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Use of Liquid Chromatography Coupled to Quadrupole Time-of-Flight Mass Spectrometry To Investigate Pesticide Residues in Fruits

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In this paper, the potential of coupling liquid chromatography with hybrid quadrupole time-of-flight mass spectrometry (LC−QTOF) for the determination of pesticides in a variety of fruit samples (orange peel and flesh, banana skin and flesh, strawberry and pear) has been explored. The quantitative application at residue levels has been proven for two insecticides (buprofezin and hexythiazox), which were satisfactorily determined at three concentration levels, 0.1, 1, and 5 mg/kg, obtaining a suitable linearity range (correlation coefficient > 0.99) of more than 2 orders of magnitude. Satisfactory recoveries have been obtained for both compounds at the three levels tested in all sample matrices, with lowest calibration levels (LCL) of 0.075 and 0.01 mg/kg. The excellent potential of QTOF for identification purposes is illustrated by the high number of identification points (IPs) earned, up to 21, at the highest concentration of 5 mg/kg, or between 11 and 21 at the 0.1 and 1 mg/kg levels. The application of LC-QTOF MS to real samples revealed the presence of several positives at concentrations close to the LCL, all of which were confirmed with more than 11 IPs. The potential of QTOF for elucidation of nontarget analytes has also been demonstrated by the finding of one transformation product (TP) of buprofezin in a banana skin sample. This TP was identified by obtaining the full scan product ion spectra at different collision energies with acceptable accurate mass deviation. The work performed in this paper illustrates the suitability and excellent confirmatory potential of LC-QTOF MS for pesticides residues analysis in food samples.

Pesticide residue analysis (PRA) in food commodities is an important field of modern analytical chemistry. Nowadays, the reliable determination of pesticide residues is a challenge for analytical chemists. Methods applied have to achieve the high sensitivity required (in many cases residues have to be accurately determined at levels close to 0.05 mg/kg in vegetables,[1] or 0.01 mg/kg in baby food[2]) for a wide variety of analyte/matrix combinations, and for compounds from different chemical families with quite distinct physicochemical characteristics. Additionally, foodstuffs are complex matrix samples, which makes the selectivity of the method very important. Thus, to develop suitable and robust methods in PRA, powerful analytical techniques able to offer the high selectivity and sensitivity needed for a wide range of analyte polarities are required.

Gas chromatography mass spectrometry (GC−MS) has usually been the technique of choice for this purpose. However, in the past 10 years there has been a clear trend to use liquid chromatography coupled to mass spectrometry (LC−MS)[3] as a powerful and effort saving technique for PRA. This trend can be explained by continuous technical improvements and by the excellent analytical characteristics of LC−MS, mainly for the determination of polar, nonvolatile analytes. Although some MS analyzers such as single quadrupole (Q) in selected ion monitoring (SIM) mode,[4,5] and ion trap (IT) in scan mode[6,7] have been applied, these methods present some limitations, mainly regarding sensitivity and/or specificity. Two alternatives have typically been considered to minimize these limitations: the use of tandem mass spectrometry, such as IT in MS^n mode[8] or triple quadrupole (QqQ);[9,10] and the use of medium/high-resolution mass spectrometry, such as time-of-flight (TOF).[11,12] These techniques have allowed a significant advance in PRA, mainly due to the notable increase in method sensitivity and selectivity. Within this field, hybrid quadrupole time-of-flight (QTOF) offers strong potential, because it combines the high resolution of TOF analyzers with the capability to perform MS/MS experiments. Thus, one of the most attractive characteristics of QTOF is the possibility of acquiring full scan spectra of product ions with accurate mass, which drastically increases the selectivity of the method due to

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the high amount and quality of the structural information produced.

In addition to the sensitivity and accuracy needed for quantification, an evident analytical requirement is to provide reliable information for a confirmation of the identity of the pesticide detected to avoid reporting false positives. The correct identification of organic pollutants has become a serious and debatable subject.[13,14] Recently, several guidelines consider confirmation as a required parameter before reporting positive findings in real-world samples. Thus, the European SANCO Document on Quality Control Procedures for PRA[15] proposes confirmation principles, although mainly based on data obtained by GC−MS analysis with electron ionization (EI). Although it opens up the possibility of using other ionization processes, such as chemical ionization (or atmospheric pressure ionization (API) in LC−MS based methods), or other MS approaches, such as high-resolution MS or tandem MS, no detailed mass spectrometric criteria are given for confirmation that take into account the different approaches used nowadays in PRA. A more detailed guideline regarding confirmation comes from the European Commission Decision 2002/657/EC, used for identification and quantification of organic residues and contaminants in foodstuffs of animal origin.[16] It is based on earning Identification Points (IPs) to evaluate the results of confirmation, as previously discussed by André et al.[17] Confirmative criteria based on IPs have been studied in depth and applied at our laboratory in environmental samples,[18,19] where QTOF has shown its great potential for confirmation of organic pollutants. As a consequence of its excellent capability to acquire wide structural information, the use of QTOF is an increasing trend for elucidation and compound confirmation (a high number of IPs can be reached), as recognized in a recent review on environmental MS.[20] The valuable structural information provided by this technique may even allow the elucidation of nontarget compounds.[21]

Although LC−TOF MS has been investigated in PRA of food samples,[11,12] the use of hybrid QTOF remains almost unexplored in this field in spite of its high potential. Most QTOF applications have been directed toward other fields such as pharmaceutical or biomedical research, with very few applications to food analysis,[22] and some work focused on environmental pollution.[23,24]

The goal of this work is to show the potential of QTOF for PRA in food samples in regard to quantification and confirmation of target analytes. In addition, the capability of QTOF for elucidation of unknowns, such as other nontarget pesticides or transformation products (TPs), and the use of the accurate mass data provided by this instrument to establish fragmentation pathways have also been explored. As an illustrative case study, two insecticides, hexythiazox and buprofezin, have been investigated in different fruit commodities.

EXPERIMENTAL SECTION

Reagents and Chemicals. Hexythiazox and buprofezin reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany). HPLC grade methanol, residue analysis grade acetone, and reagent grade formic acid were purchased from ScharLab (Barcelona, Spain). LC grade water was obtained by purifying demineralized water in a Nanopure II system (Barnstead Newton, MA).

Stock standard solutions of hexythiazox and buprofezin were prepared by dissolving 50 mg powder, accurately weighted, in 100 mL acetone, obtaining a final concentration of 500 μg/mL. These solutions were stored at −20 °C. Working solutions, used for LC-QTOF MS analysis or sample fortification, were obtained by diluting the stock solutions with acetone.

Instrumentation. A hybrid quadrupole time-of-flight (QTOF) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters, Milford, MA) was interfaced to an HPLC system, Waters Alliance 2695 (Waters) quaternary pump, for the chromatographic separation. Drying gas as well as nebulizing gas was nitrogen, generated from pressurized air in a nitrogen generator from Peak Scientific (Inchinnan, Scotland, U.K.). The nebulizer gas flow was set to approximately 15 L/h and the desolvation gas flow to 700−800 L/h. A cone voltage of 30 V and a capillary voltage of 3.5 kV were used in both positive and negative ionization modes. The nitrogen desolvation temperature was set to 350 °C and the source temperature to 120 °C. TOF MS resolution was approximately 5000 (fwhm). MS and MS/MS spectra were acquired over an m/z range 50−650. For MS/MS operation, argon (99.995%, Carburos Metálicos, Valencia, Spain) was used as a collision gas with a pressure of approximately 2×10^{-5} mbar in the collision cell. Collision energies of the different product ions were chosen depending on the method purpose. Thus, for quantification, only one collision energy was considered (20 eV), while for confirmation, three collision energies were selected (10, 20, and 30 eV). The MCP detector potential was set to 2900 V. Scan times of 1 s/spectrum were chosen. A suitable MS profile was used. Data station operating software was MassLynx v 4.0.

Mobile phases consisted of mixtures of 0.01% HCOOH in methanol/0.01% HCOOH in water, and an injection volume of 25 μL was used. For quantification purposes, the separation was performed on a short Discovery C_{18} column (50 mm × 2.1 mm, i.d. 5 μm) (Supelco, Bellfonte, PA) using gradient elution at a flow rate of 0.3 mL/min. The percentage of methanol was changed as follows: 0.0 min, 55%; 3.0 min, 55%; 4.0 min, 90%; 6.1 min, 55%; 10.0 min, 55%. For confirmation purposes, a Discovery C_{18} column (50 mm × 2.1 mm, i.d. 5 μm) was also used, but by performing a shallow gradient where the percentage of methanol changed as follows: 0.0 min, 20%; 2.0 min, 20%; 2.1 min, 60%; 17.0 min, 80%;

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17.1 min, 80%; 20.0 min, 20%. Flow rate was also selected at 0.3 mL/min. Finally, for elucidation purposes, a long X-Terra C_{18} column (250 mm × 2.1 mm, i.d. 5 μm) (Waters) was used, at a flow of 0.2 mL/min with a gradient where the percentage of methanol varied as follows: 0.0 min, 5%; 20.0 min, 95%; 23.0 min, 95%; 24.0 min, 5%; 35 min, 5%. A solvent delay of 3 min was applied in the analysis.

Calibration of the m/z-axis was performed daily using a Model 11 single syringe pump (Harvard Instruments, Holliston, MA), directly connected to the interface. Calibration in both positive and negative ESI mode was conducted from m/z 50−650 with a 1:1 mixture of 0.1 M NaOH/10% HCOOH diluted (1:25) with acetonitrile/water (80:20), at a flow rate of 20 μL/min.

Sample Procedure. Six types of samples were analyzed: orange peel and flesh, banana skin and flesh, strawberry and pear. Banana samples corresponded to field residue trials, while the rest were collected from the market. Samples were cut into small pieces without any pretreatment and were triturated with a chopper K55E (Dito Sama, Aubusson, France). An aliquot of 10 g homogenized chopped sample was accurately weighted (precision 0.1 mg) and mixed with 70 mL acetone. After extraction for 2 min with high-speed blender Ultra-Turrax T25 (Janke & Kunkel GmbH &Co., Staufen, Germany) at 8000 rpm, the entire extract was filtered through a 25−30 μm filter paper (filtros ANOIA S.A., Barcelona, Spain) and washed with 10 mL of acetone, and the volume was adjusted to 100 mL with acetone. An aliquot of the raw extract was passed through a 0.45 μm Nylon syringe filter (ScharLab, Barcelona, Spain) to remove solid particles. Then, 25 μL filtered extract was directly injected in the LC−QTOF system.

Fortification of samples was performed by delivering 1 mL mixed standard solutions of 1, 10, or 50 μg/mL in acetone to 10 g homogenized sample to obtain 0.1, 1, and 5 mg/kg levels, respectively. These samples were equilibrated, under room conditions, for 1 h prior to extraction.

MS and MS/MS Experiments. Obtaining accurate mass measurements for product ions is very helpful to establish the fragmentation pathway of the selected precursor ion. First, full scan experiments in positive ionization mode at different cone voltages, using the first quadrupole only as a RF-guide, were performed to establish the optimum cone voltage for each compound. Second, QTOF experiments at different collision energies were performed in order to obtain the more sensitive response for each product ion. At each collision energy tested, a product ion with both known exact mass and suitable abundance was selected as lock mass in order to maintain accurate mass measurements for the rest of the product ions. The accurate mass obtained for each product ion was used to confirm the proposed fragmentation pathway. Additionally, relative intensity abundances were evaluated.

Validation of the Quantitative Method. Different validation data sets were obtained for each type of sample, following the European Union SANCO working documents.[15]

The calibration curve was obtained by injecting, in triplicate, standard solutions at 12 concentration levels between 1 and 750 ng/mL. The selected calibration points were the following: 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, 500, and 750 ng/mL. Linearity acceptance criterion was that correlation coefficients were higher than 0.99.

The accuracy and the precision were calculated by analyzing blank samples spiked at three concentration levels (0.1, 1, and 5 mg/kg) and were evaluated within-day in quintuplicate at each concentration level. Acceptance criteria for accuracy were that recoveries fall between 70% and 110% and for precision that relative standard deviation (RSD) was lower than 20%.

The limit of detection (LOD), defined as the lowest concentration that analytical process can reliably differentiate from background levels, was estimated for a signal-to-noise ratio of 3 from the chromatograms of samples spiked at the lowest analyte concentration tested.

Two mass windows (i.e., 50 and 200 mDa) were tested during mass chromatogram extraction in order to have the most reliable quantitative method.

Confirmation Study. The confirmation capability of QTOF was explored and discussed on the basis of the number of Identification Points (IPs) earned, in the line of the European Commission Guideline for residue contaminants in products of animal origin.[16] A confirmation method for hexythiazox and buprofezin was applied to samples spiked at three concentrations (0.1, 1, and 5 mg/kg) in order to evaluate the QTOF confirmatory capabilities at both low and high residue levels. The relative abundance of each ion was calculated by measurement of peak areas in its eXtracted Ion Chromatogram (XIC). Theoretical abundance data were obtained as mean relative abundances calculated from the standard solutions (at different concentration levels) injected in each sample batch. The experimental value for each ion in the sample investigated was then compared with the theoretical one. Every ion, whose relative abundance fulfilled EU criteria,[16] was considered as acceptable for confirmative purposes, and the adequate number of IPs was then assigned. Experimental accurate mass values were obtained by combination of all spectra across the chromatographic peak.

Although the higher the IPs reached the more reliable the confirmation, a detected analyte can be considered as positive if, at least, 3 IPs are obtained, in the case of regulated compounds such as the majority of pesticides. For nonregulated banned compounds, a minimum of 4 IPs is required.[16]

Elucidation Study. In order to detect the presence of possible pesticide TPs in real samples, the instrument was operated first in TOF mode. To maintain mass accuracy along the time, 1 μg/mL solution of 3,5-diiodo-L-tyrosine (Sigma, Louis, MO) in methanol was used as lock mass, and introduced post-column using a model 2150 isocratic HPLC pump (LKB, Bromma, Sweden) at a flow rate of 100 μL/min by means of a PEEK T-union. To obtain accurate mass measurements, 15 spectra were combined, and an Np value of 0.6 was used. The mass data were centered using peak areas at 80% of centroid top.

The methodology applied was based on the comparison between a positive sample and a blank sample. Data were acquired in continuum mode, and subsequently centered and corrected by lock mass measurement. Differences between both samples were highlighted by means of the MetaboLynx application manager (Waters). The unknown chromatographic peaks found during this procedure might be considered, in principle, as potential TPs peaks. Their molecular formulas were calculated from their accurate mass and isotopic pattern.

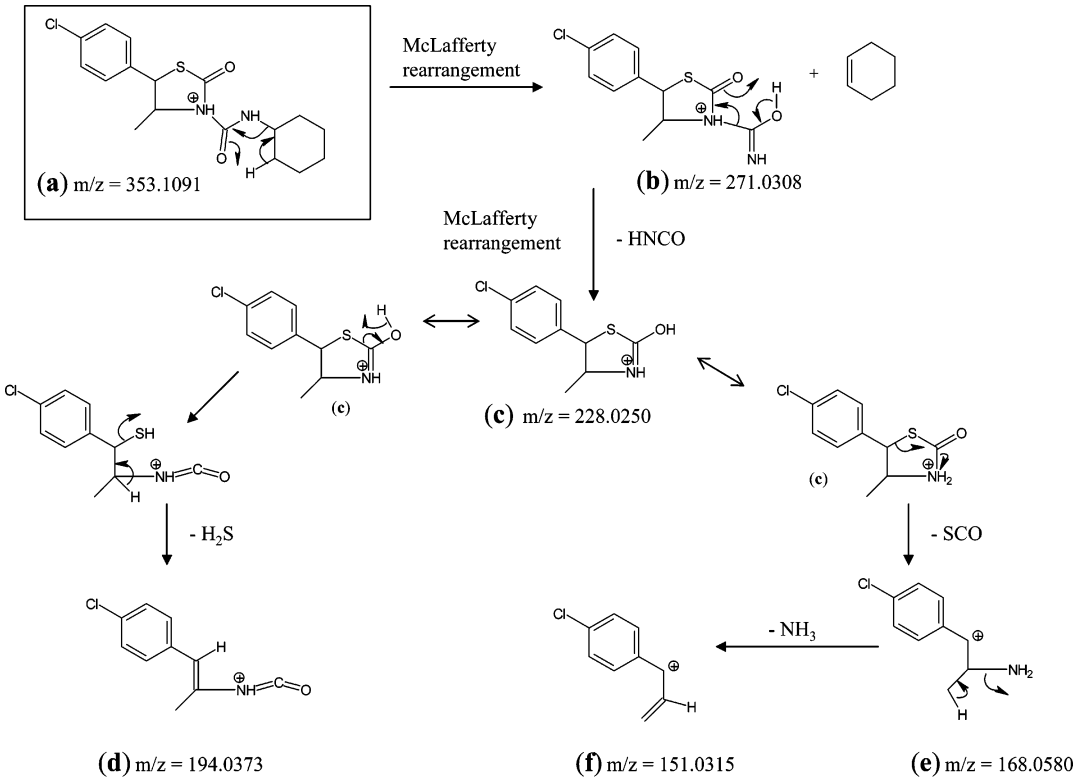

Figure 1. Fragmentation pathway proposed for hexythiazox.

Subsequently, the product ion spectrum for each presumptive TP peak was obtained operating in QTOF mode. In this case, the precursor ion, or one of the product ions at higher *m/z*, was used as a lock mass for accurate mass measurement, centering and correcting data acquisition. Possible neutral losses were evaluated as well, in order to elucidate the TP structure.

RESULTS AND DISCUSSION

MS and MS/MS Experiments. In MS experiments performed in TOF full scan mode, the best sensitivity for both compounds was obtained at a cone voltage of 30 V. In QTOF experiments, we selected the protonated molecule ($[M + H]^+$) as precursor ion (*m/z* 353 for hexythiazox and *m/z* 306 for buprofezin), and its product ion spectra were acquired at 10, 20, and 30 eV collision energies.

Data obtained after MS/MS experiments allowed us to propose the fragmentation pathways for both compounds. The pathway proposed for hexythiazox (Figure 1) starts with a McLafferty rearrangement from the precursor ion **a** to give the product ion **b** after a loss of cyclohexene. A subsequent McLafferty rearrangement leads to **c**, after a neutral loss of HNCO. From ion **c**, two possible ways of fragmentation may be considered: one to obtain **d**, after the neutral loss of H_2S, and the other, by consecutive losses of SCO and NH_3, to obtain **e** and **f**, respectively.

Figure 2 shows the proposed buprofezin fragmentation pathway, where three possible routes are considered from the precursor ion **g**. One of them includes two consecutive McLafferty rearrangements to obtain the product ions **h** and **i**, after neutral losses of propene and isobutene, respectively. Another route is proposed by a retro Diels−Alder (**g**) to generate **j** and **m**. Then, the neutral loss of $(CH_3)_2CHNCO$ from product ion **j** generates the ion **l**. From **j**, the product ions **k** and **n** are obtained by two McLafferty rearrangements with neutral losses of isobutene and SCNH, respectively. In another fragmentation route the product ion **o** is obtained directly from the precursor ion **g**.

The use of accurate mass has been reported as a useful tool to avoid misinterpretations in MS/MS fragmentation pathways.[25] In order to obtain reliable mass accuracy in TOF instruments, a reference ion with known exact mass is necessary within the spectrum. In QTOF experiments, this lock mass can be the surviving precursor ion. In our case, this approach was used at low collision energies, i.e., when the precursor ion was still present. However, at higher collision energies, the absence of precursor ion forced us to select some product ions (228.0250 for hexythiazox and 201.1062 for buprofezin) as lock mass. Using this approach, the deviations of the experimental masses from the theoretical ones were in most cases lower than 2.0 mDa (Table 1). Therefore, the proposed fragmentation pathways (Figures 1 and 2) are supported by the accurate masses obtained. The only exception was found to be the hexythiazox product ion (**f**) (deviation 3.3 mDa), probably due to its low relative abundance.

Table 1 shows the relative abundances of product ions, referenced to the highest product ion peak area at its optimum collision energy. These values depended on the collision energy applied. In general, both hexythiazox and buprofezin product ions at higher *m/z* presented optimum collision energy of 20 eV, while for lower *m/z* values 30 eV was necessary to maximize abundance. For hexythiazox, the most abundant product ion was **c**, *m/z* 228.0250, while for buprofezin the highest abundance was obtained for **j**, *m/z* 201.1062, both optimized at 20 eV. Although the number of product ions observed was higher in the case of buprofezin,

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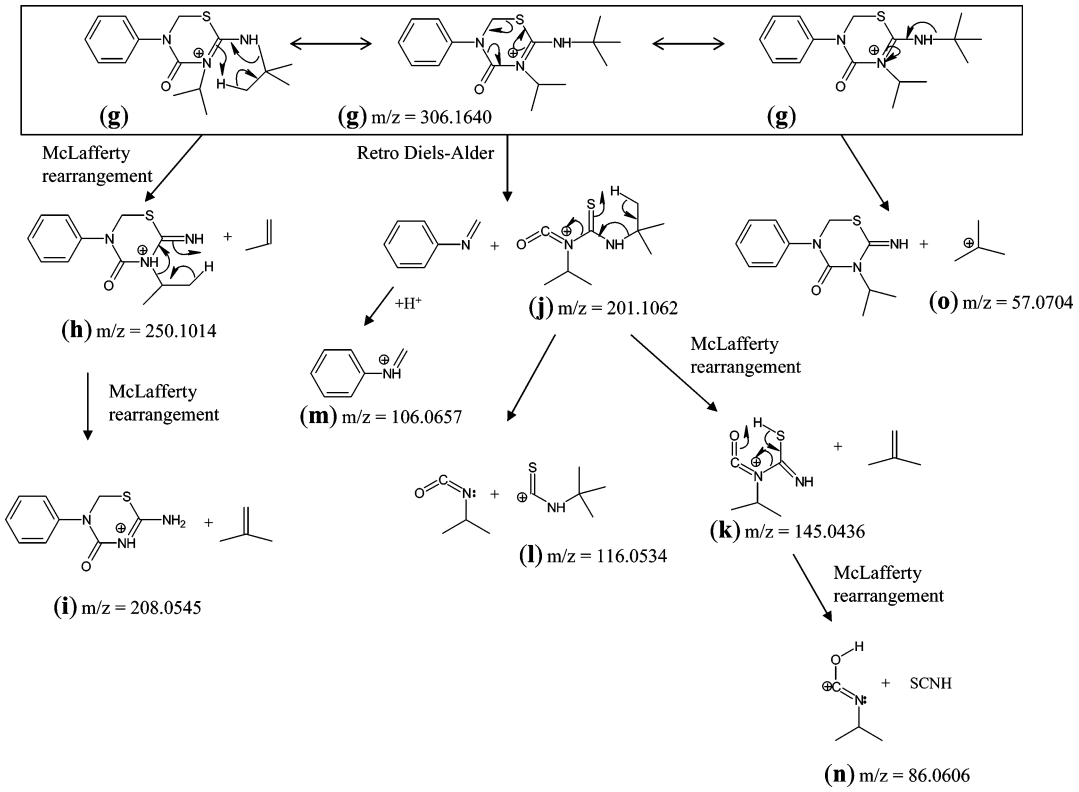

Figure 2. Fragmentation pathway proposed for buprofezin.

Table 1. Product Ions for Hexythiazox and Buprofezin[a]

analyte	product ion	theoretical *m/z*	experimental *m/z*	deviation (mDa)	abundance 10 eV	abundance 20 eV	abundance 30 eV
hexythiazox	$C_{17}H_{22}N_2O_2SCl^+$ (**a**)	353.1091			74	15	
	$C_{11}H_{12}N_2O_2SCl^+$ (**b**)	271.0308	271.0328	2.0	13	26	4
	$C_{10}H_{11}NOSCl^+$ (**c**)	228.0250	228.0250	0.0	36	100	48
	$C_{10}H_9NOCl^+$ (**d**)	194.0373	194.0391	1.8		2	17
	$C_9H_{11}NCl^+$ (**e**)	168.0580	168.0564	1.6	1	5	46
	$C_9H_8Cl^+$ (**f**)	151.0315	151.0282	3.3		0.4	2
buprofezin	$C_{16}H_{24}N_3OS^+$ (**g**)	306.1640			12	6	0.1
	$C_{12}H_{16}N_3OS^+$ (**h**)	250.1014	250.0998	1.6	1.1	4	1.4
	$C_9H_{10}N_3OS^+$ (**i**)	208.0545	208.0527	1.8	0.1	1.1	3
	$C_9H_{17}N_2OS^+$ (**j**)	201.1062	201.1045	1.7	67	100	9
	$C_5H_9N_2OS^+$ (**k**)	145.0436	145.0447	1.1	0.4	5	9
	$C_5H_{10}NS^+$ (**l**)	116.0534	116.0520	1.4	5	26	32
	$C_7H_8N^+$ (**m**)	106.0657	106.0657	0.0	0.6	5	12
	$C_4H_8NO^+$ (**n**)	86.0606	86.0611	0.5		0.3	2
	$C_4H_9^+$ (**o**)	57.0704	57.0716	1.2		1.0	2

[a] Abundances at different collision energies and deviations of experimental *m/z* values from the theoretical ones.

most of them presented relative abundances lower than 10% making their potential use for confirmative purposes troublesome in samples at concentration levels close to the LOD. Three hexythiazox ions (**b**, **c**, and **e**) exhibited relative abundances higher than 20%, facilitating their use at low analyte concentrations. It is remarkable that these relative abundances may vary depending on some parameters, such as the quantity of argon in the collision cell. Therefore, checking abundances is recommended before using them for confirmative purposes in real samples.

Quantification. After MS/MS optimization, five and eight possible product ions were plausible for determination of hexythiazox and buprofezin, respectively. For quantitative purposes, only the most sensitive product ion was selected. Thus, the method included two functions, one for buprofezin (product ions from *m/z* 353) and the other for hexythiazox (product ions from *m/z* 306), both at a cone voltage of 30 V and collision energy of 20 eV. Both compounds were quantified using different XICs at the most sensitive product ions, i.e., 228.0250 (hexythiazox) and 201.1062 (buprofezin).

Quantification was performed at two mass extraction windows (50 and 200 mDa) and at different levels of fortification in each sample type, with the aim of establishing the most suitable quantitative approach. As can be seen in Table 2, data were similar at 1 and 5 mg/kg spiking levels independently on the mass windows used. However, at 0.1 mg/kg quantitative data obtained with a microwindow XIC of 50 mDa were clearly poorer than those

Table 2. Quantification of Hexythiazox and Buprofezin in Several Sample Types Using Two Mass Windows[a]

	50 mDa			200 mDa		
recovery (%)	0.1 mg/kg	1 mg/kg	5 mg/kg	0.1 mg/kg	1 mg/kg	5 mg/kg
			Hexythiazox			
orange peel	125(35)	103(17)	90(19)	95(10)	108(19)	83(17)
orange flesh	130(145)	104(6)	114(6)	97(39)	110(7)	110(7)
banana skin	117(39)	93(19)	120(6)	127(31)	110(11)	114(4)
banana flesh	119(81)	98(23)	110(8)	114(10)	97(20)	105(9)
strawberry	122(129)	79(20)	94(12)	104(27)	88(18)	91(3)
pear	147(19)	112(16)	86(3)	109(12)	109(13)	84(5)
			Buprofezin			
orange peel	51(31)	78(5)	75(6)	57(18)	78(5)	75(6)
orange flesh	91(8)	102(4)	99(4)	93(8)	102(3)	99(5)
banana skin	84(15)	101(4)	97(2)	86(4)	101(4)	98(2)
banana flesh	90(3)	89(14)	97(3)	97(8)	93(7)	97(3)
strawberry	76(16)	82(6)	88(5)	81(11)	82(6)	89(4)
pear	79(8)	86(4)	87(5)	83(9)	86(3)	87(5)

[a] Mean recoveries and relative standard deviations (in brackets) at three concentration levels ($n = 6$, each).

of 200 mDa. Although some recoveries could be considered satisfactory, relative standard deviations at this low level were unacceptable, especially when decreasing the mass window. This fact could be explained by the need to use, as lock mass, a known ion present in the spectrum different from the quantitative one in order to calculate accurate mass. This ion is normally less abundant and, at low concentrations, can remain almost undetectable, making the assignment of accurate mass unreliable. Under these circumstances, the deviation in the measurement of each ion substantially increases, especially at mass windows lower than 200 mDa. Although this situation was only observed at the lowest concentration tested, a mass window of 200 mDa was selected for the overall concentration range to perform quantitative applications.

Traditionally, QTOF has found its main applications in qualitative analysis, as has been described in environmental analysis.[24] The main limitation for quantitative purposes comes from its low linear dynamic range. However, some authors have found satisfactory quantitative applications of TOF analyzers in the determination of pesticides in vegetable samples.[11,12] In the present work, calibration linearity was studied considering different types of adjustments. Standards for calibration were adjusted to first- and second-order equations, obtaining in both cases calibration curves about 2 (hexythiazox) or 3 (buprofezin) orders of magnitude with satisfactory correlation coefficient (higher than 0.99). When studying the tendency of both adjustments, the second-order equation fit better than the first-order one, and the estimated variance of residuals for the adjustment was lower (1.7 compared to 3.0 for hexythiazox, and 61 compared to 322 for buprofezin). Due to this behavior, a second-order adjustment was found to be more suitable, and it was finally applied for quantitative purposes.

The quantitative method led to satisfactory recoveries for both analytes in the sample matrices tested. Therefore, no relevant ionization suppression due to matrix components[26,27] was observed, even after the direct injection of the raw extracts. Relative standard deviations were typically lower than 20%, except for hexythiazox at 0.1 mg/kg in some matrices, which was assumed to be due to the lower sensitivity for this compound, together with the loss of mass accuracy explained above.

The LOD estimation may sometimes be ambiguous, due to the nonpractical existence of chemical noise in the chromatogram (Figure 3). We have used the lowest calibrated level (LCL) instead, i.e., the lowest concentration that fulfills the calibration range, which was found to be 7.5 ng/mL (hexythiazox) and 1 ng/mL (buprofezin). Thus, the reliable determination of hexythiazox and buprofezin could be made at levels down to 0.075 and 0.01 mg/kg, respectively, in samples. Although the analytes were in fact detected at concentrations lower than LCL, quantification was not considered reliable, as occasionally there may be interference from little spikes of electronic noise appearing at the analytes' retention time.

Confirmation. The developed quantitative method could be taken also as confirmative due to the additional information obtained from the product ion full scan acquired at one collision energy. From a strict point of view, confirmation requires the reanalysis of the sample, by which the second result "confirms" the first. Obviously, a second independent analysis would be preferable for more reliable confirmation, in order to rule out possible sample contamination, carry-over, mislabeled samples, or another kind of human error, this being the approach typically applied when dealing with regulatory purposes, ideally using an analytically orthogonal approach.

In the present paper, we have explored the extraordinary potential of QTOF within this field, and confirmation has been made by performing a second injection in the QTOF instrument, acquiring the complete product ion spectrum, and using a more gradual mobile phase gradient. We have adopted the criterion based on the number of IPs as a simple way to compare the amount and quality of information provided in confirmatory analyses.[16] Interesting confirmation guidelines in PRA have been proposed by the U.S. Department of Agriculture[28] and by the European Union.[29] The use of IPs as confirmatory criterion is subject to controversies and has no jurisdiction in PRA in fruits and vegetables. However, it is being increasingly applied by many authors in different fields, as it is easy to apply and is a suitable way to compare confirmation capabilities between different instruments.

The number of IPs earned per measured ion has been established according to instrumental resolution, i.e., 1.0 and 2.0 IPs for a precursor ion at low resolution (LR) and high resolution (HR), respectively, and 1.5 and 2.5 IPs for a product ion at LR and HR, respectively.[16,17] HR is defined as 10000 resolution at 10% valley, while the resolution in TOF instruments is usually expressed at 50% valley. Strictly, this means that TOF analyzers with 20000 resolution would be required to be considered as HR instruments. In spite of this, the number of IPs commonly assigned to TOF MS is the same as that of a traditional HR instrument (i.e., magnetic sector). In our opinion, the use of mass accuracy provided by TOF MS seems a more appropriate criterion for a reliable confirmation rather the instrumental resolution

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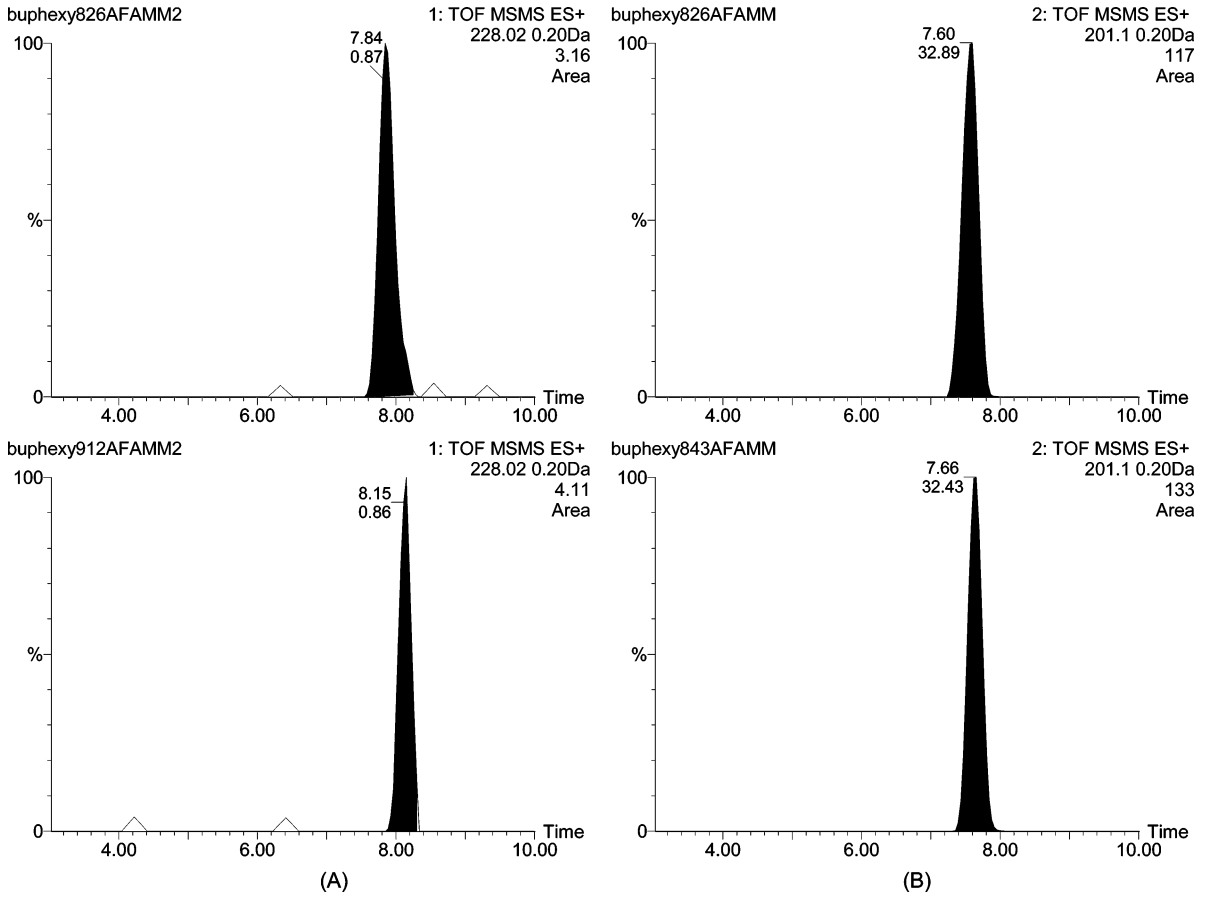

Figure 3. LC−ESI-QTOF XIC chromatograms of (A) 10 ng/mL hexythiazox standard solution (top) and pear sample fortified at 0.1 mg/kg (bottom), and (B) 10 ng/mL buprofezin standard solution (top) and orange flesh sample fortified at 0.1 mg/kg (bottom).

specifications,[18] this being surely the reason for several authors to assign the same number of IPs to TOF and to HR instruments. It is desirable that mass accuracy is also considered in future guidelines for identification/confirmation criteria due to the outstanding importance of this parameter. Although a different number of IPs has been assigned to TOF instruments depending on the experimental mass accuracy obtained,[18] the approach adopted in the present paper for QTOF measurements has been to assign 1.0 for precursor ion (isolation by the first quadrupole, at nominal mass) and 2.5 IPs for each product ion (measured with accurate mass) in order to use a homogeneous system according to previous data available in the literature. With few exceptions, in most of the examples shown in this paper, mass accuracies better than 2 mDa were obtained (5 ppm for 400 Da mass) in accordance with the specifications of our QTOF instrument. It is noteworthy that better mass accuracy could be easily observed with the new generations of QTOF instruments available at present.

A high number of IPs leads, in principle, to a satisfactory confirmation. This can be faced by different ways, such as considering complementary analysis by two techniques (for example, LC−MS and GC−MS), or the same technique but using its maximum potential (for example, LC−MS/MS). When using QTOF as the only technique, as in this paper, acquiring the complete product ion spectrum at accurate mass gives an extraordinary potential for identification purposes, as no ion shopping is required oppositely to tandem MS methods based on QqQ in SRM mode. The consequence is the earning of a high number of IPs when performing a confirmative method by QTOF. In the examples shown in this paper, we observed 5 and 8 product ions for hexythiazox and buprofezin (see Table 3), which would lead up to 13.5 and 21 IPs, respectively. To achieve the maximum intensity for each product ion, three collision energies per compound were selected. The acquisition of these six overlapped functions (three for each analyte) generated bad peak shape with the chromatographic separation employed in the quantitative method, due to the low number of points per peak when acquiring at 1scan/s. The reduction of the scan speed produced better peak shape but involved a drastic loss in sensitivity. Therefore, a slower chromatographic gradient was optimized in order to chromatographically resolve both peaks, obtaining a suitable number of points per peak for each analyte. Finally, two groups of three functions, each in MS/MS mode continuum acquisition channels (10, 20, and 30 eV per product ion), were selected with a chromatographic run time of 16 min. This problem could be solved in our case as only two analytes were involved; however, the use of relatively slow scan rates (1 scan/s) would become a limitation in the development of multiresidue methods.

The evaluation of the confirmation potential was performed on the basis of the number of IPs earned at the three concentration levels tested. The results shown in Table 3 were obtained as an average for all matrices at each level. At high analyte concentration, data for accurate mass and abundance were very satisfactory. The maximum theoretical number of IPs (13.5 and 21 for

Table 3. Relative Abundance and Accurate Mass Parameters for Each Product Ion Obtained in the Confirmation of Hexythiazox and Buprofezin at a Mass Window of 200 mDa[a]

theoretical		0.1 mg/kg					1 mg/kg					5 mg/kg				
		accurate mass		abundance			accurate mass		abundance			accurate mass		abundance		
m/z (Da)	abundance (%)	*m/z* exptl (Da)	deviation (mDa)	exptl (%) (RSD (%))	deviation (%)	IPs	*m/z* exptl (Da)	deviation (mDa)	exptl (%) (RSD (%))	deviation (%)	IPs	*m/z* exptl (Da)	deviation (mDa)	exptl (%) (RSD (%))	deviation (%)	IPs
							Hexythiazox									
353	n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	1
271.0308	20.5	271.0415	10.7	23.4(31)	14	2.5	271.0327	1.9	20.4(12)	0	2.5	271.0346	3.8	22.1(7)	8	2.5
228.0250	100.0	228.0280	3.0	100.0	n.a.	2.5	228.0258	0.8	100.0	n.a.	2.5	228.0268	1.8	100.0	n.a.	2.5
194.0373	9.7	194.0288	8.6	8.3(31)	14	2.5	194.0417	4.4	9.2(12)	6	2.5	194.0365	0.8	12.1(6)	20	2.5
168.0580	42.0	168.0497	8.3	48.4(25)	15	2.5	168.0593	1.3	43.7(9)	4	2.5	168.0576	0.4	45.7(3)	9	2.5
151.0315	2.0			n.d.		0			n.d.		0	151.0308	0.7	2.2(12)	12	2.5
						11[b]					11[b]					13.5[b]
							Buprofezin									
306	n.a.	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	1	n.a.	n.a.	n.a.	n.a.	1
250.1014	4.3			n.d.		0	250.1007	0.7	4.4(10)	3	2.5	250.1010	0.4	4.6(5)	9	2.5
208.0545	2.9			n.d.		0	208.0567	2.2	2.9(14)	2	2.5	208.0542	0.3	3.1(2)	9	2.5
201.1062	100.0	201.1005	6	100.0	n.a.	2.5	201.1053	0.9	100.0	n.a	2.5	201.1045	1.7	100.0	n.a.	2.5
145.0436	8.8	145.0424	1.2	9.4(28)	7	2.5	145.0423	1.3	8.8(8)	0	2.5	145.0424	1.2	8.8(3)	0	2.5
116.0534	26.1	116.0507	2.7	30.7(27)	18	2.5	116.0512	1.5	26.4(3)	1	2.5	116.0520	1.4	26.0(2)	0	2.5
106.0657	7.8	106.0719	6.3	6.7(28)	14	2.5	106.0633	2.4	7.4(8)	5	2.5	106.0646	1.1	8.1(4)	4	2.5
86.0606	2.1			n.d.		0	86.0587	1.9	2.0(14)	7	2.5	86.0599	0.7	2.7(6)	25	2.5
57.0704	1.6			n.d.		0	57.0730	2.6	1.3(20)	19	2.5	57.0738	3.4	2.0(6)	22	2.5
						11[b]					21[b]					21[b]

[a] Number of identification points (IPs) earned by acquiring every ion at different concentration levels. N.a. indicates not applicable; n.d.indicates not detectable. For optimized collision energies, see Table 1. [b] Total IPs.

hexythiazox and buprofezin, respectively) was earned, although two product ions (*m/z* 271.0346, hexythiazox, and *m/z* 57.0738, buprofezin) presented a slight deviation from the theoretical exact mass due to their low abundance (Table 3). The number of IPs decreased at lower concentrations as a consequence of the nondetection of some (less sensitive) product ions. Despite this limitation, a notable number of IPs was reached, allowing the reliable confirmation of the presence of target compounds at all concentrations tested.

It is interesting to comment on precision of the measurement of mass abundances from data shown in Table 3. These data were obtained as an average of 30 analyses (5 replicates in 6 different matrices). Meanwhile, at high concentrations the RSDs were normally <15% for the measurement of the experimental ions abundance; at 0.1 mg/kg the RSD values increased up to 30%. This poor precision can affect the confirmation of an individual sample at low concentrations as relative abundance may substantially vary from the theoretical one.

As an example of the confirmation potential, Figure 4 shows the spectra for a buprofezin standard and a spiked orange peel sample, both acquired at three collision energies. The unequivocal confirmation of buprofezin in the sample is guaranteed by the suitable relative abundance of the monitored product ions and their accurate mass values.

Application to Real Samples. Several banana samples from field residue trials, previously analyzed at our laboratory by LC−MS/MS with QqQ and being positives by either buprofezin or hexythiazox, were also analyzed by LC−QTOF. Quantitative data are shown in Table 4, where good agreement can be seen between values obtained by both techniques, even at low concentration levels (e.g., 0.02 mg/kg buprofezin).

The confirmation of the analyte identity was performed in the samples previously quantified, obtaining good values of deviation in both ion abundances and accurate masses. As an example, data on confirmation of hexythiazox in the banana skin 2 sample are shown in Figure 5 and Table 5. Abundance deviations varied between 4% and 17% and were calculated using quality control samples (nontreated samples spiked at 0.1 mg/kg) as reference. The experimental accurate masses were really satisfactory, with deviations lower than 2 mDa (except 5.5 mDa in the case of 194.0373 product ion). Under these circumstances, 11 IPs were reached, confirming the presence of hexythiazox in the sample. Figure 5 shows the XIC chromatograms for every ion selected (precursor ion and the four product ions that could be detected), each one at its optimized collision energy to give maximum sensitivity. This figure also shows the MS/MS spectra (precursor ion *m/z* 353) at the three collision energy values tested. An interfering compound with nominal mass *m/z* 354 seemed to coelute with hexythiazox. The presence of this interference was a consequence of the wide isolation window used in the quadrupole (about 2 Da). For this reason, the MS/MS spectra presented several additional product ions (297.8, 201.9, and 124.9), although none of them overlapping with those from hexythiazox. Correct confirmation could be easily made because an extract ion mass window of 0.2 Da was selected obtaining satisfactory chromatographic peaks, as shown in Figure 5.

Elucidation. In addition to the determination of the target analytes, the presence of nontarget TPs was also investigated.

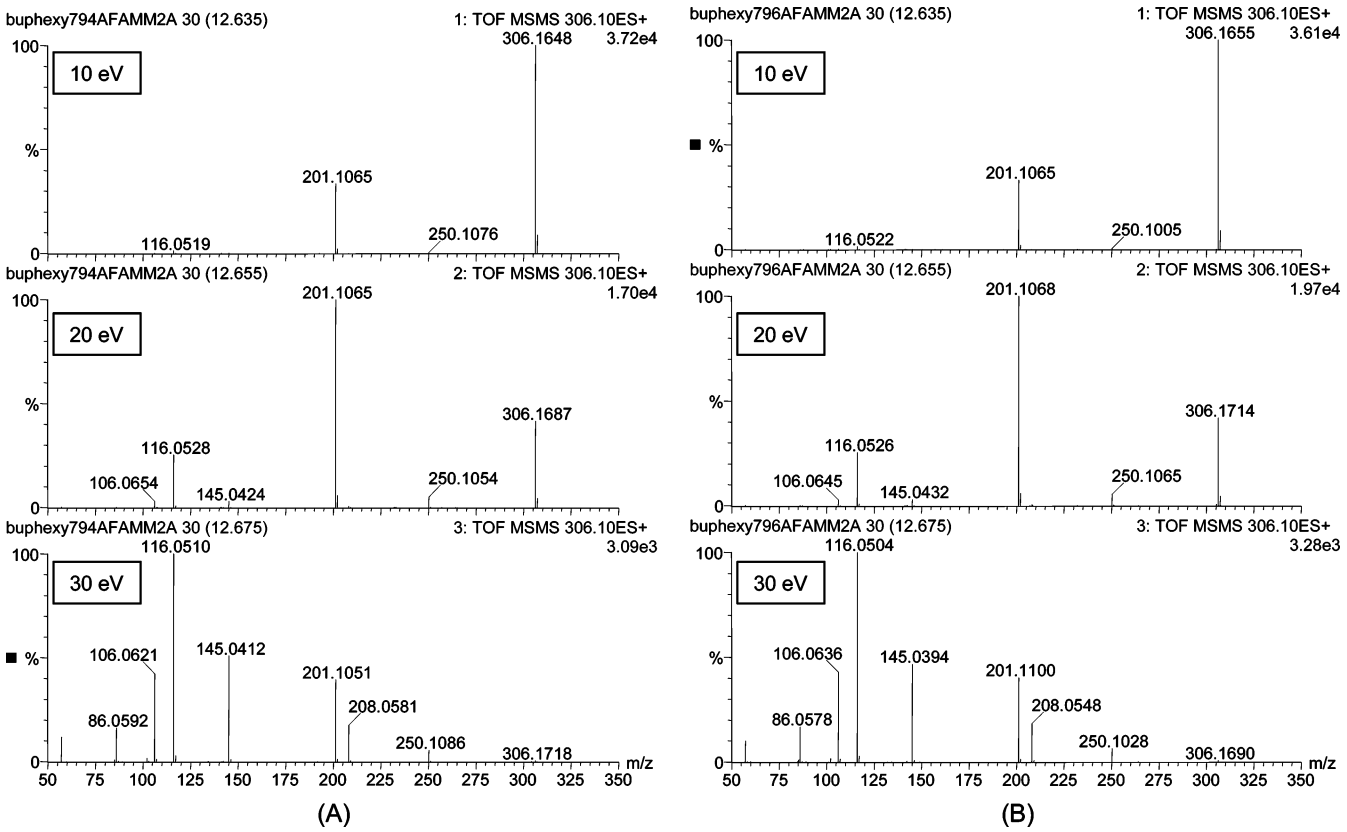

Figure 4. LC−ESI-QTOF product ion spectra used for confirmation of buprofezin (precursor ion *m*/*z* 306): (A) reference standard at 0.5 μg/mL and (B) orange peel sample fortified at 5 mg/kg.

Table 4. Quantitative Data for Hexythiazox and Buprofezin in Real Positive Banana Samples Analyzed by LC−QTOF and LC−QqQ (*n* = 2 Each)

analyte	sample	LC−QTOF (mg/kg)	LC−QqQ (mg/kg)
hexythiazox	banana skin 1	0.078	0.062
	banana skin 2	0.131	0.092
buprofezin	banana skin 3	1.110	0.960
	banana skin4	0.280	0.260
	banana flesh 1	0.019	0.012

Several positives and their corresponding blank samples were selected after application of the previously described methodology. First, an acquisition in TOF mode was performed in full scan MS at a cone of 30 V. The continuum spectra data obtained for each sample were compared by MetaboLynx software obtaining the unknown relevant peaks in positive samples that were not present in the blank samples. Using this methodology, an unknown chromatographic peak was found in banana skin samples that contained buprofezin. Accurate mass of this extra peak (322.1589) corresponded to an oxidized TP of buprofezin ($C_{16}H_{24}N_3SO_2$). Additionally, the isotopic pattern obtained supported this formula. After a bibliographic search, two possible oxidized TPs, phenol (**p**) and sulfoxide (**q**), were plausible[30] (structures shown in Figure 6). Both compounds have the exact same mass, and therefore, they would be indistinguishable by TOF instruments. Thus, the use of QTOF was essential to establish the correct chemical structure. For this purpose, the product ion full scan spectrum of the precursor ion *m*/*z* 322 was acquired at three collision energies (10, 20 and 30 eV) and compared with that of the parent buprofezin.

The previous study of the buprofezin fragmentation pathway (see Figure 2) may help to predict the feasible, differential product ions from both possible TPs. Figure 6 shows the suggested fragmentation pathways for the phenolic (Figure 6A) and the sulfoxide (Figure 6B) TPs. Regarding the phenolic one, its product ions that conserve the benzene ring present the additional value of the oxygen (15.9949) in their accurate mass (compounds **r**, **s**, and **t**). In the sulfoxide TP, the mass increment is observed in those product ions where the sulfur atom is conserved, such as **u**, **v**, **w**, **x**, and **y**. The MS/MS spectrum of Figure 6D shows that the differential product ions corresponding to the phenolic TP (i.e., **r**, **s**, and **t**) are present in the banana skin sample. Besides, other product ions from buprofezin (**j**, **k**, **l**, **n**, and **o**) were also observed, assuring the presence of the phenolic TP. Mass errors observed for the remaining precursor ion and for product ions appearing in the spectrum (Figure 6D) were considerable, up to 6 mDa, surely due to the use a light product ion (*m*/*z* 201) as lock mass. This TP has been reported in the leaf/stem and root extracts of tomato samples after hydroponics' treatment, but it has not been found after foliar treatment.[30] In order to fully verify the structure of the TP found, its chemical synthesis and further analysis by LC−QTOF MS would be helpful.

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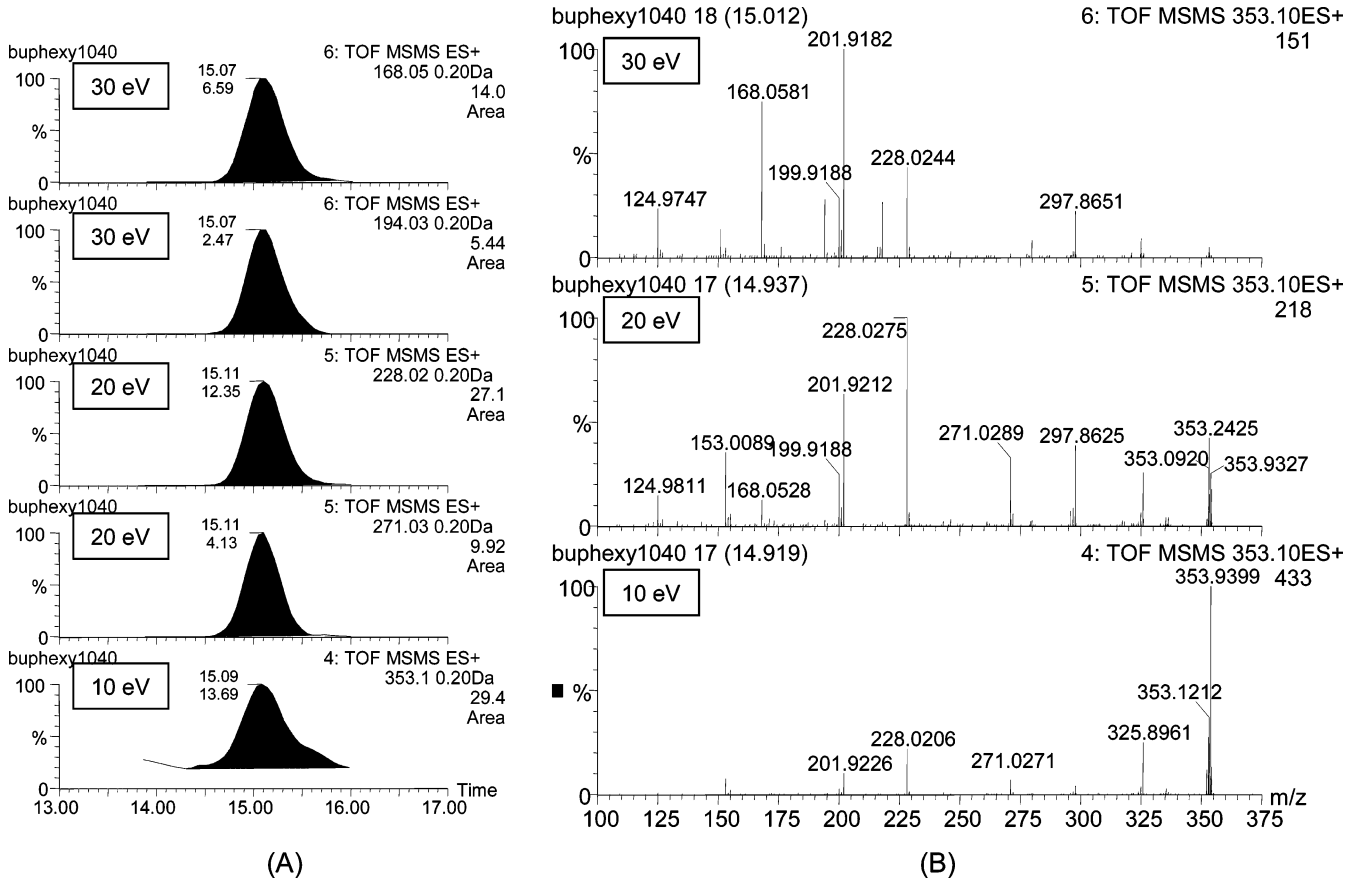

Figure 5. Confirmation of hexythiazox in banana samples by LC−ESI-QTOF: (A) XIC chromatograms at the optimized collision energy for each ion and (B) MS/MS spectra from real banana skin sample 2 containing hexythiazox (0.1 mg/kg).

Table 5. LC-QTOF Confirmation of Hexythiazox in a Positive Banana Skin Sample (Concentration 0.1 mg/kg) by Measurement of Abundance and Accurate Mass of Each Product Ion (n = 2)

		banana skin sample 2				
quality control at 0.1 mg/kg		abundance		accurate mass		
m/z (Da)	abundance (%)	experimental (%)	deviation (%)	m/z experimental	deviation (mDa)	IPs[a]
353	n.a.[b]	n.a.	n.a.	n.a.	n.a.	1
271.0308	32	28.5	11	271.0298	1.0	2.5
228.0250	100	n.a.	n.a.	228.0259	0.9	2.5
194.0373	12	14.0	17	194.0318	5.5	2.5
168.0580	58	55.5	4	168.0565	1.5	2.5

[a] Total IPs: 11. [b] N.a. indicates not applicable.

CONCLUSIONS

QTOF has strong potential in pesticide residue analysis of food samples. Besides its capability to develop quantitative, but especially confirmative, methods for target compounds, QTOF is very helpful in the elucidation of nontarget analytes as well as in the proposal of fragmentation pathways.

The study performed using two model insecticides, buprofezin and hexythiazox, in six different fruit matrices has shown that QTOF can be used for quantification purposes with sufficient dynamic linear range and satisfactory sensitivity, comparable to that obtained with TOF analyzers[11] although still lower than that of QqQ instruments in SRM mode, where no preconcentration is normally needed to reach low limits of detection.[31] Thus, at present LC−QqQ far surpasses LC−QTOF for quantification analysis. The most valuable added characteristic of QTOF is undoubtedly its strong potential for unequivocal confirmation of the analyte identity, as it combines the capabilities of accurate mass measurements and the use of tandem mass spectrometry. As a consequence, a high number of IPs is earned, unachievable by other mass analyzers such as TOF, QqQ, or IT.

Hybrid QTOF also has strong potential for elucidation of unknown (nontarget) compounds, such as most pesticide TPs in real samples. This potential has been highlighted in this paper by the differentiation of isobaric compounds which would probably

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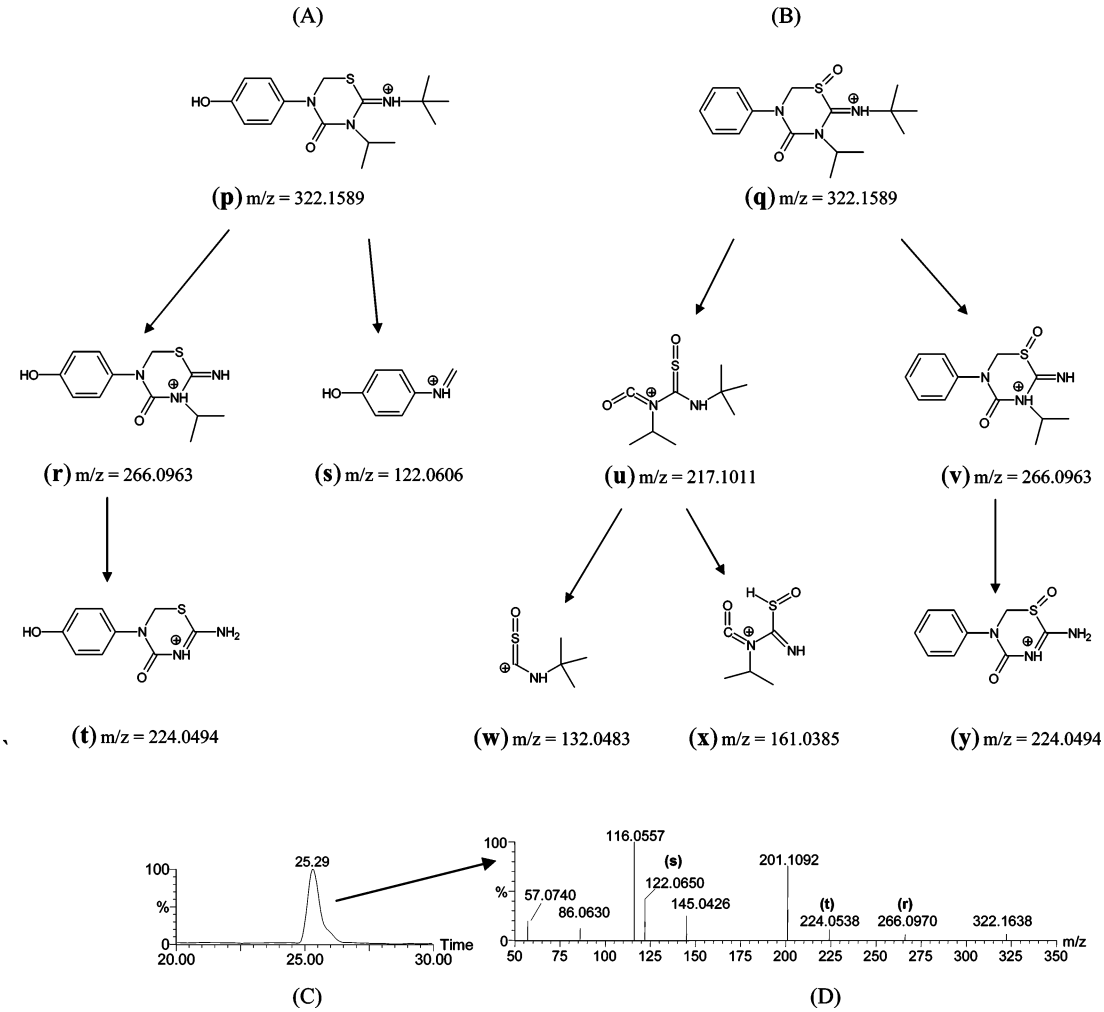

Figure 6. Suggested fragmentation pathway for (A) phenolic and (B) sulfoxidic metabolites of buprofezin. (C) LC−ESI-QTOF chromatogram and (D) MS/MS spectrum (precursor ion *m/z* 322) from a positive buprofezin banana skin sample (0.28 mg/kg) containing the phenolic metabolite.

be indistinguishable by the use of a TOF instrument. The measurement of accurate mass for product ions also provides valuable information to establish fragmentation pathways, demonstrating the improved characteristics of QTOF in comparison to QqQ instruments, where unequivocal assignments are more difficult to be made.

Some drawbacks observed in this paper in the use of QTOF for PRA, such as the need for selecting a lock mass coming from the analyte in MS/MS mode or the high scan time (1 scan/s), will be surely overcome in new QTOF instrument generations. Considering the present state-of-the-art within LC−MS techniques, it is reasonable to expect that QTOF will become one of the most valuable tools in PRA in the near future.

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