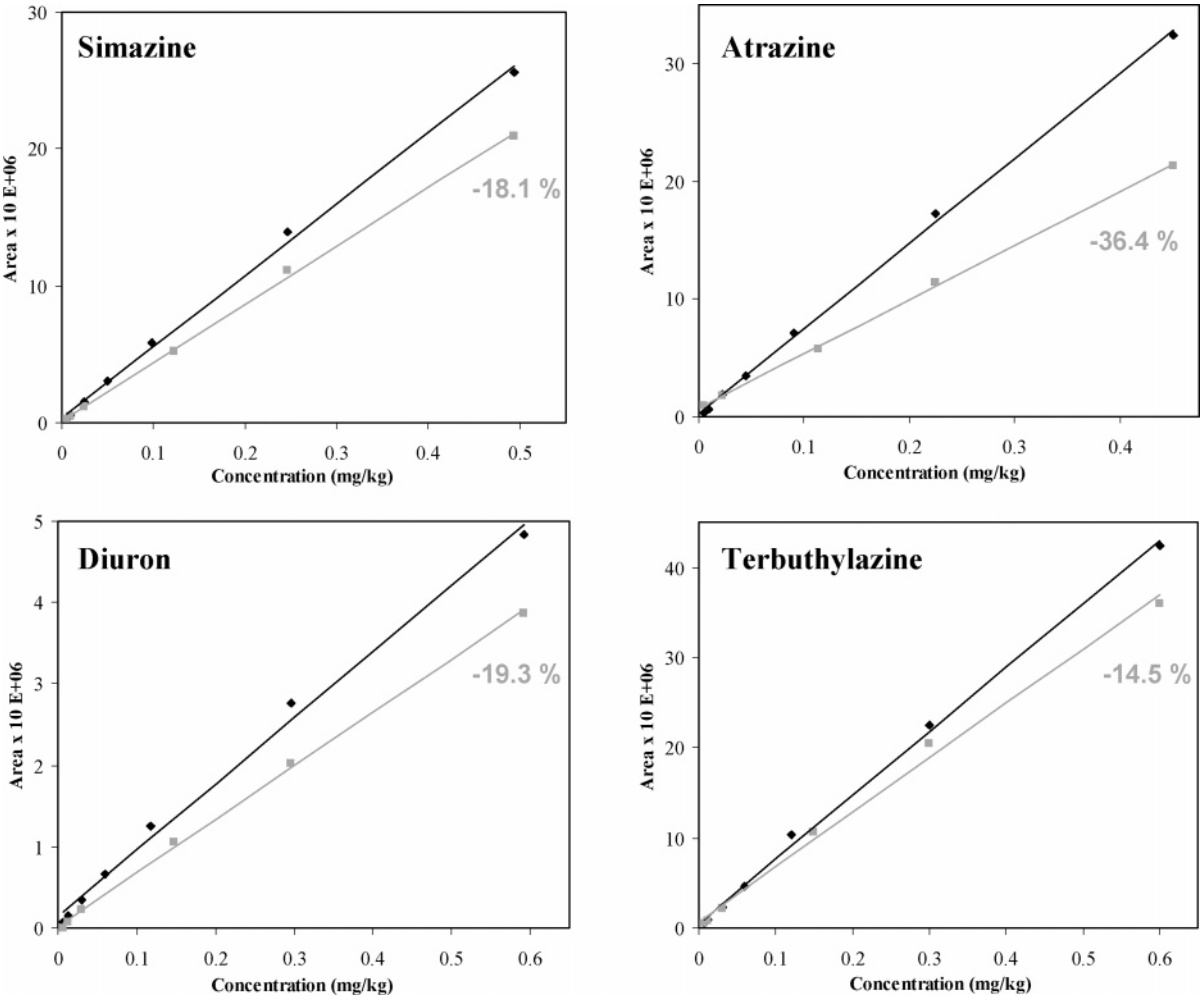

Figure 3. Calibration plots obtained from spiked olive oil samples (matrix-matched standards) versus solvent samples by LC/TOF-MS. The percentage of matrix-induced signal suppression is also included.

Table 2. LC/TOF-MS Accurate Mass Measurements for the Protonated Molecules and the Main Fragment Ions for the Herbicides Studied in Olive Oil Matrix-Matched Standard[a]

compound	empirical formula	theoretical mass	measured mass	error mDa	error ppm
simazine	$C_7H_{13}N_5{}^{35}Cl$	202.0854	202.0851	–0.3	–1.5
	$C_7H_{13}N_5{}^{37}Cl$	204.0824	204.0821	–0.4	–1.7
	$C_5H_9N_5{}^{35}Cl$	174.0541	174.0543	0.2	1.1
	$C_4H_7N_3{}^{35}Cl$	132.0323	132.0324	0.1	0.7
atrazine	$C_8H_{15}N_5{}^{35}Cl$	216.1010	216.1015	0.5	2.1
	$C_8H_{15}N_5{}^{37}Cl$	218.0980	218.0981	0.0	0.1
	$C_5H_9N_5{}^{35}Cl$	174.0541	174.0538	–0.3	–1.7
diuron	$C_9H_{11}N_2O^{35}Cl_2$	233.0243	233.0245	0.2	0.9
	$C_9H_{11}N_2O^{35}Cl^{37}Cl$	235.0213	235.0217	0.4	1.5
	$C_9H_{11}N_2O^{37}Cl_2$	237.0183	237.0187	0.3	1.3
	$C_9H_{10}N_2O^{35}Cl_2Na$	255.0062	255.0067	0.5	1.8
	C_3H_6NO	72.0444	72.0448	0.4	5.7
terbuthylazine	$C_9H_{17}N_5{}^{35}Cl$	230.1167	230.1169	0.2	0.9
	$C_9H_{17}N_5{}^{37}Cl$	232.1137	232.1140	0.3	1.1
	$C_5H_9N_5{}^{35}Cl$	174.0541	174.0543	0.2	1.2

[a] Fragmentor voltage 190 V. Spiking level 0.025 mg/kg. Chlorine isotope signals are also included in the table.

(*11*), was used for the extraction of the selected herbicides from olive oil samples. This method added a preliminary liquid−liquid extraction before the MSPD step.

(a) Liquid−Liquid Extraction Step (LL). An aliquot of about 5 g (ca. 5.5 mL) of olive oil sample was weighed in a 50 mL beaker where 15 mL of petroleum ether saturated with acetonitrile was added. The mixture was then transferred to a 100 mL separatory funnel, in which a two-step liquid−liquid extraction was undertaken. The first step was carried out by adding 25 mL of acetonitrile saturated with petroleum ether. The funnel was shaken vigorously for 4 min, and the acetonitrile phase was separated from the petroleum ether phase. Afterward, another 10 mL of acetonitrile saturated with petroleum ether was added to the petroleum ether extract. The mixture was shaken for 3 min again, and the acetonitrile phase was collected and added to the previous one. Finally, a 7 mL aliquot of the acetonitrile extract was transferred to a 10 mL glass test tube. The extract was then carefully evaporated up to a final volume of about 2 mL. This remaining extract was transferred to a glass mortar to be subjected to matrix solid-phase dispersion.

(b) Matrix Solid-Phase Dispersion (MSPD). The extract obtained in the previous liquid−liquid extraction step was homogenized with 2 g of aminopropyl-bonded silica (Bondesil-NH_2, 40 μm particle size, Varian Inc., Middleburg, The Netherlands) until a fine powder was obtained. The mixture was transferred to a commercially available minicolumn containing 2 g of florisil (12 mL Bond-Elut-Varian minicolumn, Varian Inc). This minicolumn was connected to a vacuum system for solid-phase extraction adjusting the flow to 3 mL/min. An elution step was carried out with 2 × 5 mL of acetonitrile. The final extract was evaporated until near dryness, being then dissolved with 1:1 acetonitrile/water. Prior to LC/TOF-MS analysis, the extract was filtered through a 0.45 μm PTFE filter (Millex FG, Millipore, Milford, MA).

Spiked olive oil samples at different concentrations were used for assessment of recoveries in a previous work (*11*). With this extraction methodology (LL and MSPD), recoveries for all the herbicides from olive oil were in the range from 81% to 111% (*11*).

LC/TOF-MS Analyses. The separation of the selected herbicides was carried out using an HPLC system (consisting of a vacuum degasser, an autosampler, and a binary pump; Agilent Series 1100, Agilent Technologies, Santa Clara, CA) equipped with a reversed phase

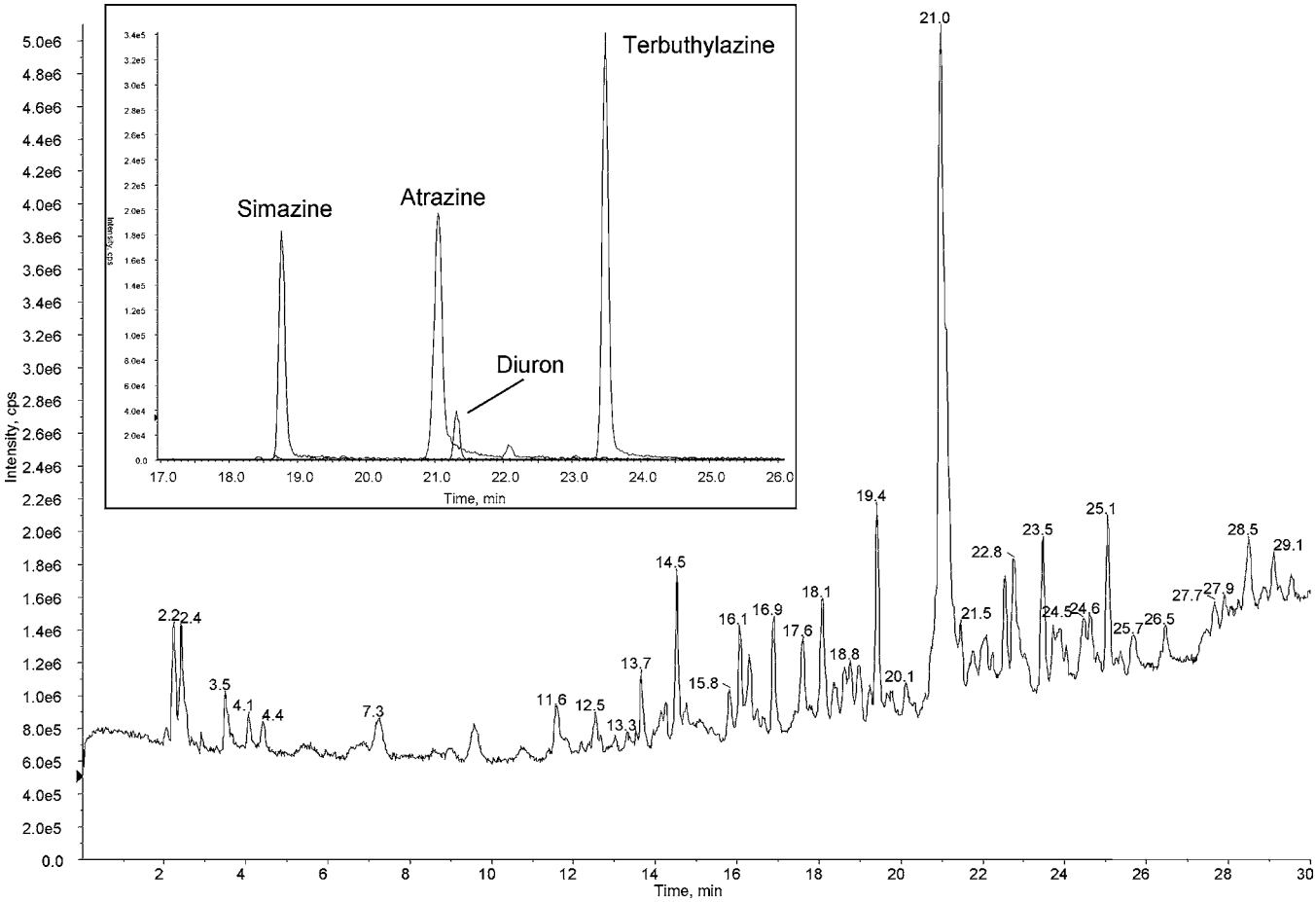

Figure 4. Total ion chromatogram corresponding to the analysis of a spiked olive oil sample with herbicides (0.025 mg/kg) by LC/TOF-MS. The extracted ion chromatogram (XIC) for each corresponding protonated molecule is also shown; window = 0.1 Da.

Table 3. Analytical Parameters for the Analysis of Herbicides in Olive Oil Samples by LC/TOFMS.

compound	concentration range (mg/kg)	linearity (R^2)	LOD (μg/kg)	RSD (%) $N = 5$
simazine	0.005–0.5	0.9990	1.5	3
atrazine	0.005–0.5	0.9996	1	2
diuron	0.005–0.5	0.9992	5	4
terbuthylazine	0.005–0.5	0.9948	1	2

C_8 analytical column of 150 mm × 4.6 mm and 5 μm particle size (Zorbax Eclipse XDB-C8). Column temperature was maintained at 25 °C. The injected sample volume was 50 μL. Mobile phases A and B were acetonitrile and water with 0.1% formic acid, respectively. The optimized chromatographic method held the initial mobile phase composition (10% A) constant for 5 min, followed by a linear gradient to 100% A after 30 min. The flow-rate used was 0.6 mL/min. A 12-min post-run time was used after each analysis. This HPLC system was connected to a time-of-flight mass spectrometer, Agilent MSD TOF (Agilent Technologies, Santa Clara, CA), equipped with an electrospray interface operating in positive ion, using the following operation parameters: capillary voltage 4000 V; nebulizer pressure 40 psig; drying gas 9 L/min; gas temperature 300 °C; fragmentor voltage 190 V; skimmer voltage 60 V; octopole DC 1 37.5 V; octopole RF 250 V. LC/MS accurate mass spectra were recorded across the range 50−1000 *m*/*z*. The data recorded was processed with Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software. The instrument performed the accurate-mass internal mass calibration automatically using a dual-nebulizer ion source combined with an automated calibrant delivery system, which introduced the internal reference masses (121.0509 and 922.0098) at approximately 600 μL/h.

RESULTS AND DISCUSSION

LC/TOF-MS Analyses of Chlorinated Herbicides. Olive oil is one of the most difficult food matrices due to the presence of numerous interferences that show up in full-scan mode. For this reason, LC/TOF-MS parameters were optimized by exploring the fragmentation of the analytes studied. Under the optimized instrumental parameters (nebulizer and drying nitrogen flow rates, vaporizer, and drying temperatures), the effect of the fragmentor voltage was studied in order to obtain additional information from characteristics fragments of the studied compounds. The in-source collisionally induced dissociation (CID) fragmentation is greatly enhanced at high fragmentor voltages. This provides highly valuable structural information since the accurate mass of the characteristic fragment ion can be used along with that of the protonated molecule for confirmation purposes. The relative abundances for both the protonated molecules and the main fragments of the herbicides studied are summarized in **Table 1** at three different voltages: 160 (low), 190 (medium), and 230 V (high). Triazines in general require high fragmentor voltages for fragmentation as we can observe in this table. Atrazine shows fragmentation at a voltage of 230 V. However, even at a higher fragmentor voltage of 230 V, the relative abundance of the main fragment ions for simazine (at *m*/*z* 174 and *m*/*z* 132) was low. Terbuthylazine shows good fragmentation at a medium voltage. On the other hand, diuron is similar to atrazine, high voltages are necessary to obtain significative abundance for the frag-

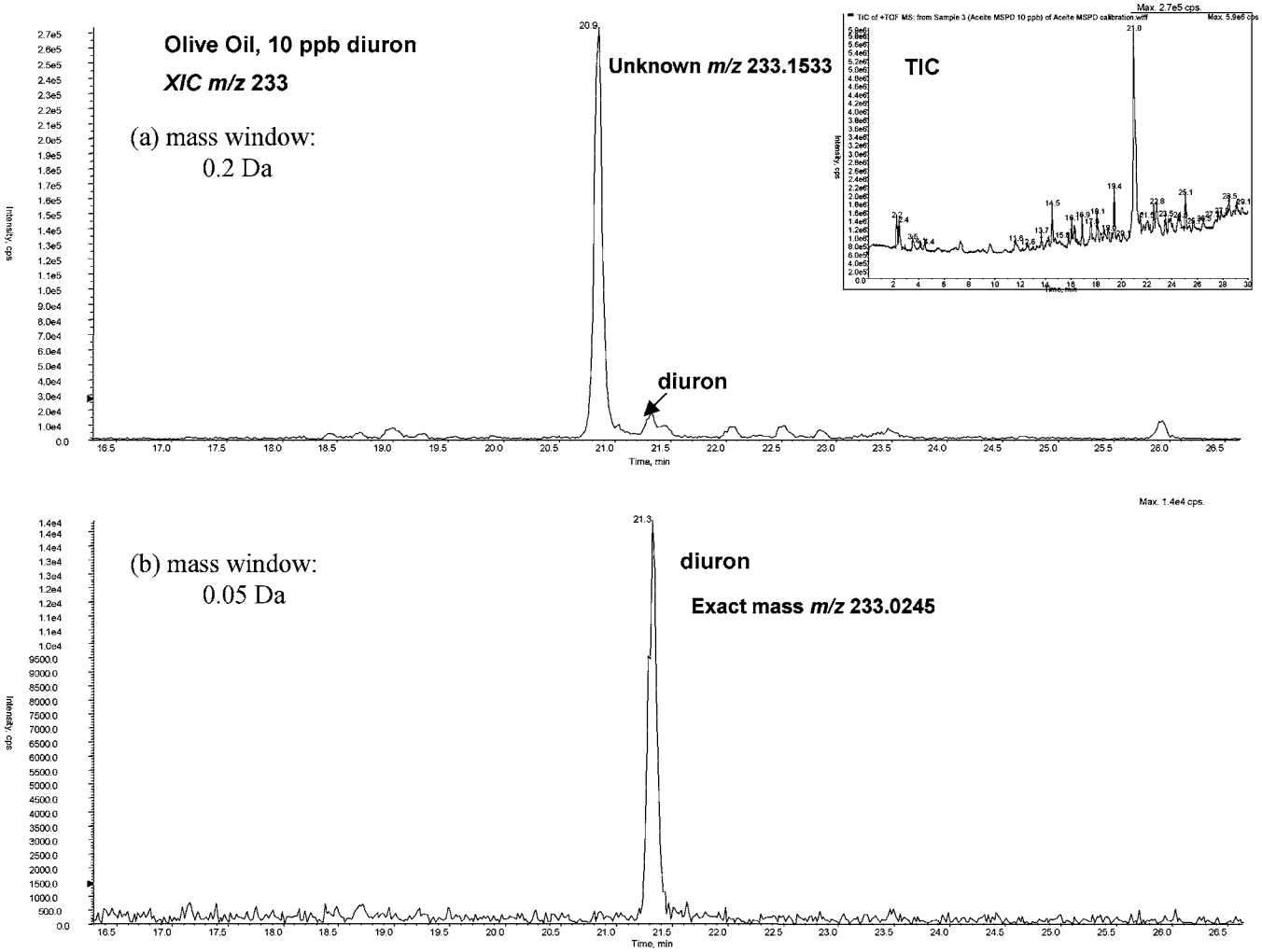

Figure 5. Extracted ion chromatogram for diuron at *m*/*z* 233 in an olive oil extract using two different accurate mass windows: (a) 0.2 and (b) 0.05 Da. Total ion chromatogram (TIC) is also shown in the top right corner.

ments. Using the information provided by the fragmentor voltage study, the fragments for every ion were identified matching the elemental composition generated from the accurate mass measurements of each fragment. Both atrazine and terbuthylazine gave the same characteristic ion (*m*/*z* 174). Diuron presented a minor sodium adduct ($[M + Na]^+$ at *m*/*z* 255), and a characteristic fragment ion at *m*/*z* 72. In order to obtain sufficient sensitivity for quantitative purposes (using the protonated molecule) and additional qualitative spectra information, provided by the fragments ions generated by in-source fragmentation, a value of 190 V was chosen for further analyses. All the fragments were previously confirmed by ion-trap MS/MS as reported in a previous work from our group (*11*).

Besides the accurate mass of the protonated molecule and the information provided by the fragment ions obtained with an optimized in-source fragmentation, these four herbicides present another feature which enables the unequivocal identification/confirmation of these chemical species. It is the presence of at least one chlorine atom in the chemical structures. The signal intensity pattern of the ^{37}Cl isotope signal evidences that the peak contains chlorine atom(s) unequivocally. In addition to this, the relative abundance of the isotopic signal for ^{37}Cl will suggest whether the compound contains a unique chlorine atom, such is the case of terbuthylazine, simazine, and atrazine, or two atoms as in the case of diuron. The chlorine isotopic profile of the four selected herbicides can be observed in **Figure 2**. Moreover, not only the chlorine isotopic profiles are useful in this sense, but also the accurate mass obtained for the ^{37}Cl isotopes, which is one of the characteristic features of time-of-flight when applied to halogen containing pesticides (see Figure 2), is useful. Every one of the chlorine 37 isotope accurate mass represents one more identification point in regards to the mass spectrometric criteria for positive identifications. Therefore, the accurate mass of each protonated molecule along with the characteristic fragment ion, the corresponding generated elemental compositions, the presence of the chlorine signature, and the characteristic retention time represent enough information to unequivocally identify and confirm members of this class of herbicides in such complicated matrices. In this way, the method based on accurate mass measurements meets the European Commission (EC) criteria for the spectrometric identification and confirmation of organic residues and contaminants based on the use of identification points (IPs) (*17*−*19*).

The mass accuracies obtained for both the protonated molecules and their characteristic fragment ions for the selected herbicides are shown in **Table 2** (using an olive oil matrix-matched standard spiked at 0.025 mg/kg of each pesticide). In this table, the accurate masses for the molecules containing the chlorine 37 isotope are also shown, providing extra information for each one of the herbicides studied. In this way, we can achieve accurate mass information for both the protonated molecule, which contains the chlorine 35, and the one containing the chlorine 37 isotope. Since diuron contains two chlorine

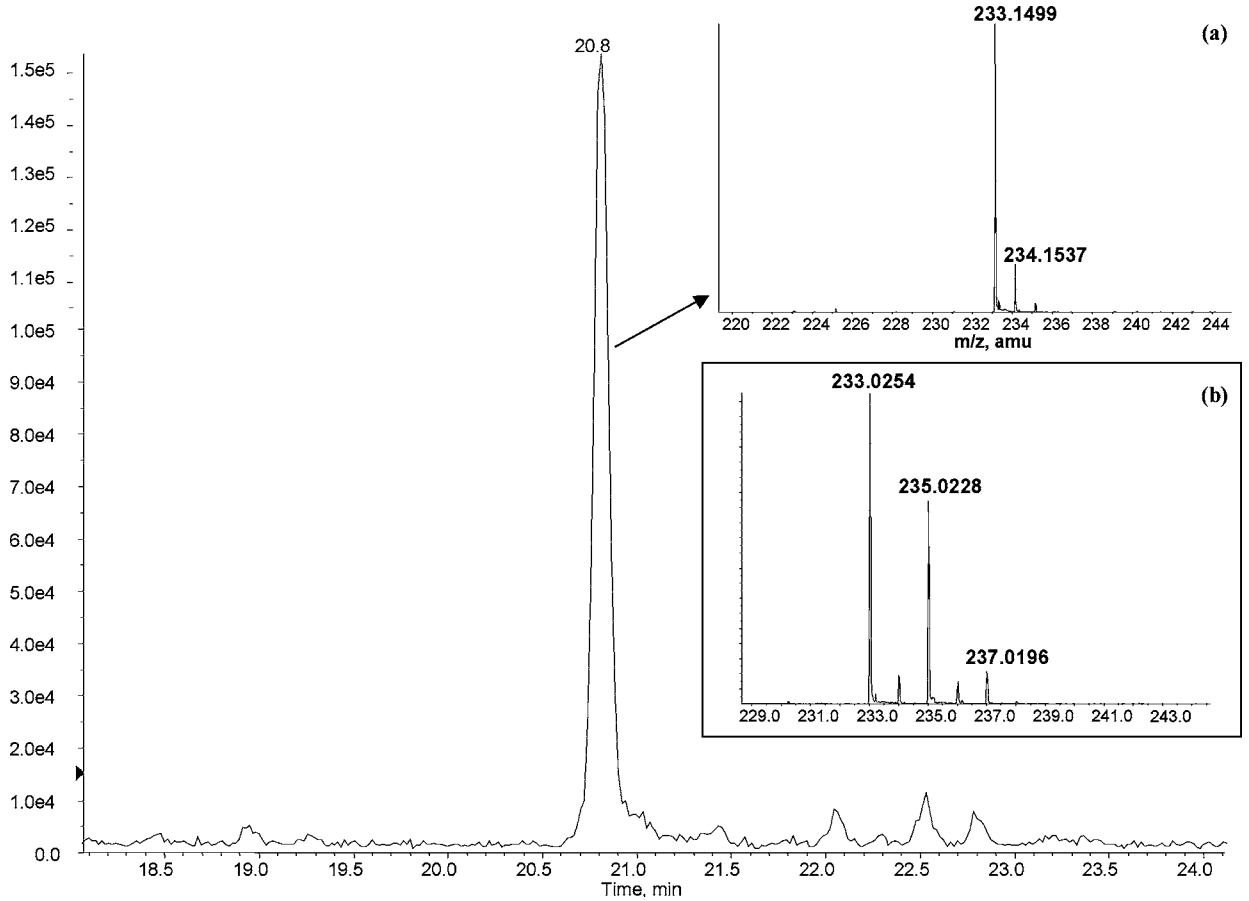

Figure 6. Case of a "false positive" of diuron in an olive oil sample. Extracted ion chromatogram at $m/z = 233$ (mass window 0.2 Da): (a) accurate mass spectra of the suspected peak; (b) accurate mass spectra of a diuron standard (for details, see text).

atoms, we can get up to 3 ions and their respective accurate masses in this case, which is much wider information than that obtained from single quad and selected ion monitoring techniques.

The effect of the concentration level on the accuracy of the mass measurements of the selected herbicides was evaluated at different concentration levels within the range 0.005−0.5 mg/kg. No significant differences were observed in the mass accuracy obtained in the matrix-matched standards compared with that obtained with standards in pure solvent, with average mass accuracy values better than 2 ppm of error for all the herbicides. This fact illustrates the capability of performing accurate mass measurements for the unequivocal confirmation of these species in olive oil matrices at diferent concentration levels.

Analytical Performance. In order to explore the feasibility of LC/TOF-MS for quantitative analyses in olive oil, as a complex matrix with a high content of fat, the analytical performance of the proposed methodology was studied.

(a) Linearity. The calibration was carried out using spiked matrix-matched standards prepared by the extraction method based on MSPD described in the Experimental Procedures section. In this sense, a "blank" olive oil was extracted, and herbicides were spiked in the final extract at different concentration levels. Linearity was then evaluated by analyzing olive oil spiked with standards at seven different concentration levels in the range from 0.005 to 0.5 mg/kg. Quantitation was carried out using the peak area from the extracted ion chromatograms (XIC) of the protonated molecule with a mass window of 0.1 Da. **Figure 3** shows the linear calibration curves obtained by LC/TOF-MS for the selected herbicides in an olive oil matrix compared to the curve obtained in pure solvent. As it can be observed, the linearity of the analytical response within the studied range of 2 orders of magnitude is excellent, with correlation coefficients higher than 0.99 in all cases. The results obtained are summarized in **Table 3**.

(b) Matrix Effects. The occurrence of matrix effects in LC/MS is well-known, especially when electrospray ionization is used. Matrix effects can either reduce or enhance the response when compared with "solvent" standards. To evaluate the signal suppression, the slopes obtained in the calibration with solvent-based standards for each pesticide were compared with those obtained with matrix-matched standards. As it can be observed in **Figure 3,** olive oil samples suffer from considerable matrix-induced suppression with a decrease in the signal ranging from 14% to 36%. In this case, matrix-matched calibration, using the same matrix as the samples analyzed, must be used for quantitation purposes.

(c) Limits of Detection. The limits of detection (LODs) were estimated from the injection of matrix-matched standard solutions with concentration levels giving a signal-to-noise ratio of about 3. The results are shown in **Table 3**. The limits of detection obtained are remarkably low since they are far below the maximum residue level regulations established for these herbicides. For example, the maximum residue level established for terbuthylazine in olives is 0.05 mg/kg. Due to the high complexity of this matrix, these values are difficult to fulfill with other techniques (i.e., GC/MS). In this sense, LC/TOF-MS analyses benefit from the use of narrow mass windows for quantitation purposes, which results in enhanced signal-to-noise ratio, thus providing lower detection limits. This fact illustrates the analytical potential of the proposed method based on MSPD

and LC/TOF-MS for the analyses of herbicides in complex matrices with high content of fat. In addition, the relative standard deviation (RSD) values obtained from a run-to-run repeatability study were below 5% for all the compounds.

All these analytical features can compare very well against what can be achievable using single quadrupole or triple quadrupole instruments in selected ion monitoring (SIM) and multiple reaction monitoring (MRM), respectively, which are the most widely accepted techniques for routine analyses (*20*−*22*). As an example, a typical total ion chromatogram of a spiked olive oil sample at 0.025 mg/kg together with the extracted ion chromatogram used for quantification purposes is shown in **Figure 4**.

Selectivity Using LC/TOF-MS. Selectivity for the target compounds is enhanced when using a high-resolution technique. Selectivity is the ability to separate or isolate the response of the target compounds from matrix ions, and this is achieved using the high resolving power of time-of-flight measurements. This feature reinforces the usefulness of benchtop TOF mass spectrometers applied to analyses of pesticides in food. **Figure 5** shows an example of the selectivity achieved by TOF-MS. When a wide amu window (0.2 Da) is selected in the extracted ion chromatogram for $m/z = 233$ (diuron), other interferences might be present in the sample matrix as it is observed from the peak at 20.9 min. When the same window is narrowed down to 0.05 Da, the main interference disappears leading to a more selective identification for the target compound. Furthermore, this selectivity improves the signal-to-noise ratio leading to better method detection limits overall.

Analyses of Olive Oil Samples. To evaluate the effectiveness of the proposed method, it was applied to the analysis of a total of eight samples of olive oil. Fortunately, in most cases, pesticide residues were not found. Only in one olive oil sample, terbuthylazine was found at concentration levels near the limit of detection (below the authorized maximum residue level). The other olive oil samples analyzed did not contain any of the targeted herbicides. However, one of the samples, which was used as a reference sample in an interlaboratory comparison test for pesticide residue analysis (undertaken by 12 Spanish laboratories) gave a singular result: 7 out of 12 laboratories (which used GC methods) found traces of diuron at concentration values between 0.03 and 0.05 mg/kg. When the LC/TOF-MS methodology developed in this work and a quantitation mass window of 0.1 Da was used, diuron was not found. However, when the width of the mass window was increased to 0.2 Da (233.0−233.2), a large peak at a retention time of 20.8 min, which is very close to the retention time for diuron (the retention time expected for the diuron peak is 21.3 min) was seen, as it is shown in **Figure 6**. As we would expect, if we take a look at the accurate mass spectra of this peak, it is clear it is not diuron. The accurate mass spectra of the suspected peak (**Figure 6a**) and that of a standard of diuron (**Figure 6b**) are completely different with a mass difference of about 0.1 Da. However, the "positive" results for diuron obtained by the other laboratories can be attributed to the use of a GC/MS method in selected ion monitoring mode (where only nominal mass is monitored). Most of the laboratories participating in the interlaboratory exercise used this method, with less spectral resolution, which cannot avoid this type of interferences, yielding thus a possible number of false positives.

This is a real example that illustrates the usefulness of routine accurate mass measurement capabilities of LC/TOF-MS, with a unique feature, which can aid this kind of "daily" analytical problem. In fact, when one deals with this kind of samples with the possibility of isobaric interferences due to the complexity of matrix, the use of mass spectrometric techniques with high selectivity is absolutely necessary. In this sense, the selectivity of LC/TOF-MS relies on the resolving power of the instrument on the *m*/*z*-axis, which enables the discrimination between the target species and "isobaric" interferences within 0.05 Da of mass difference (using 350 *m*/*z*, as example) (*23*). Therefore, an isobaric interference in LC/TOF-MS analyses would arise only if an interfering species with the same time retention of the target species had the same exact mass (differences far below 50 mDa). In the example shown for diuron, it could be also easily circumvented using the isotopic signal of chlorine for quantitation purposes, thus avoiding this hypothetical isobaric interference, without a remarkable lack of sensitivity.

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