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Direct Determination of Paclobutrazol Residues in Pear Samples by Liquid Chromatography-Electrospray Tandem Mass Spectrometry

JUAN V. SANCHO, ÓSCAR J. POZO, TATIANA ZAMORA, SUSANA GRIMALT, AND FÉLIX HERNÁNDEZ*

Analytical Chemistry, Experimental Sciences Department, University Jaume I, E-12071, Castellón, Spain

A rapid and sensitive liquid chromatography/electrospray ionization/tandem mass spectrometry (LC-ESI-MS-MS) method has been developed for the determination of the plant growth regulator paclobutrazol in pear samples. Extraction was performed with methanol by using a high-speed blender Ultra-Turrax, and 10 μL of pear extract was directly injected in the LC-ESI-MS-MS system without any previous sample treatment. The highest sensitivity of the method was achieved under MS-MS conditions obtaining a limit of detection of 0.7 μg/kg and a quantification limit of 5 μg/kg, with a run time of only 5.5 min. Recoveries for paclobutrazol from spiked pear samples at 0.005, 0.05, and 0.5 mg/kg were around 82−102% with relative standard deviations between 2 and 7%. The method was applied to real treated and untreated samples of pears, using quality control samples as a evaluation of the method reliability. Two MS-MS transitions were selected, one for quantification (294 > 70) and the other for confirmation of the analyte (296 > 70). All the experiments were performed in compliance with good laboratory practices.

KEYWORDS: Paclobutrazol; pesticides; fruit samples; liquid chromatography; tandem mass spectrometry

INTRODUCTION

Paclobutrazol is a plant growth regulator registered for the reduction of terminal growth and pruning volume in trees, the inhibition of gibberellin and sterol biosynthesis, and hence the rate of cell division. (*1*). The maximum residue limit (MRL) for paclobutrazol in pome fruits is at the sub-ppm level, and it has been set up at 0.05 mg/kg in several European countries, such as Spain and Germany, while in others it is about 10 times higher (between 0.3 and 0.5 mg/kg). When the MRL is established at the low level, a sensitive analytical methodology has to be developed and a limit of quantification (LOQ) of 10 times lower than the MRL would be advisable in order to gain reliability in the results obtained.

Traditionally, gas chromatography (GC) has been the most common technique used for the determination of pesticide residues in fruits but usually includes time-consuming steps, such as solvent extraction and suitable cleanup steps (*2*). On the other hand, liquid chromatography (LC) has been applied for polar, thermolabile, or low volatility compounds and also allows one to perform direct injection of raw extracts.

In relation to paclobutrazol, few analytical methods have been reported. A laborious method by GC-NPD for apple tree leaves was published, which involved two cleanup steps by solid phase extraction, a solvent exchange, and preparative high-performance liquid chromatography (HPLC) in order to obtain recoveries >90%, even using diclobutrazol as the internal standard (*3*). More recently, HPLC-based methods have been published in plant and soil samples (*4*) and apple and pear pulps (*5*) using UV detection and different cleanup steps in order to get recoveries ≥70% at the 0.01 mg/kg level (*5*). Thus, using these conventional techniques, it is necessary to perform one or more cleanup steps to decrease interferences and preconcentration steps in order to obtain adequate detection levels.

Nowadays, the LC-MS technique has been applied to residue analysis of polar pesticides in vegetables, due to its inherent benefits in sensitivity and selectivity (*6*, *7*). Moreover, LC coupled to tandem MS (MS-MS) has also been applied in this field as a powerful confirmation tool, improving the sensitivity and reducing the sample pretreatment steps, such as solvent partitioning or solid phase extraction. Methods published using LC-MS-MS achieve satisfactory results even without making use of cleanup treatments (*8*, *9*). However, in the analysis of complex matrixes, coeluting interferences could inhibit or enhance the analyte ionization, decreasing or increasing its signal and, therefore, avoiding a correct quantification (*9*−*14*). This matrix effect depends on the combination analyte/sample and obviously could be likely present when performing direct injection of raw extracts, if no chromatographic optimization is carried out.

The purpose of this work is to develop a rapid, sensitive, and selective method for paclobutrazol residue analysis in pear,

* To whom correspondence should be addressed. Tel: +34 964 728100. Fax: +34 964 728066. E-mail: hernandf@exp.uji.es.

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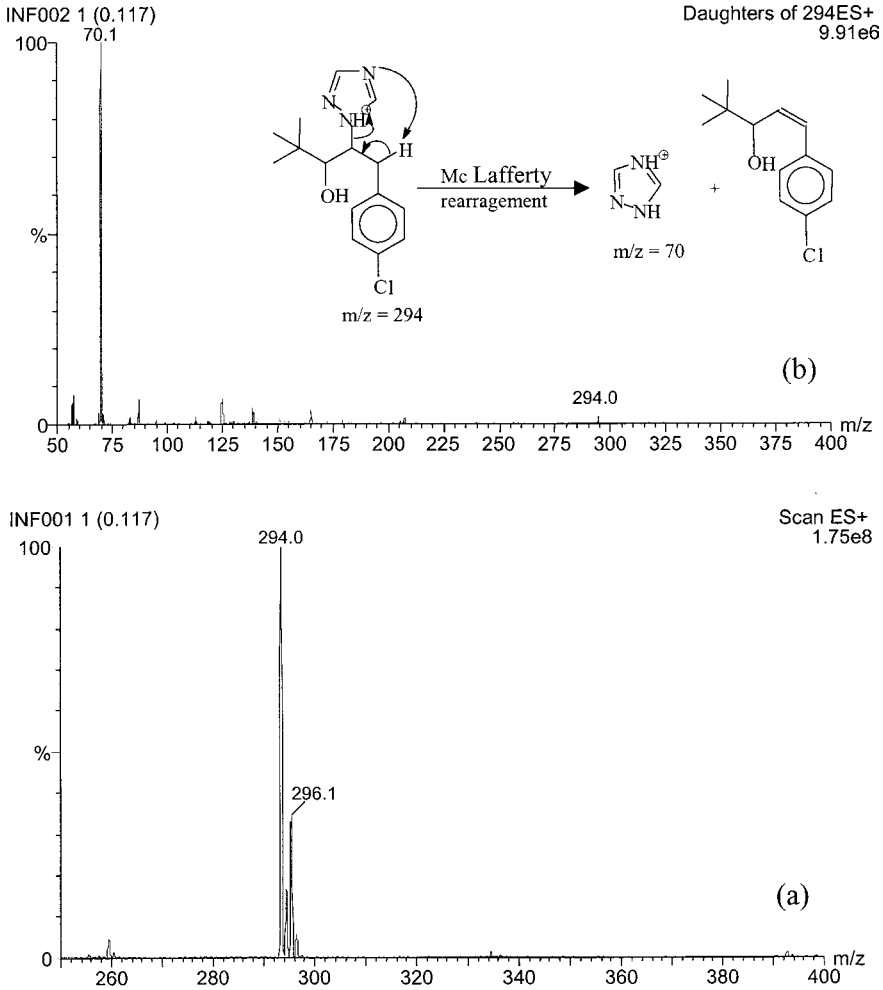

Figure 1. Positive ion electrospray full scan mass spectra (**a**) and product ion spectra (**b**) of paclobutrazol acquired by infusion of 5 μg/mL standard solution and proposed fragmentation pathway for paclobutrazol by McLafferty rearragement.

avoiding any type of sample treatment except from extraction and, consequently, minimizing the analytical errors associated with this step. The LOQ pursued is 1 order of magnitude lower than the lowest MRL setup in several EU countries. The method has been developed in compliance with good laboratory practice requirements in order for it to be applied for registration purposes.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. Paclobutrazol reference standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany). HPLC grade acetonitrile and methanol were purchased from ScharLab (Barcelona, Spain). LC grade water was obtained by purifying distilled water in a Nanopure II system (Barnstead, Newton, MA). Formic acid (98%) was supplied by ScharLab (Barcelona, Spain).

A standard stock solution of paclobutrazol was prepared dissolving 50 mg of powder, accurately weighted, in 100 mL of HPLC grade acetonitrile obtaining a final concentration of 500 μg/mL, which was stored at −20 °C. Working solutions, used for LC-MS analysis or for sample fortification, were obtained by diluting stock solution with HPLC grade acetonitrile and with LC grade water for solutions lower than 1 μg/mL.

Sample Preparation. Two types of samples were obtained as follows: commercial samples taken from the market and field samples from residue trials that were treated with paclobutrazol and collected at different times after pesticide application.

Pear samples were cut into small pieces without any pretreatment, such as washing or removing their skin, and were triturated. Homogenized pear sample (25 g) was accurately weighted (precision 0.1 mg) and mixed with 80 mL of methanol. After the samples were extracted for 2 min with a high-speed blender Ultra-Turrax T25 (Janke & Kunkel GmbH & Co., Staufen, Germany) at 8000 rpm, the entire extract was filtered through a filter paper and washed with 15 mL of methanol. Finally, the volume was adjusted to 100 mL with methanol.

To remove solid particles, an aliquot of extract was passed through a 0.45 μm Nylon filter. Then, 10 μL of the methanolic raw extract was directly injected in the LC-MS-MS system.

Fortification of homogenized pear samples was performed by delivering appropriate volumes of standards in acetonitrile (between 0.5 and 2 mL), to yield concentration levels of 0.005, 0.05, and 0.5 mg/kg. These samples were equilibrated, under dark conditions, for 1 h prior to extraction.

LC-Electrospray Ionization (ESI)-MS-MS. A HPLC system Waters Alliance 2690 (Waters, Milford, MA) was interfaced to a Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) with an orthogonal Z spray electrospray interface. The LC separation was performed by injecting 10 μL and using a Discovery C_{18} column (50 mm × 2.1 mm i.d. particle size of 5 μm) (Supelco, Bellefonte, PA) by running an isocratic mobile phase consisting of mixtures of acetonitrile:water (35:65, v/v) with 0.01% formic acid in 5.5 min at a flow rate of 300 μL/min.

The drying gas and the nebulizing gas were nitrogen generated from pressurized air in a NG-7 nitrogen generator (Aquilo, Etten-Leur, NL). The nebulizer gas flow was set to approximately 80 L/h, and the desolvation gas flow was set to 800−900 L/h. Infusion experiments were performed using a model 11 single syringe pump (Harvard, Holliston, U.S.A.), directly connected to the interface.

For operation in MS-MS mode, the collision gas was argon (99.995%; Carburos Metálicos, Valencia, Spain) with a pressure of

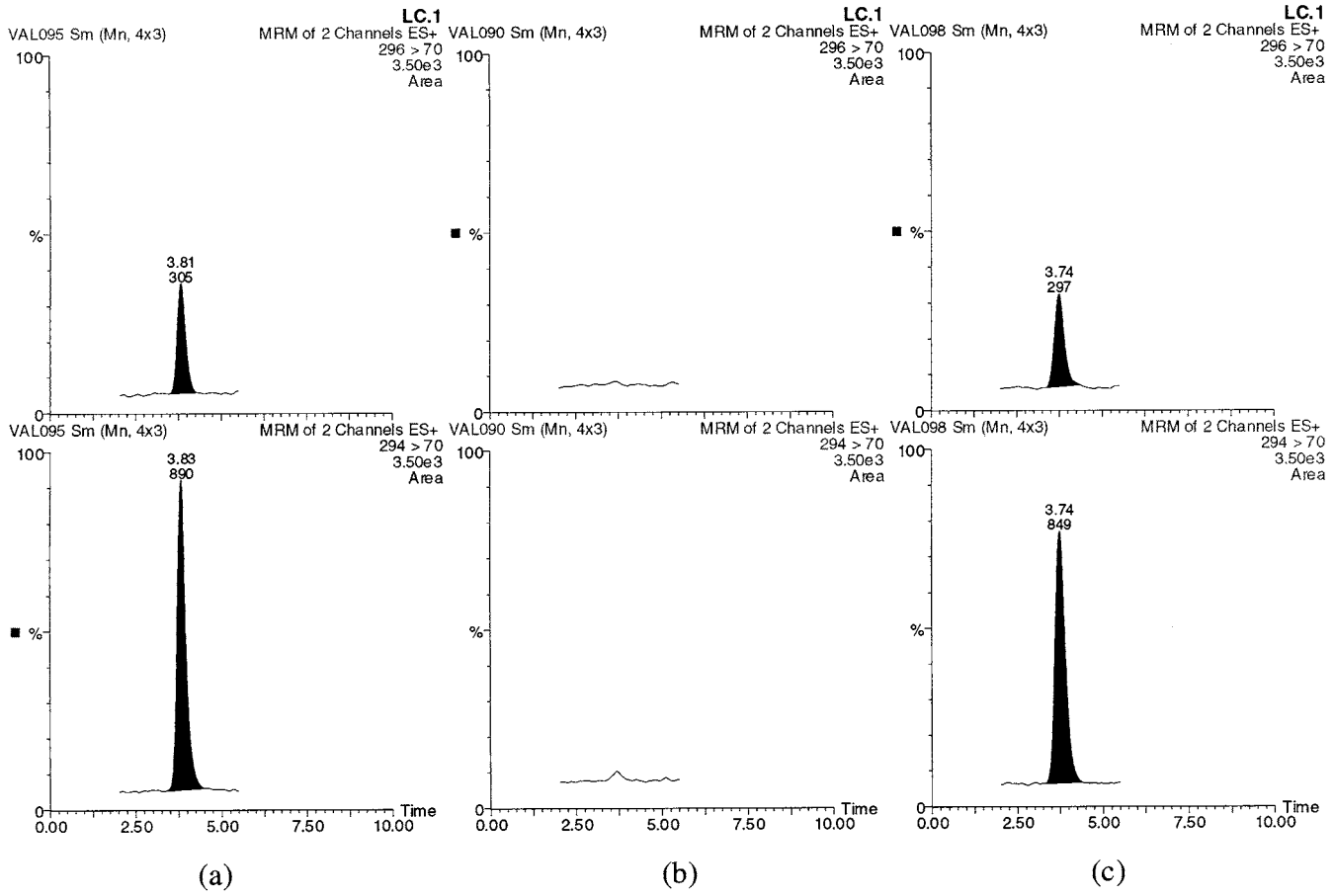

Figure 2. LC-ESI-MS-MS chromatograms for (**a**) standard (1.25 μg/L), (**b**) blank pear sample, and (**c**) pear sample fortified at 0.005 mg/kg with paclobutrazol. Top, confirmation channel (296 > 70), and bottom, quantification channel (294 > 70).

2×10^{-3} mbar in the collision cell. Capillary voltages of 3.5 kV were used in the positive ionization mode. The interface temperature was set to 350 °C, and the source temperature was set to 120 °C. Dwell times of 0.2 s/scan were selected. A solvent delay of 2 min was chosen in order to have an additional cleanup using the built-in divert valve controlled by the Masslynx software.

Validation Study. The calibration curve was obtained by analyzing in triplicate 13 standard solutions at concentrations between 0.5 and 1250 ng/mL.

The recoveries and the precision were obtained by analyzing paclobutrazol in pear samples spiked at three concentration levels (0.005, 0.05, and 0.5 mg/Kg) and were evaluated within the day in quintuplicate. Additionally, the precision between 5 days was also estimated at two concentration levels (0.005 and 0.05 mg/kg) in quintuplicate.

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was obtained when the signal was three times the average of background noises of 10 chromatograms at the lowest analyte concentration assayed. The LOQ was established as the lowest concentration assayed that gave acceptable recoveries (>70%) and precision (<15%).

The use of MS-MS detection allowed us to improve the selectivity and led to the unequivocal confirmation of the analyte in the samples analyzed. In the present work, a qualification channel was possible due to the presence of the chlorine atom in the molecule of paclobutrazol, yielding an isotopic ratio $^{35}Cl/^{37}Cl$ of around 3. For a reliable peak assignment, a 15% tolerance was admitted in the samples analyzed. Besides, the specificity of the method was evaluated, by injecting the procedure blank, a pear sample blank, and a blank sample spiked at the lowest concentration. The response obtained should not exceed 30% of LOQ. Masslynx NT v 3.5 (Micromass) software was used to process the quantitative data obtained from calibration standards and from pear samples.

Data Evaluation. For quantification of paclobutrazol, two external standard calibration curves were used with at least three points within the calibration range in each set of samples. To ensure the quality of the analysis, samples were injected in duplicate and blank samples fortified at two levels (0.05 and 0.005 mg/kg) were used as a quality control (QC) alternately inserted every four samples. The quantification of the sample list was considered satisfactory if the QC recoveries were in the range of 70−110%.

RESULTS

MS Optimization−Infusion Experiments. The full scan mass spectra and the MS-MS spectra of paclobutrazol are shown in **Figure 1**. They were obtained from infusion of 5 μg/mL solutions (50:50 acetonitrile:water, v:v) at a flow of 10 μL/min. As a result of the basic character of the triazol group, paclobutrazol shows positive ionization. The full scan mass spectra present two peaks at *m/z* 294 and 296 corresponding to the pseudomolecular ion $[M + H]^+$ with the characteristic isotopic pattern in agreement with the presence of a chlorine atom. The spectrum was optimized at a cone voltage of 40 V.

The MS-MS spectra of paclobutrazol obtained with a collision energy of 20 eV show only an important fragment at *m/z* 70, which could be explained by the McLafferty rearrangement shown in **Figure 1**. As the paclobutrazol fragmentation leads to the loss of the chlorine atom, both precursor ions, *m/z* 294 and 296, share the same fragmentation at *m/z* 70. Summarizing, a MS-MS qualification channel and a MS-MS quantification channel were selected at 296 > 70 and 294 > 70, respectively, using a cone voltage of 40 V and collision energy of 20 eV.

Extraction Solvent and LC Optimization. Paclobutrazol is very soluble in organic polar solvents (between 100 and 150

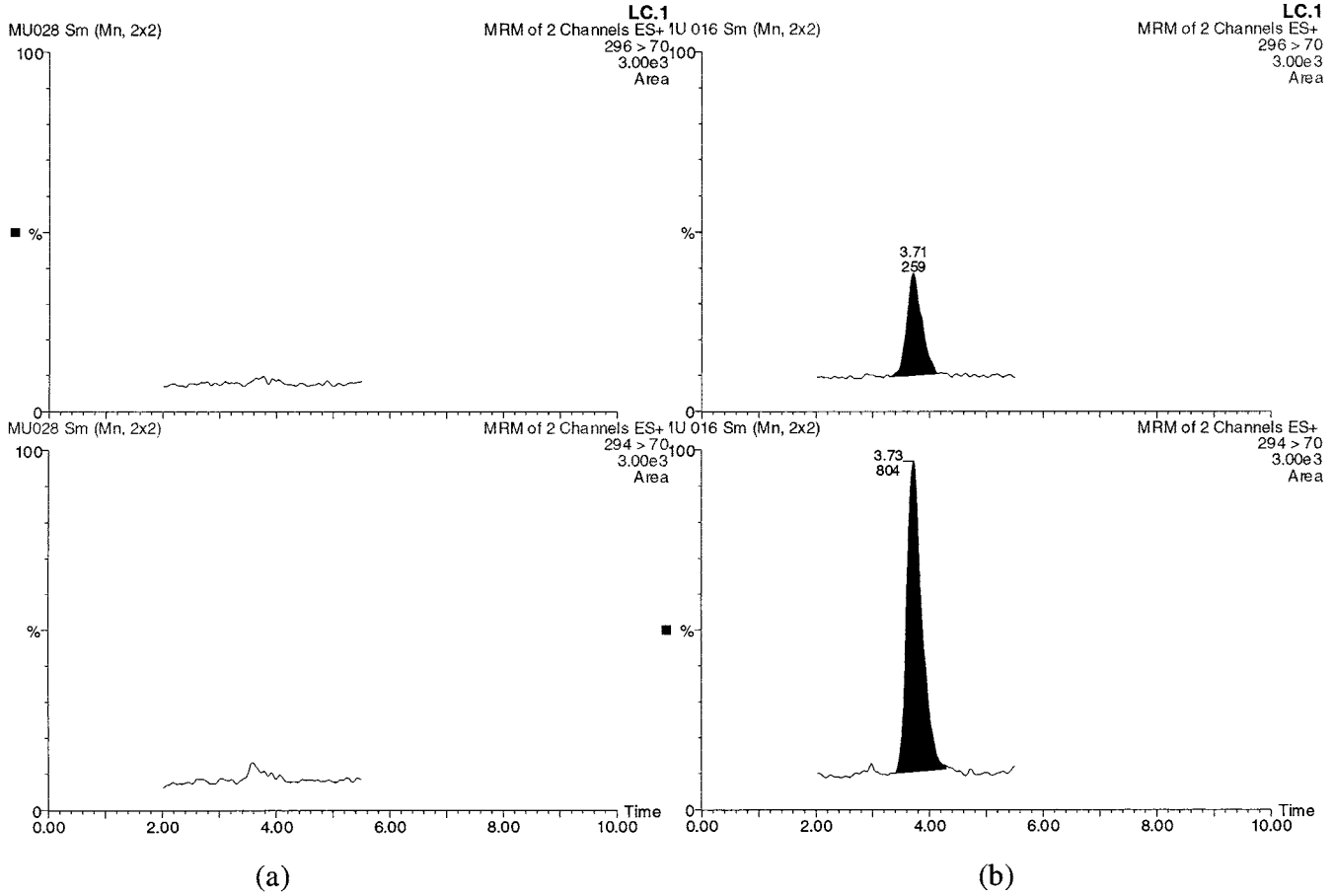

Figure 3. LC-ESI-MS-MS chromatograms corresponding to the residue analysis of (**a**) commercial pear sample and (**b**) field sample that contained 5.1 μg/kg of paclobutrazol. Top, confirmation channel (296 > 70), and bottom, quantification channel (294 > 70).

g/L at 20 °C) (*1*). To inject the pear extract without any pretreatment or solvent exchange, methanol, one of the most typical organic eluents in LC, was used as the extractant.

As the analysis was performed by positive ionization in electrospray mode (ES+), a higher signal could be expected after addition of acid to the mobile phase. However, the concentration of acid should be optimized in order to avoid a signal decrease due to matrix effect. In this paper, we selected 0.01% formic acid in the mobile phase as a compromise between adequate ionization and matrix effect.

Keeping in mind the rapid determination of paclobutrazol, an isocratic 65:35 water:acetonitrile (v/v) mobile phase was assayed in two columns, Nucleosil C_{18} and Discovery C_{18}. After direct injection of 10 μL of the methanolic raw extract, a good peak shape was obtained only with the Discovery C_{18} column, which was finally selected for subsequent experiments.

In all experiments, the first part of the chromatogram was sent to waste by using the built-in divert valve in the mass spectrometer controlled by the Masslynx software. This solvent delay gave an additional cleanup step, which avoided the overload of the interface with early-eluting interferences that could decrease the analyte ionization. This simple cleanup, together with the selectivity inherent to the MS-MS technique, led to the adequate quantification by external standards prepared in methanol due to the absence of matrix interference.

Validation Study. Calibration curves showed good linearity between 0.5 and 1250 μg/L, with a correlation coefficient $\geq$0.999. The method was precise and accurate at the three fortification levels studied. The recovery values as well as the precision within and between days, expressed as relative standard deviation (RSD), are shown in **Table 1**. Data show satisfactory recoveries, between 82 and 102%, at the three concentration levels assayed and excellent precision with RSD always lower than 10%. The LOQ corresponded to the lowest fortification level assayed, i.e., 0.005 mg/kg, for which satisfactory recovery and adequate precision (RSD < 15%) were obtained. Moreover, a LOD as low as 0.7 μg/kg was estimated (S/N > 3) from chromatograms at the lowest concentration level assayed. This very low value illustrates the high sensitivity of MS-MS detection in pesticide residue analysis.

Table 1. Recovery and Precision (as RSD) of the Method at Different Concentration Levels of Paclobutrazol in Pears (n = 5)

	repeatability		intermediate precision
level of fortification (mg/kg)	recovery (%)	RSD (%)	RSD (%)
0.005	102	6	9
0.05	90	2	8
0.5	82	7	*a*

[a] Not assayed.

In all experiments performed, both standards and spiked samples showed an ion ratio $^{35}Cl/^{37}Cl$ within the accepted tolerance, using the transition 296 to 70 as the confirmation channel. In the study of the specificity, no responses were detected for either the procedure blank or the pear sample blank, showing the high specificity of MS-MS detection.

Typical LC-MS-MS chromatograms of standard solutions and pear sample extracts (blank and fortified at the lowest level

assayed) are shown in **Figure 2**. They were obtained after direct injection of a raw extract sample with a total chromatographic run of only 5.5 min.

Application to the Method to Real Pear Samples. The validated method was applied to 48 pear samples: eight commercial samples taken from the market and 40 samples from field residue trials. Every six samples, two QC, were inserted (one at the LOQ level and the other at the 10 × LOQ level) obtaining an overall response between 79 and 106%, demonstrating the robustness of the method. All experiments were made in compliance with good laboratory practices.

Paclobutrazol was not detected in any of the commercial samples analyzed, while treated samples presented different concentration levels according to the collection date after application in the field. As an example, **Figure 3** shows a blank sample (**Figure 3a**) and a positive sample containing paclobutrazol at a level very close to the LOQ (concentration found, 5.1 μg/kg) (**Figure 3b**). As it can be seen, the presence of paclobutrazol was satisfactorily confirmed at these low concentration levels, using the transition 294 > 70.

CONCLUSION

This work has shown that LC-ESI-MS-MS is a powerful analytical technique for the rapid determination of paclobutrazol residues in pear samples. The high selectivity and sensitivity of LC-MS-MS allow the direct injection of the raw methanolic extracts achieving a detection limit 80 times lower than the MRL set up for this pesticide in pome fruits in several European countries. The isocratic separation on a Discovery C_{18} column permits the correct quantification without matrix effects as well as short chromatographic runs of only 5.5 min, rendering a throughput of around 250 samples/day. The method was validated and implemented for routine analysis of pears in compliance with good laboratory practices, demonstrating its speediness and robustness.

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