Analysis of Forchlorfenuron in Vegetables by LC/TOF-MS after Extraction with the Buffered QuEChERS Method

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This paper describes the application of liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS), with electrospray ionization, for residue analysis of forchlorfenuron in tomato, zucchini and watermelon. The assessed method includes a sample preparation step based on the buffered QuEChERS approach. The TOF-MS fragmentation pattern of forchlorfenuron was studied at different fragmention voltages in the range of 120−270 V. Analyses were carried out under full-scan conditions by using the extracted ion chromatogram (XIC) of the *m/z* 248 ion with a 0.2 Da window. The linearity of the analytical response across the studied range of concentrations (10−500 μg/kg) was excellent, obtaining correlation coefficients higher than 0.999, and relative standard deviations of the response factors lower than 14%, for the 15 linear calibration curves of forchlorfenuron evaluated along the complete validation study. No significant matrix effects were observed. The signal-to-noise ratios obtained for the 10 μg/kg forchlorfenuron in matrix matched standards were >70 for all three matrices. Recovery studies were carried out on spiked tomato, zucchini and watermelon blank samples, at three concentration levels (10, 50, and 200 μg/kg) performing five replicates at each level. Forchlorfenuron mean recoveries ranged between 80% and 87% in watermelon and zucchini, and between 65% and 71% in tomato, obtaining in all cases relative standard deviation values lower than 10%. The method readily achieved a lowest validated level of 10 μg/kg, which was fit-for-purpose in residue monitoring applications.

KEYWORDS: Analysis; forchlorfenuron; LC/TOF-MS; QuEChERS; fruits and vegetables

INTRODUCTION

Plant growth regulators, such as auxins, gibberellins and cytokinins, are used to improve fruit set and development in many crops, overall when the pollination and fertilization conditions are unfavorable (*1*). Forchlorfenuron, 1-(2-chloro-4-pyridyl)-3-phenylurea, is a relatively new plant growth regulator belonging to the synthetic cytokinins group (*2*), extensively used in recent years for increasing fruit size in many crops, particularly in grapes and kiwifruit (*3*, *4*). It is also very effective to induce artificially parthenocarpy (the development of the ovary into fruit without fertilization or seed formation), being an alternative to natural pollination in some crops, such as melon, watermelon and pumpkin (*5*, *6*). During the present decade, forchlorfenuron has been registered in many countries and, according to the USDA database for Maximun Residue Limits (MRLs), the tolerances established for its residues around the world range between 10 μg/kg and 100 μg/kg, depending on crop and country. Particular examples of forchlorfenuron MRLs for kiwifruit are 10 μg/kg in Australia, 40 μg/kg in USA, 50 μg/kg in EU, or 100 μg/kg in Japan (*7*).

By now, only a few papers have been published on the analysis of forchlorfenuron residues in agricultural products. Hu and Li reported the determination of forchlorfenuron residues in watermelon by solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) (*8*), Sharma and Awasthi studied the behavior of forchlorfenuron residues in grape by using liquid chromatography with UV detector as analytical technique (*9*), and Valverde et al. used liquid chromatography with tandem mass spectrometry (LC/MS−MS) to evaluate the persistence of forchlorfenuron residues in watermelon after applying an individual spray treatment to the flower ovary at the anthesis stage (*10*). Finally, in 2008, Suarez-Pantaleon et al. described the development of immunoassays for the detection of forchlorfenuron (*11*), but this technology has not been yet validated for the analysis of its residues in vegetables.

Liquid chromatography with time-of-flight mass spectrometry (LC/TOF-MS) has been recently demonstrated to be a sensitive and selective technique for the determination and identification/confirmation of pesticide residues in fruits and vegetables, showing a great potential to be used in routine multiresidue analysis (*12−15*). However, the use of LC/TOF-MS for the identification and quantitation of forchlorfenuron in vegetables has not been yet reported. The objective of this work was to develop and evaluate a rapid method for determining forchlorfenuron residues in vegetables by using LC/TOF-MS and the quick,

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easy, cheap, rugged, and safe (QuEChERS) buffered approach (*16, 17*) in the sample preparation step. It was also an objective of this work to introduce the analyte forchlorfenuron in a multiresidue analytical methodology for pesticides in fruits and vegetables, this being the reason for which the LC column and chromatographic conditions used in this study were the same as those already described by Ferrer et al. in a previous paper (*12*).

EXPERIMENTAL PROCEDURES

Reagents and Materials. HPLC grade methanol and acetonitrile were supplied by Merk (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). HPLC grade water was obtained in a Milli-Q-Plus ultrapure water system from Millipore (Milford, MA). Acetone was high purity grade solvent for pesticide residue analysis from Panreac (Barcelona, Spain). PRS grade anhydrous sodium acetate and acetic acid were also obtained from Panreac. Anhydrous magnesium sulfate (AR grade, 99% purity) was obtained from VWR-Prolabo (Fontenay sous Bois, France), and before use, it was baked for 5 h at 500 °C in a muffle furnace to remove phthalates and residual water. Primary-secondary-amine (PSA) sorbent was supplied by Varian (Harbor City, CA). Certified analytical standard of forchlorfenuron, 99.2% purity, was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Forchlorfenuron stock standard solution (1,000 μg/mL) was prepared in acetone. Working standard solutions (1−100 μg/mL) were prepared in methanol by suitable dilution of the stock standard solution. Standard solutions for LC/TOF-MS analysis (10−500 μg/L) were prepared in water: methanol (9:1) and in tomato, zucchini or watermelon blank extracts. Pure standard and standard solutions were stored in dark at −18 °C.

LC/TOF-MS Analysis. Electrospray ionization (ESI) was applied in LC/TOF-MS in positive mode for analysis of forchlorfenuron. Chromatographic separation was achieved 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 C8 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 sample injection volume was 50 μL. The mobile phase was composed of solvent A (acetonitrile) and solvent B (water with 0.1% formic acid) at a constant flow of 0.6 mL/min. The chromatographic method held the initial mobile phase composition (10% A) constant for 5 min, followed by a linear gradient to 100% after 30 min. A 12 min postrun time back to the initial mobile phase composition was used after each analysis. This HPLC system was connected to a TOF-MS instrument, Agilent MSD TOF (Agilent Technologies), equipped with ESI operating in positive ion, using the following operation parameters: capillary voltage 4000 V; nebulizer pressure 40 psig; drying gas flow 9 L/min; drying gas temperature 300 °C; fragmentor voltage 190 V; skimmer voltage 60 V; octapole DC-1 37.5 V; octapole RF 250 V. LC/TOF-MS accurate mass spectra were recorded across the range 50−1000 *m/z*. Data processing was carried out with Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software. Accurate-mass internal mass calibration was performed automatically using a dual-nebulizer ion source combined with an automated calibrant delivery system, which introduced continuously the internal reference solution at approximately 600 μL/h. The refence masses were 121.0509 and 922.0098 *m/z* (resolution: 9500 ± 500 at 922.0098 *m/z*).

Analyses were carried out under full-scan conditions by using the extracted ion chromatogram (XIC) of the protonated molecule of forchlorfenuron (*m/z* 248) from the total ion chromatogram (TIC) with a 0.2 Da window. The accurate mass spectrum of forchlorfenuron was obtained once the background of the XIC was subtracted. The accurate mass of the protonated molecule was used for both confirmation and quantitation purposes, peak areas of the extracted ion being used for quantitation.

Sample Preparation. Blank watermelon, tomato and zucchini samples (produced without using forchlorfenuron) were collected from integrated pest managament plantations, in three different experimental greenhouses belonging to the "University of Almería-Anecoop Foundation", located in Nijar, Almería (Spain). Extraction of forchlorfenuron from spiked watermelon, tomato and zucchini samples was carried out according to the original buffered QuEChERS (*16*) method, but final acetonitrile extracts were evaporated to dryness and reconstituted in water:

methanol (9:1) before LC/TOF-MS analysis. A brief description of the extraction procedure used is as follows: (a) Weigh 15 g of thoroughy homogenized sample into a 50 mL centrifuge tube; (b) add 15 mL of 1% acetic acid in acetonitrile, 6 g of anhydrous magnesium sulfate, and 1.5 g of anhydrous sodium acetate; (c) seal the tube, shake vigorously for 1 min by hand, and centrifuge at 3700 rpm (2600 rcf) for 2 min; (e) transfer 5 mL of the extract (upper layer) to a 15 mL centrifuge tube containing 250 mg of PSA and 750 mg of anhydrous magnesium sulfate; (f) cap the tube, shake vigorously for 20 s by hand, and centrifuge at 3700 rpm (2600 rcf) for 2 min; (g) transfer 1 mL of the extract to a LC/TOF-MS autosampler vial, evaporate to dryness using a nitrogen stream, and reconstitute with 1 mL of water:methanol (9:1). This final extract contained 1 g of sample/mL and was ready to be analyzed by LC/TOF-MS.

Method Validation. The linearity in the response of forchlorfenuron was studied by using water:methanol (9:1) and tomato, zucchini or watermelon blank extracts solutions to evaluate possible matrix effects. For this purpose, for each one of the three matrices, both matrix matched and solvent standard solutions of 10, 50, 100, 200, and 500 μg/kg (μg/L for solvent standards), were analyzed in the same LC/TOF-MS injection sequence. Recovery studies were carried out on spiked tomato, zucchini and watermelon blank samples, at three concentration levels (10, 50, and 200 μg/kg) performing five replicates at each level. Extracts obtained for the same matrix and spiking level were analyzed in the same chromatographic sequence, using bracketing matrix matched standards for calibration. Chromatographic sequences for the analysis of the 10 μg/kg spike samples consisted of (1) matrix blank; (2) 10 μg/kg standard; (3−7) 10 μg/kg spiked samples; (8) 10 μg/kg standard; (9) 50 μg/kg standard; (10) 100 μg/kg standard; (11) 200 μg/kg standard; (12) 500 μg/kg standard; and (13) solvent blank. Similar sequences were used for the analysis of the 50 and 200 μg/kg spiked samples, which were bracketed between standards of the same concentration.

RESULTS AND DISCUSSION

Fragmentation of Forchlorfenuron by LC/TOF-MS. The fragmentation pattern of forchlorfenuron was studied by using different fragmention voltage values, in the range from 120 to 270 V, under the previously optimized instrumental parameters (nebulizer and drying nitrogen flow rates, vaporizer and drying temperatures and capillary voltage) (*13*). The effect of the fragmentor voltage on the in-source collisionally induced dissociation (CID) fragmention for forchlorfenuron is showed in **Table 1**, where the relative abundances of the protonated molecule and the main fragments obtained for a 500 μg/L solvent standard at 120, 150, 190 and 270 V are indicated. **Figure 1** shows the chemical formula of forchlorfenuron and the proposed fragmentation pattern, and **Figure 2** includes the mass spectrum obtained with a fragmentation voltage of 190 V. At this medium voltage value of 190 V, forchlorfenuron showed a good fragmentation, with the protonated molecule (*m/z* 248) as the base peak in the spectrum, being also present a characteristic fragment ion (*m/z* 129) with a relative abundance higher than 30%. Therefore, the value of 190 V was selected for further analyses in order to obtain both sufficient sensitivity for quantitative purposes (using the protonated molecule) and additional qualitative spectra information. Once the fragmentation pattern was established under the optimized conditions, the accurate mass of the molecular ion and characteristic fragment ion together with their elemental

Table 1. Effect of the Fragmentor Voltage on the Fragmentation of Forchlorfenuron

m/z fragment	relative abundance				
	120 V	150 V	190 V	230 V	270 V
248	100	100	100	7.7	0.4
129		7.4	48	100	98
93			6.8	20	100
155			6.7	12	13

composition, along with the retention time, can be used for confident identification criteria (*15*). On the other hand, as can be seen in **Figure 2**, the intensity of the ^{37}Cl isotope signal (*m/z* ions 250 and 131) provides unequivocal evidence that both the molecular ion and the characteristic fragment ion contain one chlorine atom in their chemical structures. That means that the chlorine isotopic pattern in the spectrum and the accurate mass obtained for the ^{37}Cl isotope of the protonated molecule and the characteristic fragment can be used as additional criteria for unequivocal identification or confirmation of forchlorfenuron in a sample.

Accurate Mass Measurements. Accurate mass measurements of the protonated molecule of forchlorfenuron ($C_{12}H_{10}ClN_3O$ + H) were performed on the extracted ion chromatograms (*m/z*
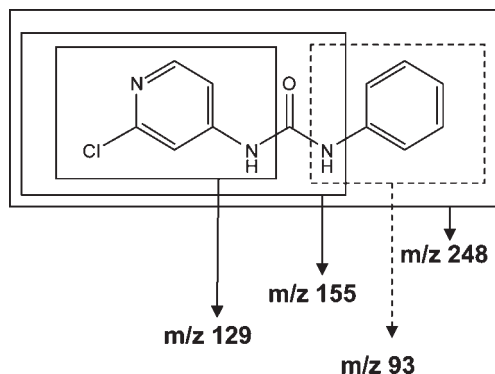
248.0−248.2) obtained for all the standards and spiked samples analysed during the validation study. The mass error was calculated by using the mass value of 248.05851 Da (given by the TOF softward) as reference. In all cases, the error obtained ranged between −1.68 ppm and +0.34 ppm (−0.66 mDa and +0.08 mDa), being kept far below the widely accepted accuracy threshold of 5 ppm for confirmation of elemental compositions (*15, 18*). No significant differences were observed in the accuracy obtained in the three types of matrix-matched standards (tomato, zucchini and watermelon) and solvent standards at the studied concentration levels (10−500 μg/kg).

Analytical Performance. Figure 3 shows the extracted ion chromatogram (XIC) obtained for a zucchini matrix matched standard of forchlorfenuron of 10 μg/kg and the corresponding TOF mass spectrum at 20.89 min. Retention time values obtained for forchlorfenuron in the LC/TOF-MS analysis of all the standards and extracts injected along the complete validation study ranged between 20.8 and 20.9 min. This retention time is very similar to that previously determined by Ferrer et al. (*12*) for the pesticide spinosyn A using the same equipment and chromatographic conditions. The linearity of the analytical response obtained for forchlorfenuron across the studied range of concentrations (10−500 μg/kg) was excellent. **Table 2** includes the results obtained for the linear correlation coefficients (r^2), slope, and relative standard deviations (RSD) of the response factors (n = 5 or 6) from the 15 different linear calibration curves evaluated along the complete validation study (3 in solvent and 12 in blank matrix extracts). Correlation coefficients of the linear calibration curves of forchlorfenuron in both solvent and matrix extracts were higher than 0.999 in all cases, with relative standard deviations of the response factors in the range of 4−14%. The

Figure 1. Molecular formula of forchlorfenuron and the proposed fragmentation pattern.
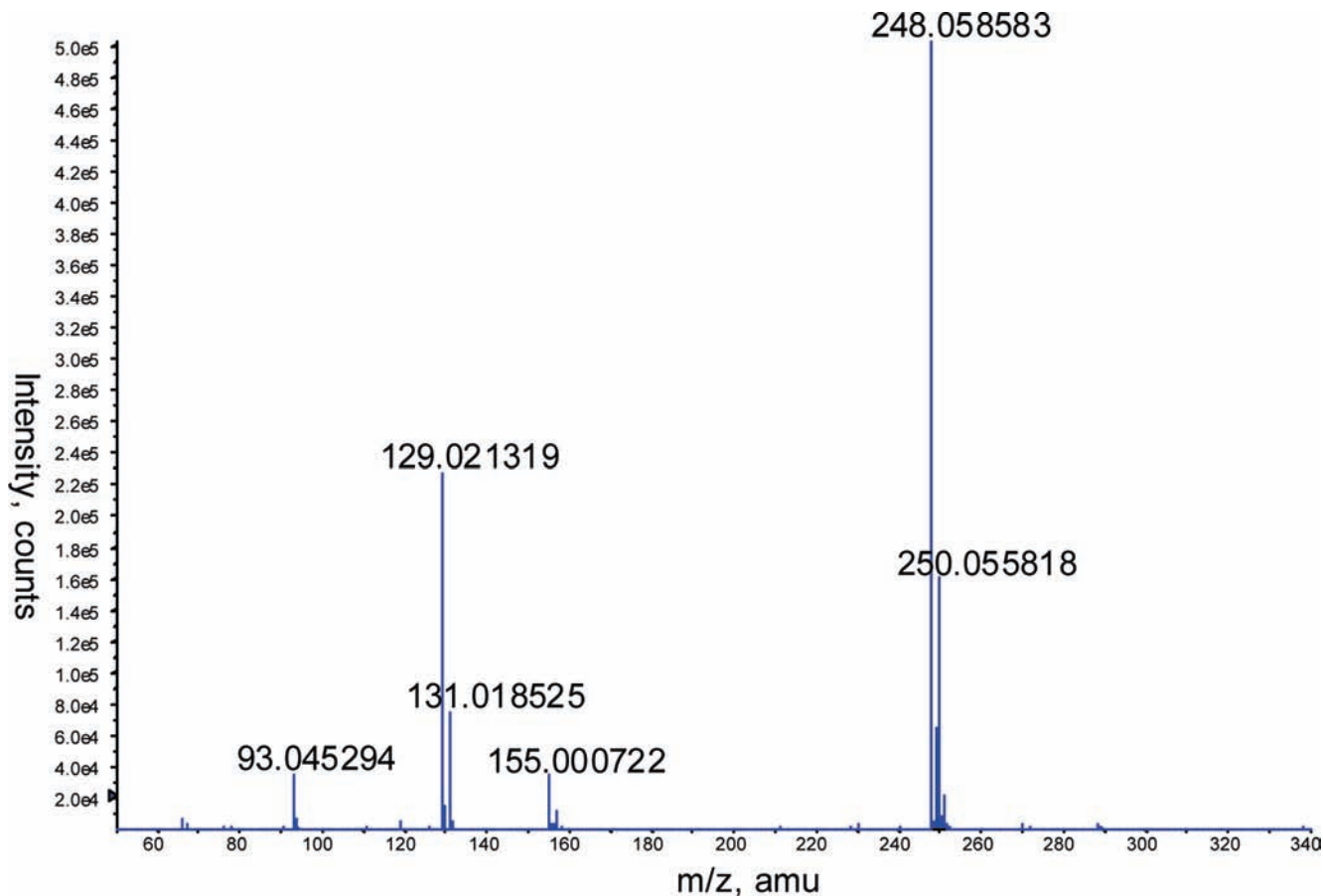
Figure 2. Mass spectrum of forchlorfenuron obtained with a fragmentation voltage of 190 V.
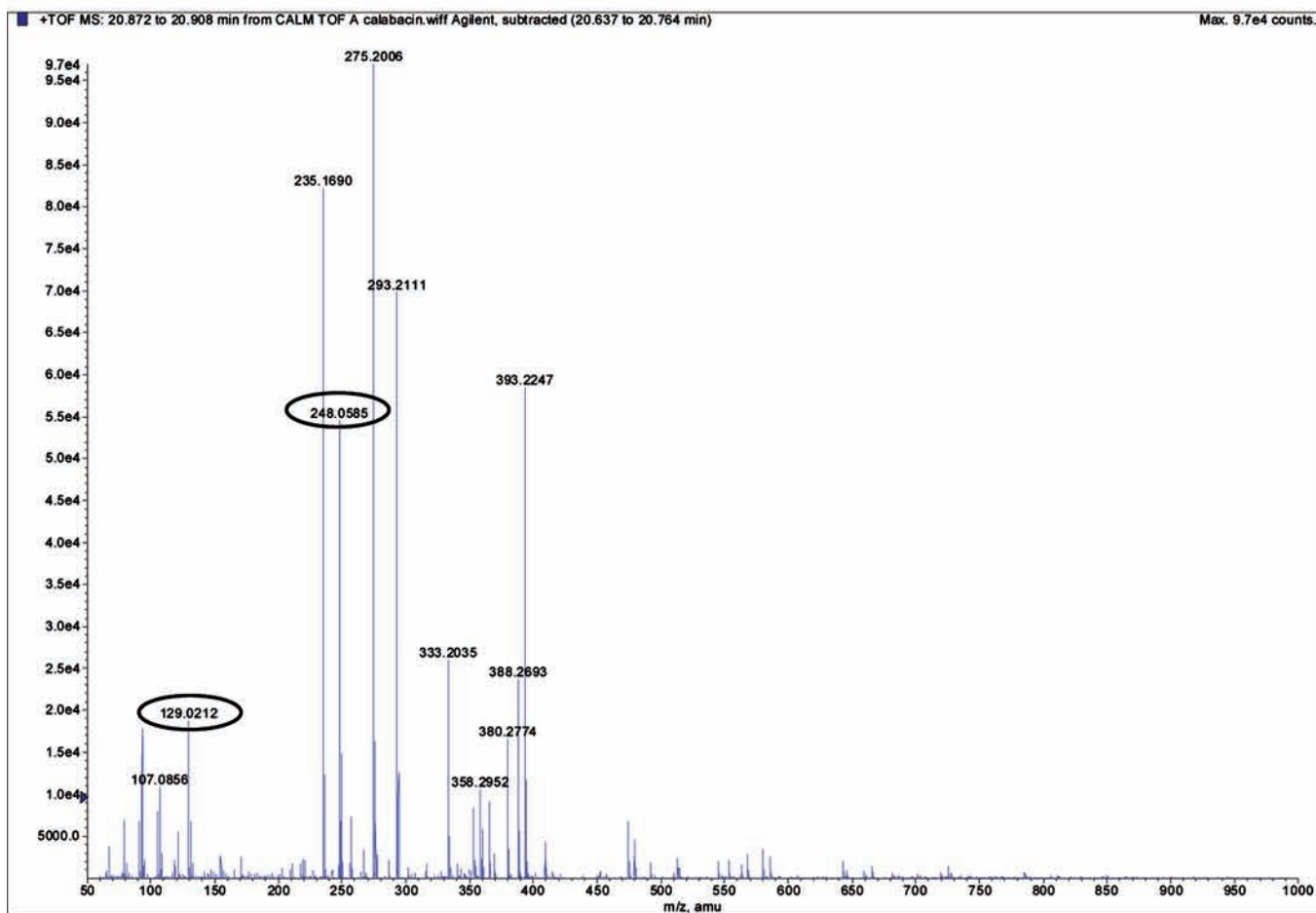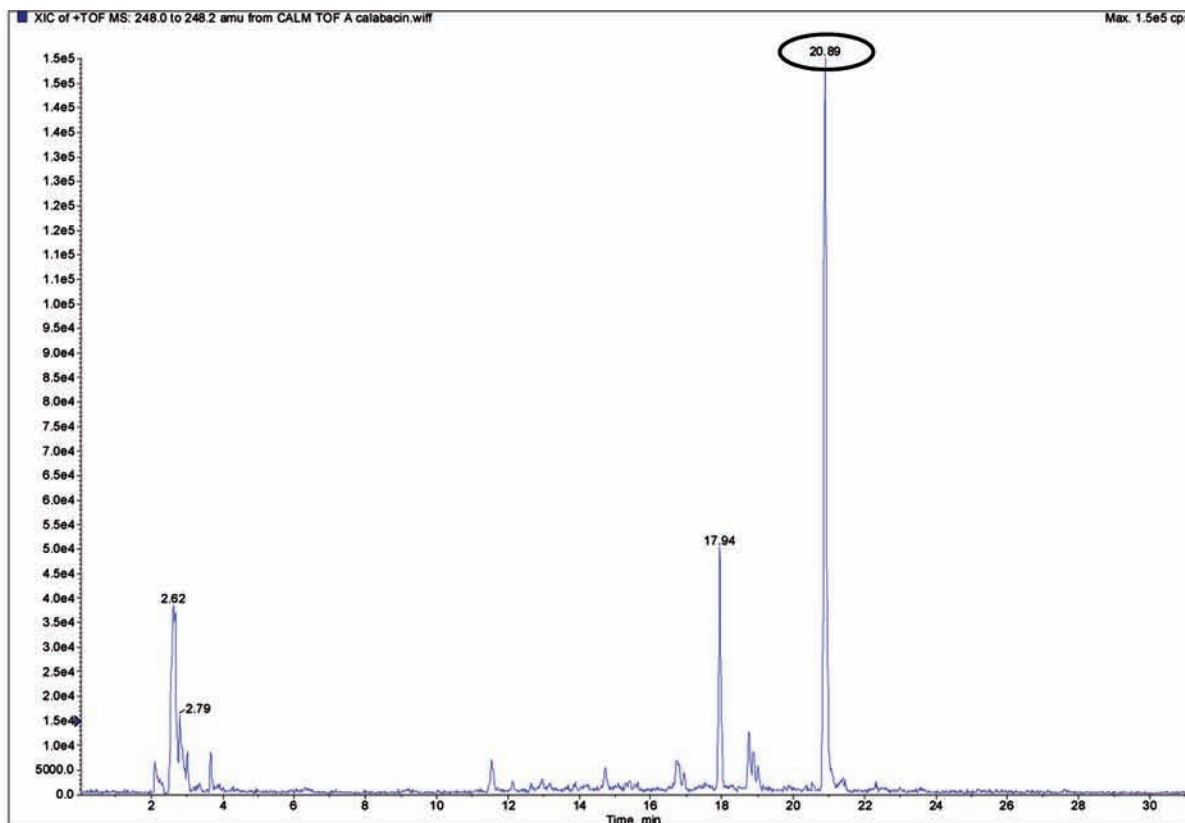
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Figure 3. Extracted ion chromatogram (XIC) for a 10 μg/kg zucchini matrix matched standard of forchlorfenuron (above) and the corresponding TOF mass spectrum obtained at 20.89 min (below).

Table 2. Forchlorfenuron Linear Calibration Curves Obtained along the Complete Validation Study

chromatographic sequence/matrix	calibration points	slope × 10⁵ (area units/μg kg^{-1})	r^2	RSD of response factors (%)
1/solvent	5	1.14	0.9998	4
1/watermelon	5	1.08	0.9998	11
2/watermelon	6	1.07	0.9999	6
3/watermelon	6	1.09	0.9997	6
4/watermelon	6	1.10	0.9998	9
5/solvent	5	1.16	0.9992	5
5/zucchini	5	1.12	0.9998	10
6/zucchini	6	1.11	0.9998	10
7/zucchini	6	1.11	0.9999	11
8/zucchini	6	1.13	0.9996	8
9/solvent	5	1.14	0.9999	8
9/tomato	5	1.23	0.9997	14
10/tomato	6	1.18	0.9991	13
11/tomato	6	1.18	0.9998	11
12/tomato	6	1.16	0.9999	10

Table 3. Forchlorfenuron Recoveries from Spiked Blank Samples (n = 5)

matrix	spike level (μg/kg)	mean recovery (%)	RSD (%)
watermelon	10	87	8
	50	82	6
	200	84	3
zucchini	10	80	10
	50	82	8
	200	82	9
tomato	10	71	9
	50	65	5
	200	68	7

day-to-day and matrix-to-matrix reproducibility of the chromatographic response was also excellent, obtaining a RSD value of 4% for the slopes of the matrix matched calibration curves (n = 12). The ratios "slope matrix/slope solvent" for the calibration curves obtained in the same chromatographic sequence were 1.08 for tomato, 0.95 for watermelon, and 0.97 for zucchini. From these results, LC/TOF-MS analysis of forchlorfenuron in the three studied matrices was found to be precise and non-matrix dependent. The signal-to-noise ratios (S/N) obtained for the 10 μg/kg forchlorfenuron matrix matched standards resulted to be in the range of 70−110 for tomato, 110−130 for zucchini, and 350−600 for watermelon. It is important to note that there is no reason that watermelon gives higher S/N than tomato or zucchini if there are no matrix effects, unless there was direct matrix interference, which is unlikely. The S/N was probably increasing from watermelon to tomato as the instrument was contaminated or tuned differently over time. This issue could also justify the worsening recoveries obtained in tomato (see below) and shows the importance of instrument maintenance and quality control activities in this type of analysis. The S/N results obtained indicate that the LC/TOF-MS method has enough sensitivity to be applied for residue analysis of forchlorfenuron in vegetables, the limit of detection (LOD) being expected to be lower than 1 μg/kg. A preliminary and approximate value for the LOD could be calculated as the concentration of forchlorfenuron giving a S/N between 3 and 5 in tomato extracts (the less favorable matrix). Using this conservative approach, a LOD about 0.5 μg/kg is obtained and proposed for future works to validate the LC/TOF-MS method for qualitative analysis of forchlorfenuron in vegetables.

The accuracy and intralaboratory reproducibility of the assessed quantitative method (QuEChERS sample preparation and LC/TOF-MS determination) were evaluated by means of recovery tests on blank tomato, zucchini and watermelon samples spiked at three concentration levels, with five replicates at each level. Mean recovery values, and the corresponding relative standard deviations, obtained on each matrix and spiking level are indicated in **Table 3**. Forchlorfenuron mean recoveries ranged between 80% and 87% in watermelon and zucchini, and between 65% and 71% in tomato, obtaining in all cases RSD values lower than 10%. On the other hand, the 45 single recovery values obtained in this validation study ranged between 60% and 95%, and the overall mean recovery resulted to be 78% with a RSD of 12%. All these values are satisfactory according to the validation criteria usually established for pesticide residue analysis (*19, 20*)

with the exception of the mean recovery values obtained for tomato at spiking levels of 50 and 200 μg/kg, which were close to but lower than 70%. These results indicate that, during the routine application of this methodology for the analysis of florchlorfenuron in tomato samples, additional quality control recovery tests on tomato blank samples should be included in the analytical sequence, with the aim of confirming and complementing the results obtained in the initial validation on this matrix. Taking into account the results obtained along all the validation study for the 10 μg/kg spikes and matrix matched standards, which include mean recoveries in each matrix ≥71% with RSD ≤ 10%, signal-to-noise ratios ≥ 70 and accurate mass errors ≤ 0.5 ppm, the limit of quantification (LOQ) of the method was established at this lowest validated level of 10 μg/kg.

Following the practical guidelines proposed by Eurachem (*21*), Eurolab (*22*), and Codex Alimentarius (*23*) for estimating measurement uncertainty of chemical tests, which include some specific guidelines for multirresidue analysis of pesticides, the combined relative standard uncertainty associated to the determination of forchlofenuron residues with the validated QuEChERS−LC/TOF/MS method (for high water content vegetable samples and in the range of concentrations of 10−200 μg/kg) can be estimated as the overall RSD value of 12% (precision component) obtained for the 45 individual recovery results determined in the validation study. Note that, with an overall mean recovery of 78%, based on 45 measurements, the bias component of the relative standard uncertainty is about 3% ($[(100 - 78)/(45^{1/2})\%]$), which is much lower than the precision component and does not affect, in practice, the final result of the combined relative standard uncertainty. With this estimation, and using a coverage factor of 2 (which gives a level of confidence of approximately 95%), the value for the relative expanded uncertainty of these tests was estimated as 24% (2 × 12%), but this value does not include either the uncertainty component associated with the sample homogenization step of the analytical method or the possible corrections/no corrections of the analytical results by using recovery factors.

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