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Identification of Pesticide Transformation Products in Food by Liquid Chromatography/ Time-of-Flight Mass Spectrometry via "Fragmentation−Degradation" Relationships

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The identification of transformation products of pesticides in foodstuffs is a crucial task difficult to tackle, due to the lack of standards and scarce information available. In this work, we describe a methodology for the identification and structural elucidation of pesticide transformation products in food. The proposed strategy is based on the use of liquid chromatography electrospray time-of-flight mass spectrometry (LC/TOFMS): accurate mass measurements of (molecule and fragment) ions of interest are used in order to establish relationships between fragmentation of the parent pesticides in the instrument (in-source CID fragmentation) and possible degradation products of these pesticides in food. Examples of this strategy showing the potential of LC/TOFMS to determine unknown pesticides in food are described in two different real samples, suggesting that pesticides often are transformed into degradation products in the same fashion that they are fragmented in the instrument. Using the proposed approach and without using standards a priori, based solely on accurate mass measurements of ions and "fragmentation−degradation" relationships, we have identified two parent pesticides (amitraz and malathion) along with six degradation products, *m/z* 253 (*N*,*N*′-bisdimethylphenylformamidine), 163 (*N*-2,4-dimethylphenyl-*N*-methyl formamidine), 150 (2,4-dimethylformamidine), and 122 (2,4-dimethylaniline) from amitraz, and *m/z* 317 and 303, due to ether hydrolysis of methyl and ethyl groups from malathion. Structures for these species were proposed, and the potential of the proposed approach was critically discussed.

Pesticide residue analysis is a discipline of paramount importance, not only for the protection of human health but also for trade and official control purposes. Currently, more than 1400 active substances against pests belonging to many different chemical classes are applied to agricultural crops in order to control undesirable molds, insects, or weeds.[1] Since the presence of trace amounts of both pesticide residues and their transformation products (TPs) could be a potential health hazard, their determination in fruits and vegetables is a priority objective to evaluate food quality to avoid possible risk for the human health.[2] For this reason, numerous regulations such as the European Union directives have set maximum residue limits for pesticides and their relevant metabolites in food. This has fostered the development of new techniques, in order to establish methods in compliance with these regulations. Up-to-date, liquid chromatography/(tandem)/mass spectrometry, with quadrupole analyzers, and GC/MS/MS have become the most used methods for the analysis of target pesticides.[3,4] Analytical food laboratories, all over the world, carry out extensive research, monitoring pesticide residues in fruits and vegetables using these methodologies.

The scope and capabilities of these (multiresidue) methods used in these monitoring programs are restricted to a limited number of selected pesticides and metabolites (target analysis), normally the most widely used or detected. However, along with these common pesticide residues, other xenobiotics also can be present at relevant concentrations, which may be as toxic as the target compounds. The main sources of these unknown compounds are as follows: (i) impurities present in commercial formulations, originated in the synthesis process of the pesticides,[5] (ii) transformation products originated during storage or application of the commercial pesticide formulation, or (iii) transformation products originated in environmental conditions. The contribution of these suspected unknown compounds might be very relevant, if we consider, for instance, that impurities in commercial formulations are typically in the 10−15% range. All these species are not being controlled by the current routine monitoring programs, which are based mainly on target analysis of "known

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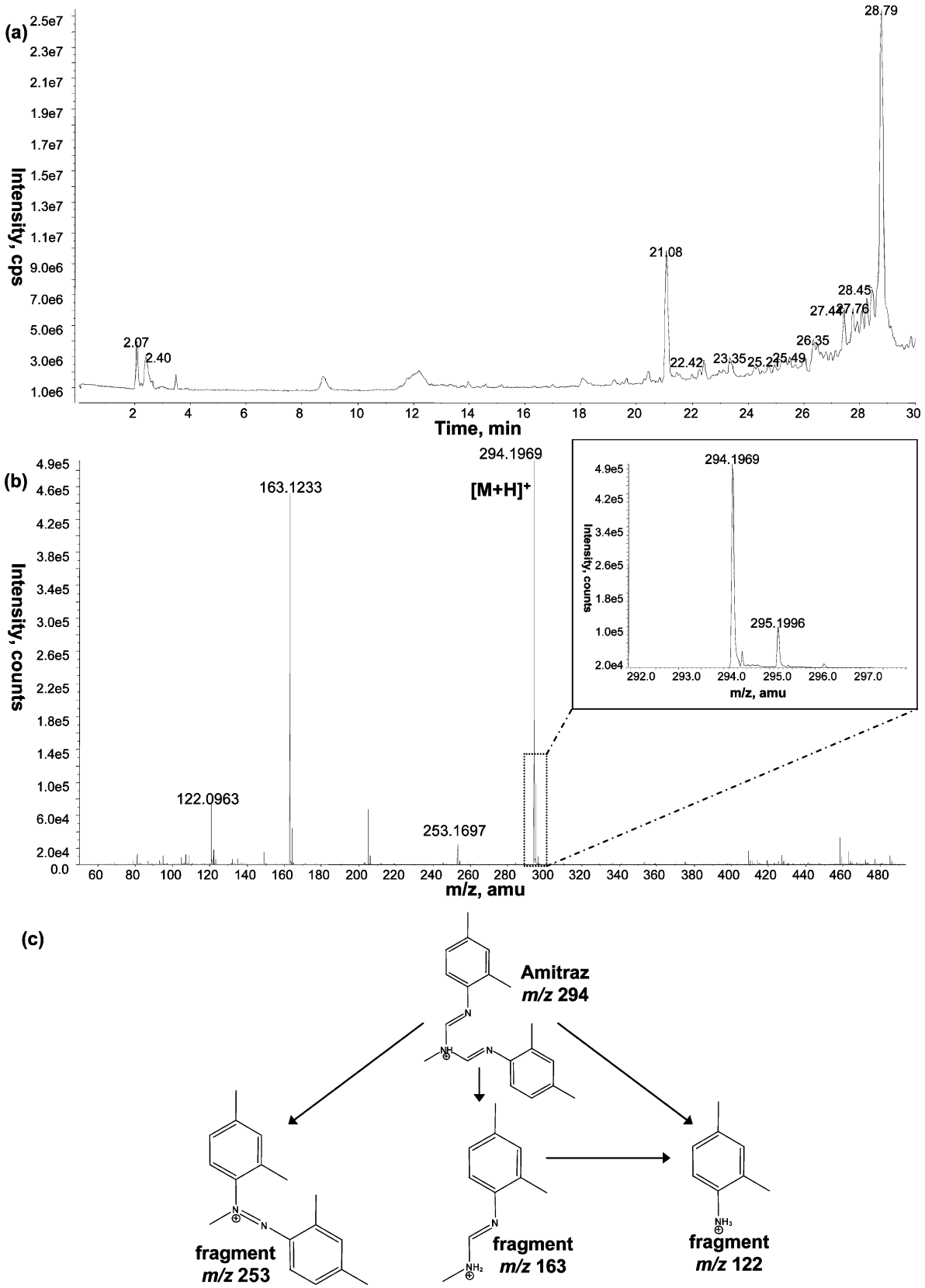

Figure 1. (a) Total ion chromatogram obtained from the LC/TOFMS analysis of the studied pear extract in which amitraz was detected at retention time 28.8 min; (b) accurate mass spectrum of the peak at 28.8 min; (c) proposed fragment pathways of amitraz obtained from in-source CID fragmentation.

species", in which specific masses of analytes must be predefined before data acquisition. This is the main drawback of these techniques, only a limited number of target compounds is covered (generally the most commonly detected pesticides), and thus, no information about possible transformation products is obtained. Therefore, there is a need of advanced mass spectrometric

Table 1. Accurate Mass Analysis of ESI-(+)-TOFMS Mass Spectrum of Peak at 28.8 min in a Pear Extract

m/z_{exptl}	elemental compositions[a]	m/z_{calcd}	error, mDa	error, ppm	DBE[b]
amitraz $[M + H]^+$ 294.1969	$C_4H_{20}N_{15}O$	294.196 97	−0.077	−0.26	2.5
	$C_{19}H_{24}N_3$	294.196 47	0.42	1.44	9.5
	$C_{11}H_{28}N_5O_2S$	294.195 82	1.08	3.66	0.5
	$C_{17}H_{29}NOP$	294.198 12	−1.23	−4.18	4.5
	$C_8H_{24}N_9O_3$	294.199 66	−2.76	−9.4	1.5
	$C_{12}H_{29}N_3O_3P$	294.196 97	2.9	4.4	6.5
amitraz fragment 1 (m/z 253) 253.1697	$C_{17}H_{21}N_2$	253.169 92	−0.22	−0.89	8.5
amitraz fragment 2 (m/z 163) 163.1233	$C_{10}H_{15}N_2$	163.122 97	0.32	1.99	4.5
amitraz fragment 3 (m/z 122) 122.0963	$C_8H_{12}N$	122.096 42	−0.12	−1.03	3.5

[a] Elemental composition calculator tool (minimum and maximum number of atoms): C [0−50]; H [0−100]; O [0−25]; N [0−20]; S [0−2]; P [0−1]; C1 [0−0]. [b] Double bond and ring equivalent.

techniques that can combine high-performance (high sensitivity and selectivity) target analysis capabilities with the ability of identifying not only "nontarget" compounds, which could be later included as targets of the monitoring programs, but also possible transformation products or impurities from commercial pesticide formulations. In this sense, few studies have been on the identification and routine analysis of their main TPs, which can be rather persistent and as hazardous as their parent compounds.[6] The detection and characterization of the formed TPs in food is a task of paramount importance, in order to evaluate their formation, kinetics, stability, and toxicity in foodstuffs.

Nowadays, the studies concerning the identification of TPs usually rely on the use of "laboratory-simulated degradation models", such as photoirradiation typically mediated by OH radical attacks on different organic structures, using large concentrations of the studied parent species in solvent or clean matrices, being the main transformation products identified, elucidated and characterized by means of hyphenated techniques.[7] However, impurities formed in the synthesis can be hardly simulated, and furthermore, the conditions in which the experiments are accomplished hardly suits with those common in produce, where very low concentrations of the studied species are present. In addition, the behavior of the pesticides in the simulated experiments under extreme conditions may be remarkably different from those common in foodstuffs. Scarcely any literature has been reported on studies of degradation directly performed in food samples. The polar nature of degradation products makes their analysis by liquid chromatography well suited since many of them lack the thermal stability and volatility required in gas chromatographic methods. The need to analyze and identify unknown relatively polar compounds at low concentrations levels means that methods in which liquid chromatography is coupled to mass spectrometry have a large playing field, which combines trace determination and structure elucidation.[8]

Liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) benefits from the high resolving power of signals on the m/z axis, enabling the measurement of accurate masses of ions within mass accuracies approaching FT-MS instruments, when dynamic accurate mass calibration systems are used.[9,10] In addition, the sensitivity offered by this type of instrument is typically 1−2 orders of magnitude higher than quadrupole systems operated in full-scan mode. This advantage is important since this kind of analysis is carried out under full-scan conditions.

In this work, we describe analytical methodology to study and elucidate the most common degradation products of pesticides in food, taking advantage of these unique features of TOF (high sensitivity (in full scan) and accurate mass measurements), which enables acquiring a full-scan accurate mass spectrum of any peak in a chromatogram, and represents a high value for the identification of unknowns in the samples because we get information of all the ions (molecular + fragments) generated by a specific compound. With this ability, we propose a strategy for the identification of degradation products, based on the use of "fragmentation−degradation" relationships. From a given parent species, the fragmentation patterns occurred in-source (by CID) could be used as a reference or model to predict possible degradation products. Examples of this strategy will be shown for the identification of six degradation products of amitraz and malathion on different food extracts, showing the unique potential of LC/TOFMS for the identification of unknown degradation products in food without the use of standards a priori.

EXPERIMENTAL SECTION

Chemicals and Materials. HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Formic acid was obtained from Fluka (Buchs, Switzerland). PSA (primary−secondary amine) Bond Elut was obtained from Varian, Inc. (Palo Alto, CA). 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.

Sample Treatment. Fruit and vegetable samples were purchased from different local markets. The pesticides were extracted using two different extraction procedures:

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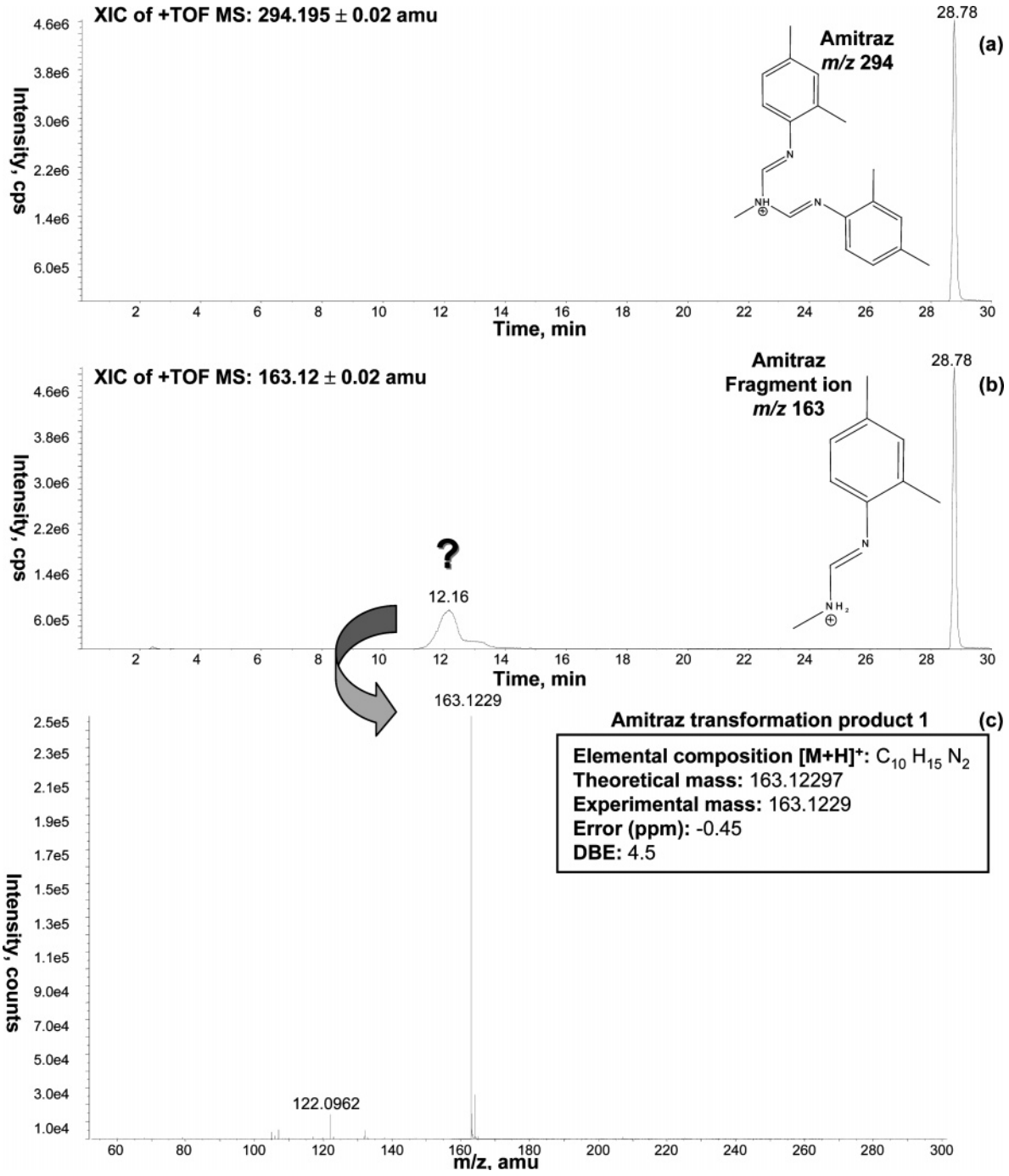

Figure 2. Extracted ion chromatograms obtained from the LC/TOFMS analysis of the studied pear extract: (a) *m*/*z* 294.19 ± 0.02 Da and (b) *m*/*z* 163.12 ± 0.02 Da; (c) accurate mass spectrum of the peak at 12.16 min, which corresponds to amitraz transformation product 1 (*N*-2,4-dimethylphenyl-*N*-methylformamidine). For details, see text.

(a) Standard Vegetable Extraction. The employed procedure (so-called "QhEChERS", described elsewhere[11,12]) is, to our knowledege, the "most universal" extraction method available. It covers a wide range of compounds, from polar to apolar, for both GC/MS and LC/MS analyses and with satisfactory recoveries close to 100% in most cases. Therefore, this extraction protocol is the best suited to this kind of analysis, in which we are searching for unknown unexpected transformation products. The proposed procedure comprised the following steps. A representative 15-g portion of previously homogenized sample was weighed in a 200-mL PTFE centrifuge tube. Then 15 mL of acetonitrile was added, and the tube was vigorously shaken for 1 min. After this time, 1.5 g of NaCl and 4 g of $MgSO_4$ were added, and the shaking process was repeated for 1 min. The extract then was centrifuged (3700 rpm) for 1 min. At this point, three different phases could be distinguished: an aqueous phase (at the bottom),

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(12) Lehotay, S. J.; De Kok, A.; Hiemstra, M.; Van Bodegraven, P. *J. AOAC Int.* **2005**, *88*, 595−614.

the intermediate phase containing part of the vegetable matrix, and the upper phase of acetonitrile. Five milliliters of the supernatant (acetonitrile phase) was then taken with a pipet and transferred to a 15-mL graduated centrifuge tube containing 250 mg of PSA and 750 mg of $MgSO_4$, which was then energetically shaken for 20 s. The extract was then centrifuged again (3700 rpm) for 1 min. Finally, an extract containing the equivalent of 1 g of sample/mL in 100% acetonitrile was obtained. Two milliliters of this extract was then evaporated to near dryness and reconstituted to a final composition that contained the same organic solvent content as that of the initial mobile phase (10% MeOH). Prior to LC/MS analysis, the extract was filtered through a 0.45-μm PTFE filter (Millex FG, Millipore).

(b) Rapid Fruit/Vegetable-Skin Extraction. The following procedure, which was used to prepare the studied pear extract, yields very clean and noncomplex extracts with very low chemical noise, which fosters the detection of unknown degradation products even at very low concentration, which could not be detected if the whole piece is processed by the method described above. The obtained extracts contain higher pesticide concentrations (since pesticides are often concentrated on the peel of the fruit/vegetable), making easier the elucidation of possible degradation products. The skin of the piece was carefully washed with a fixed amount of methanol (between 2 and 5 mL, depending on the vegetable size), using a pipet to distribute homogeneously the solvent on the whole surface of the piece. The solvent used to extract the peel was captured in a 250-mL beaker. The procedure was repeated three times using the same solvent recovered on the beaker. An aliquot of 250 μL was mixed with 750 μL of mQ water, and the obtained extract was filtered through a 0.45-μm PTFE filter.

Chromatography. The separation of the species from the whole fruit or peel extracts was carried out using an HPLC system (consisting of vacuum degasser, autosampler, and binary pump) (Agilent Series 1100, Agilent Technologies, Santa Clara, CA) equipped with a reversed-phase C_8 analytical column of 150 mm $\times$ 4.6 mm and 5-μm particle size (Zorbax Eclipse XDB-C8). A 50-μL aliquot of fruit extract was injected in each study. Mobile phases A and B were water with 0.1% formic acid and acetonitrile, respectively. The chromatographic method held the initial mobile-phase composition (10% B) constant for 5 min, followed by a linear gradient to 100% B at 30 min. The flow rate used was 0.6 mL min^{-1}.

Liquid Chromatography/Time-of-Flight Mass Spectrometry. The HPLC system was connected to a time-of-flight mass spectrometer Agilent MSD TOF (Agilent Technologies) equipped with an electrospray interface operating in positive ion mode, using the following operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas, 9 L min^{-1}; gas temperature, 300 °C; skimmer voltage, 60 V; octapole dc 1, 37.5 V; octapole rf, 250 V; fragmentor voltage (in-source CID fragmentation), 190 V. LC/MS accurate mass spectra were recorded across the range 50−1000 m/z. Accurate mass measurements of each peak from the total ion chromatograms were obtained using an automated calibrant delivery system to provide the correction of the masses. The instrument performed the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low flow of a calibrating solution that contains the internal reference masses (m/z 121.0509 and 922.0098), complemented with a software package, which is autocalibrating and continuously recording the results of the internal reference masses along with the raw data. This strategy provides enhanced accuracy in relation to previous TOF instruments, in which the mass calibration was accomplished with a compound present in the sample. The instrument worked providing a typical resolution of 9500 ± 500. The full-scan data recorded were processed with Applied Biosystems/MDS-Sciex Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software.

Table 2. Accurate Mass Analysis of ESI-(+)-TOFMS Mass Spectrum of Peaks at 12.16 (m/z 163), 8.7 (m/z 122), 18.07 (m/z 150), and 18.13 min (m/z 253), Corresponding to the 4 TPs Detected in the Studied Pear Extract

m/z_{exptl}	elemtl compstns[a]	m/z_{calcd}	error, mDa	error, ppm	DBE[b]
amitraz TP 1 (m/z 163) $[M + H]^+$ 163.1229	$C_{10}H_{15}N_2$	163.122 97	−0.07	−0.46	4.5
fragment 1 (m/z 122) 122.0962	$C_8H_{12}N$	122.096 42	−0.22	−1.85	3.5
amitraz TP-2 (m/z 122) $[M + H]^+$ 122.0963	$C_8H_{12}N$	122.096 42	−0.12	−1.03	3.5
fragment 1 (m/z 105) 105.0697	C_8H_9	105.069 87	−0.17	−1.68	4.5
fragment 2 (m/z 107) 107.0728	C_7H_9N	107.072 95	−0.15	−1.6	4
amitraz TP-3 (m/z 150) $[M + H]^+$ 150.0912	$C_9H_{12}NO$	150.091 34	−0.14	−0.93	4.5
fragment 1 (m/z 122) 122.0963	$C_8H_{12}N$	122.096 42	−0.12	−1.0	3.5
fragment 2 (m/z 105) 105.0694	C_8H_9	105.069 87	−0.38	−3.59	4.5
fragment 3 (m/z 107) 107.0727	C_7H_9N	107.072 95	−0.25	−2.3	4
amitraz TP-4 (m/z 253) $[M + H]^+$ 253.1695	$C_{17}H_{21}N$	253.169 92	−0.42	−1.68	8.5

[a] Elemental composition calculator tool (minimum and maximum number of atoms): C [0−50]; H [0−100]; O [0−25]; N [0−20]; S [0−2]; P [0−1]; C1 [0−0]. [b] Double bond and ring equivalent.

RESULTS AND DISCUSSION

Different extracts from market-purchased fruit and vegetables were studied following the procedure described above. A total of 30 peel extracts of different fruit and vegetables (citrus fruits, apple, tomato, pear, grape, etc.) and ~25 fruit and vegetable samples (pepper, tomato, broccoli, citrus, apples) extracted by QuEChERS were studied in order to evaluate the proposed approach. In "positive" samples, we found various cases where the elucidation of transformation products could be accomplished by LC/TOFMS accurate mass measurements and degradation−fragmentation relationships. As an example, we discuss in detail the case of two widely used pesticides (malathion and amitraz) because of their importance and because of the total number of

(13) Thurman, E. M.; Ferrer, I.; Fernández-Alba, A. R. *J. Chromatogr., A* **2005**, *1067*, 127−134.
(14) García-Reyes; Ferrer, I. ; Thurman, E. M.; Molina-Díaz, A.; Fernández-Alba, A. R. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2780−2788.

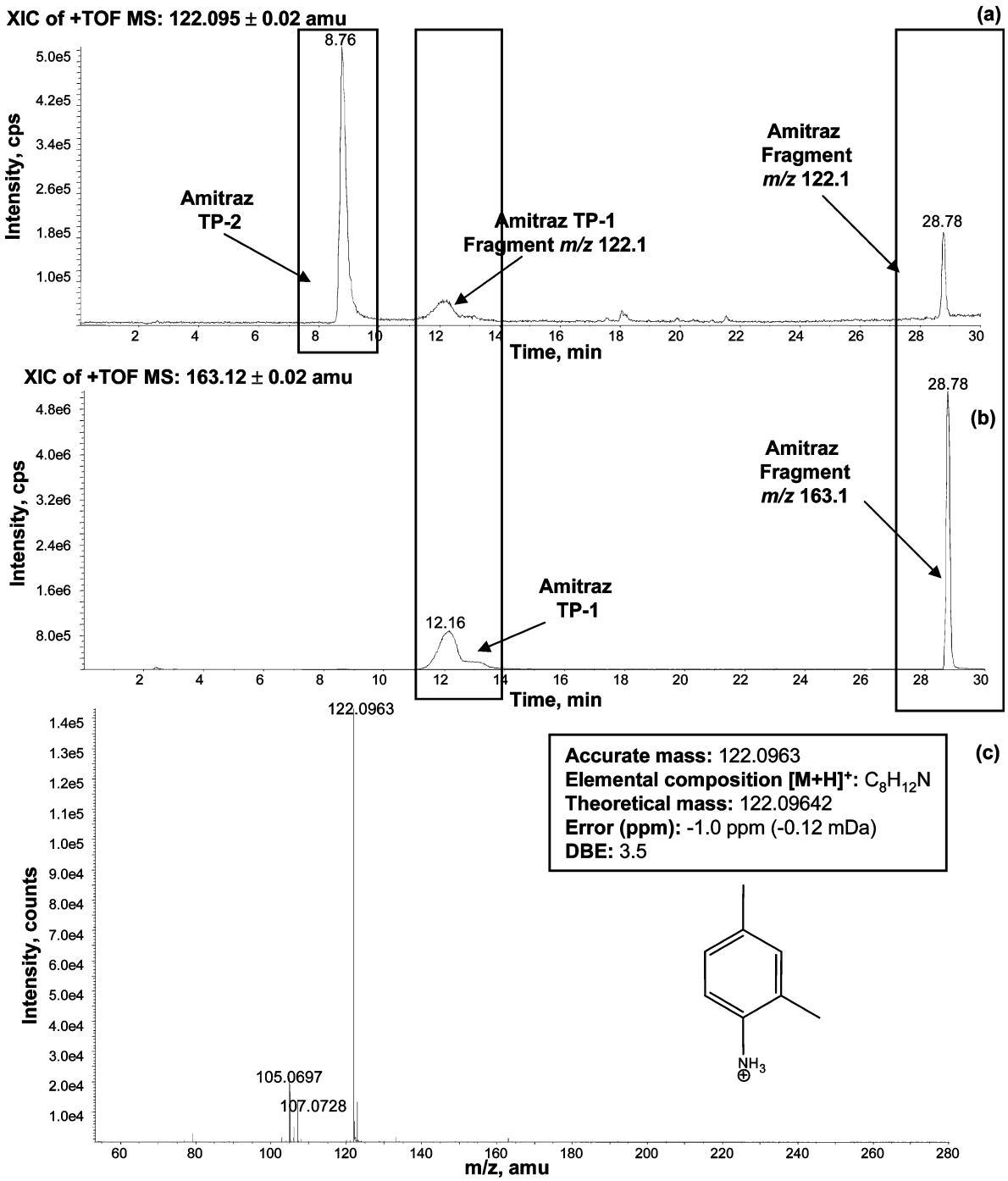

Figure 3. Extracted ion chromatograms obtained from the LC/TOFMS analysis of the studied pear extract: (a) *m*/*z* 122.09 ± 0.01 Da and (b) *m*/*z* 163.12 ± 0.01 Da; (c) accurate mass spectrum of the peak at 8.7 min, which corresponds to amitraz transformation product 2 (2,4-dimethylaniline). For details, see text.

transformation products elucidated as well. Following are the discussions of the identification of two pesticides (amitraz and malathion) and six of their transformation products.

Identification of Amitraz and Its Transformation Products in Pears. (a) Parent Compound Identification. The identification and confirmation of amitraz was accomplished by the use of the elemental composition information for both the protonated molecule and characteristic fragment ions provided by LC/TOFMS accurate mass measurements.[13,14] Amitraz was detected in a pear extract using the procedure described elsewhere based on identification by elemental composition database search, without the use of standards a priori.[14,15] The TIC of the pear extract is shown in Figure 1a. The accurate mass spectrum of the large peak at 28.8 min is also shown in Figure 1b. The experimental accurate mass for that peak with *m*/*z* 294 was 294.1969. The detected *m*/*z* 294 isotopic signature revealed the absence of both chlorine and sulfur atoms. Accurate mass analysis was performed on *m*/*z* 294 and is shown in Table 1. The more probable elemental compositions for *m*/*z* 294 ion were obtained

(15) Thurman, E. M.; Ferrer, I.; Zweigenbaum, J. A.; García-Reyes, J. F.; Woodman, M.; Fernández-Alba, A. R. *J. Chromatogr., A* **2005**, *1082*, 71−80.

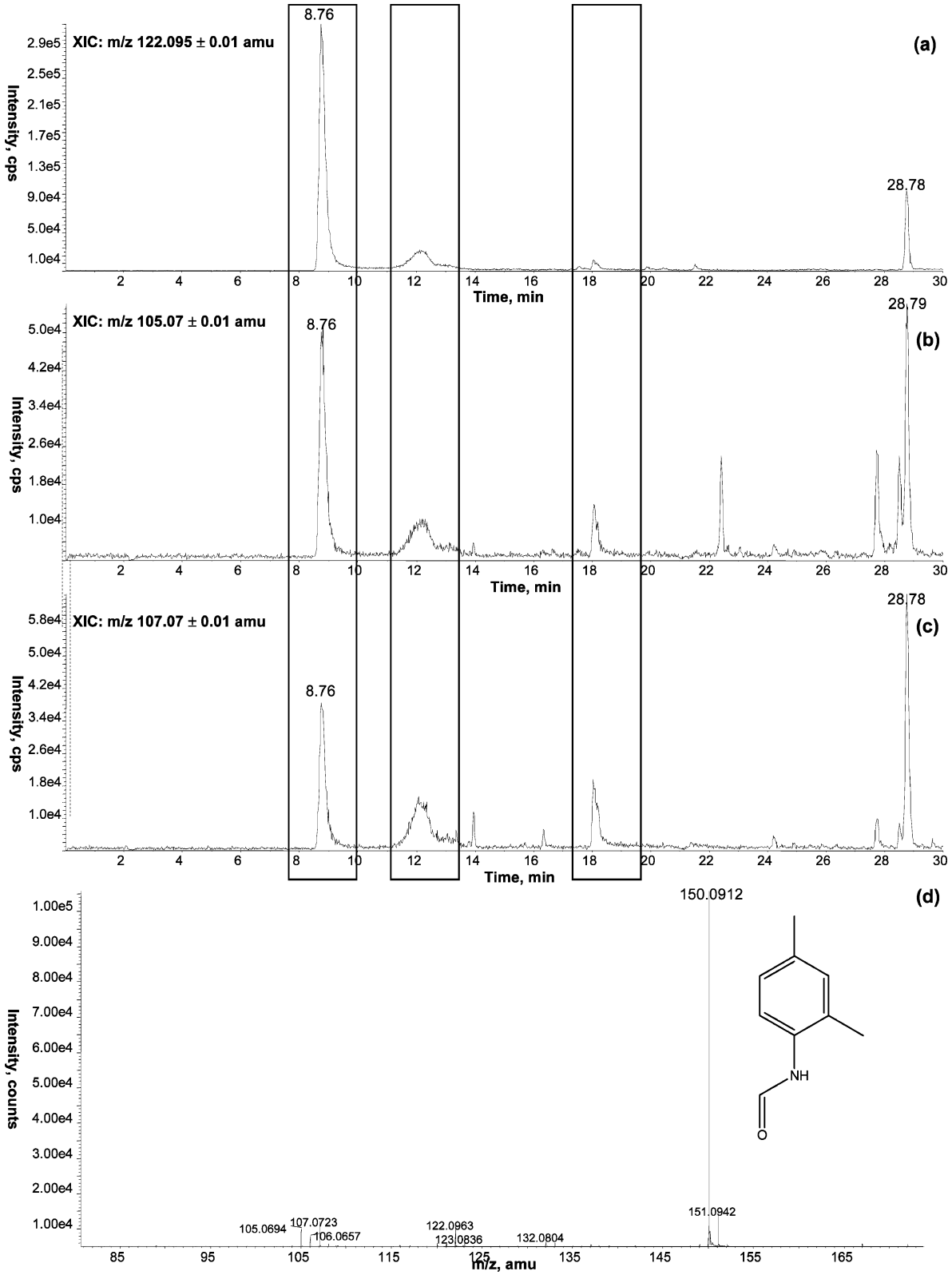

Figure 4. Extracted ion chromatograms obtained from the LC/TOFMS analysis of the studied pear extract: (a) *m*/*z* 122.09 ± 0.01 Da and (b) *m*/*z* 105.07 ± 0.01 Da; (c) *m*/*z* 107.07 ± 0.01 Da; (d) Accurate mass spectrum of the peak at 18.1 min. For details, see text.

and used to search for pesticides in their parent form, using databases (The Merck Index, Chem-Index, Sigma-Aldrich electronic catalogue, etc.) and elemental composition as searching criterion. The presence or absence of isotopic signatures can be also used to reduce the potential number of elemental compositions that fits the experimental data.

In this case, the search yielded a positive, amitraz an insecticide. For confirmation purposes, the in-source CID fragmentations of amitraz were examined. The proposed elemental composition of each fragment ion was investigated with respect to the structure of the suspected species (amitraz) proposed in the previous step. The fragment ions with *m*/*z* 163 and 122 drawn also in Figure 1

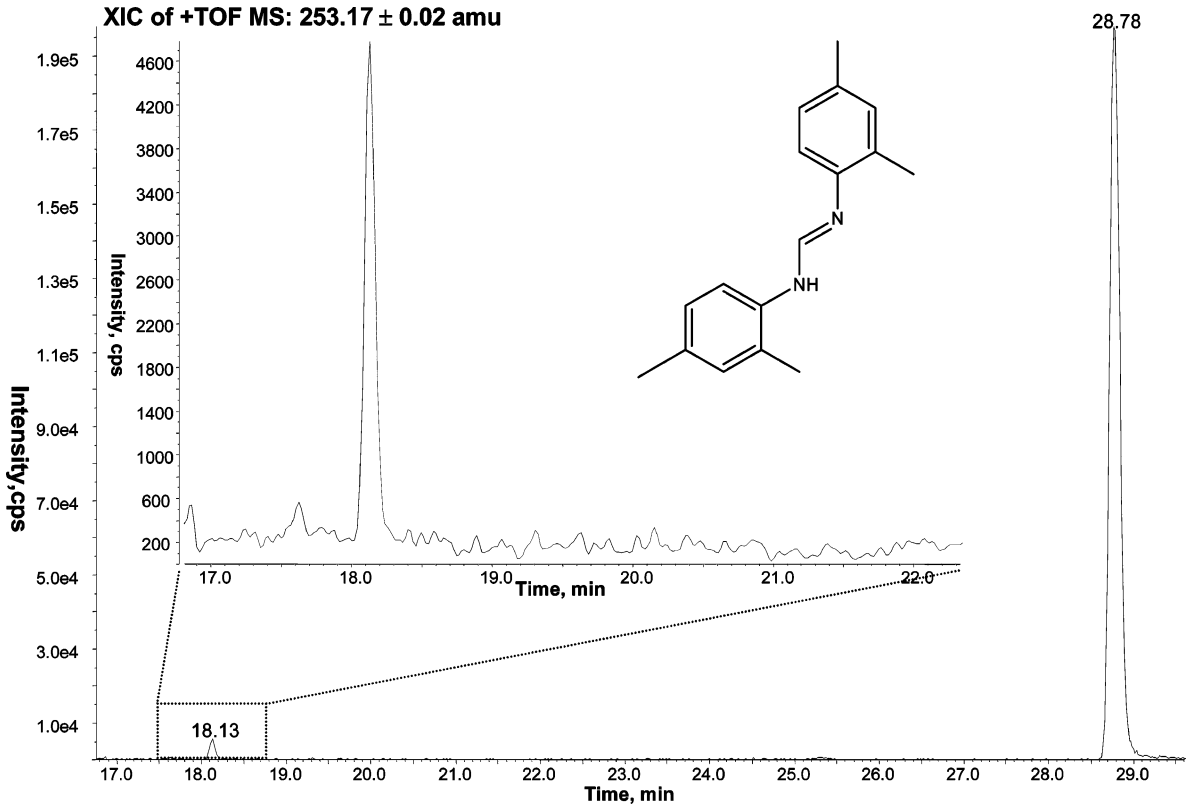

Figure 5. Extracted ion chromatogram of *m*/*z* 253.17 (peak at retention time 28.78 min correspond to amitraz fragment ion and zoomed peak at 18.1 min corresponds to amitraz metabolite with *m*/*z* 253 (*N*,*N*′-bisdimethylphenylformamidine). For details, see text.

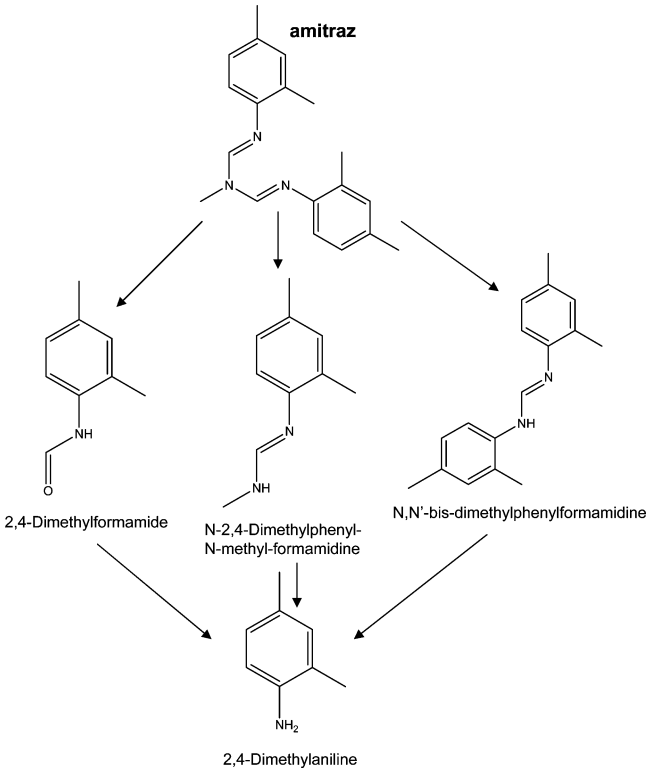

Figure 6. Proposed transformation pathways of amitraz in vegetables revealed by the data collected by LC/electrospray TOFMS.

matched with the proposed pesticide structure, thus confirming the positive identification of amitraz. The accurate mass analysis for these fragment ions is also shown in Table 1.

(b) Identification of Amitraz Transformation Products by Fragmentation−Degradation Relationships. The characteristic fragmentation of amitraz is shown in Figure 1c, according to the data collected by accurate mass analysis of fragment ions. The strategy proposed in this work consists of using the fragmentation as a guide or reference to detect possible transformation products, since the moieties or bonds or weaker parts of the molecule that can react or be removed easily are also the more probable to be broken during in-source fragmentation. An example is shown with amitraz. For this compound, the main fragment ion was *m*/*z* 163. In Figure 2a and b, the extracted ion chromatograms of *m*/*z* 294.195 ± 0.02 and 163.12 ± 0.02 Da, respectively, are shown. The peak at 28.78 min corresponds to the amitraz molecule (protonated molecule and fragment ion). However, an additional peak can be seen in Figure 2b (retention time 12.16 min), and the accurate mass spectrum for that peak is shown in Figure 2c. As can be seen, the experimental accurate mass for this unknown species (*m*/*z* 163.1229) is really close to that obtained for the amitraz fragment ion with *m*/*z* 163 (accurate mass, 163.1233). Therefore, the accurate mass analysis yields the same possible elemental composition ($[M + H]^+$ $C_{10}H_{15}N_2$, experimental accurate mass, 163.1229; −0.45 ppm error). In addition, this mass spectrum corresponding to the peak with *m*/*z* 163 at 12.16 min also contained the fragment ion *m*/*z* 122, also characteristic of amitraz ($C_8H_{12}N$, experimental accurate mass, 122.0962; −1.85 ppm error). It clearly reveals that amitraz degrades in the same fashion it fragments. The accurate mass analysis performed on this transformation product is included in Table 2. The fragment ion resulting from in-source by collision-induced dissociation has exactly the same structure of one of its main degradation products.

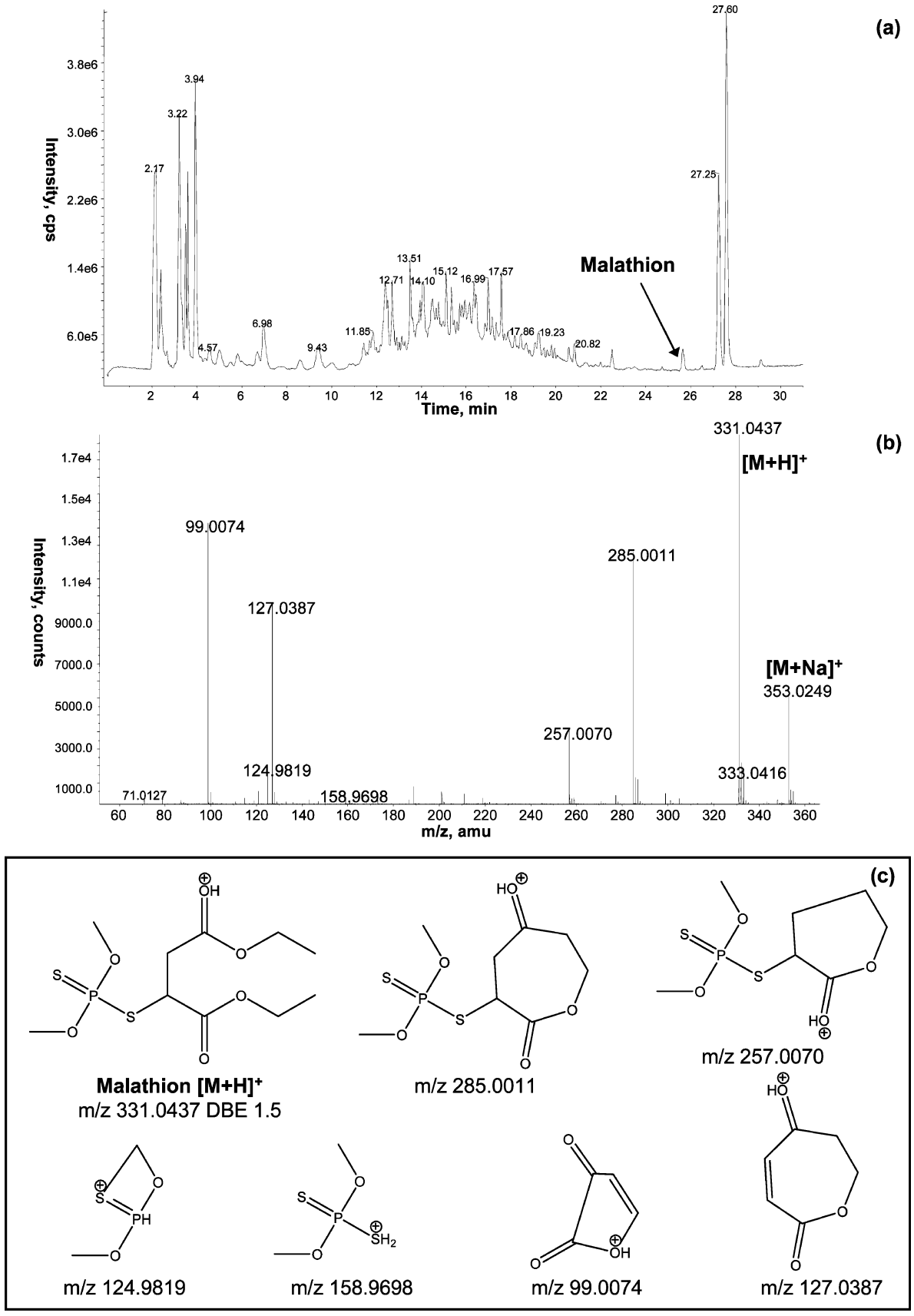

Figure 7. (a) Total ion chromatogram obtained from the LC/TOFMS analysis of the studied pepper extract, in which malathion was detected (peak at 25.6 min); (b) accurate mass spectrum of malathion; (c) proposed structures of in-source CID fragment ions of malathion. For details, see text.

Table 3. Accurate Mass Analysis of ESI-(+)-TOFMS Mass Spectrum of Peak at 25.6 min in a Pepper Extract: Accurate Mass Analysis of Malathion and CID Fragment Ions Detected in the Studied Pepper Extract

m/z_{exptl}	elemtl compstns[a]	m/z_{calcd}	error, mDa	error, ppm	DBE[b]
malathion $[M + H]^+$ 331.0437	$C_{10}H_{12}N_4O_7P$	331.043 81	−0.11	−0.34	7.5
	$C_{13}H_3N_{10}O_2$	331.043 49	0.20	0.62	17.5
	$C_{10}H_{20}O_6PS_2$	331.043 34	0.35	1.07	1.5
	$C_6H_3N_{16}S$	331.0441 8	−0.48	−1.46	13.5
	$C_8H_{15}N_2O_{10}S$	331.044 19	−0.49	−1.49	2.5
	$C_{13}H_{11}N_6OS_2$	331.043 02	0.67	2.03	11.5
	$C_3H_{12}N_{10}O_5PS$	331.044 5	−0.80	−2.41	3.5
	$C_5H_7N_{12}O_4S$	331.042 84	0.85	2.58	8.5
	$C_{11}H_{16}N_4O_2PS_2$	331.044 68	−0.98	−2.97	6.5
	$C_{16}H_{11}O_8$	331.044 84	−1.14	−3.45	11.5
	$C_9H_{16}O_{11}P$	331.042 47	1.22	3.70	2.5
	$C_7H_4N_{14}OP$	331.042 46	1.23	3.73	13.5
	$C_{20}H_{11}O_3S$	331.042 34	1.36	4.10	15.5
	$C_{11}H_8N_8O_3P$	331.045 15	−1.45	−4.38	12.5
	$C_{12}H_7N_6O_6$	331.042 15	1.54	4.65	12.5
	$C_{19}H_{12}N_2PS$	331.045 33	−1.63	−4.94	15.5
	$CH_7N_{12}O_9$	331.045 34	−1.65	−4.97	4.5
malathion $[M+Na]^+$ (m/z 353) 353.0249	$C_{10}H_{19}O_6PNaS_2$	353.025 2	−0.39	−1.10	1.5
malathion fragment 1 (m/z 285) 285.0011	$C_8H_{14}O_5PS_2$	285.001 48	−0.38	−1.34	2.5
malathion fragment 2 (m/z 158.9) 158.9698	$C_2H_8O_2PS_2$	158.969 78	0.012	0.07	−0.5
malathion fragment 3 (m/z 127) 127.0387	$C_6H_7O_3$	127.038 97	−0.27	−2.73	3.5
malathion fragment 4 (m/z 124.9) 124.9819	$C_2H_6O_2PS$	124.982 06	−0.17	−1.33	0.5
malathion fragment 5 (m/z 99) 99.0074	$C_4H_3O_3$	99.007 67	−0.27	−2.1	3.5

[a] Elemental composition calculator tool (minimum and maximum number of atoms): C [0−50]; H [0−100]; O [0−25]; N [0−20]; S [0−2]; P [0−1]; C1 [0−0]. [b] Double bond and ring equivalent.

This is an example of using fragmentation−degradation relationships to detect unknown pesticide transformation products. This identification was accomplished without the use of standards and may help to develop methods for quantitative analysis of pesticide residues, including not only pesticides in their parent form but also transformation products to provide a more comprehensive view on the real overall concentration levels of pesticides present in commodities.

Another example of this strategy is shown in Figure 3a and b, where the extracted ion chromatograms of m/z 122.095 ± 0.02 and 163.12 ± 0.02 Da are shown. The peaks at 28.78 and 12.16 min correspond to amitraz molecule (fragment ions m/z 122 and 163) and amitraz transformation product 1 (TP-1) (m/z 163)m respectively. An additional peak at retention time 8.76 min can be seen in Figure 3a, and the accurate mass spectrum for that peak is shown in Figure 3c. The experimental accurate mass for this unknown species (m/z 122.0963) matches with that obtained for the amitraz fragment ion with m/z 122 (accurate mass, 122.0963), and obviously, the accurate mass analysis yields the same possible elemental composition as well. The accurate mass analysis performed on this transformation product (m/z 122) is included in Table 2. This is another transformation product of amitraz, which has the same structure as the characterized fragment ion with m/z 122. In addition, this compound at 8.76 min also contains two less abundant fragment ions (m/z 105 and 107), which are also common to amitraz. This is another report on the potential use of fragmentation−degradation relationships to detect transformation products and to understand the way pesticides are degraded or metabolized in vegetables, all without using standards, which may not be available, especially for transformation products.

Figure 4 shows the extracted ion chromatograms of three characteristic fragment ions of the amitraz family (m/z 122.095 ± 0.01 Da (Figure 4a); m/z 105.07 ± 0.01 Da (Figure 4b); and m/z 107.07 ± 0.01 Da (Figure 4c). As can be seen, these three ions are common to species at 28.78 (amitraz), 12.16 (amitraz-TP-1), and 8.76 min (amitraz-TP-2), but also to an unknown compound at 18.1 min. The shape of the peak may suggest the possibility this is a mixture of two compounds with common fragment ions. The accurate mass spectrum of the peak at 18.1 min is shown in Figure 4d. A major ion is shown there with m/z 150.091. The presence of m/z 122, 107, and 105 confirms that it belongs to the family of amitraz, although as can be seen in Table 2, the main possible elemental composition contains an oxygen atom ($[M + H]^+$, $C_9H_{12}NO$; accurate mass, m/z 150.091 34; −0.9 ppm error), absent in both the parent species and the rest of degradates. Anyhow, the accurate mass analyses on ions with m/z 122, 107, and 105 are a proof of this species being a real amitraz transformation product. Accurate mass analysis for this transformation product is included in Table 2. The information on fragment ions and the elemental composition helped us to propose the structure included in Figure 4, which corresponds to 2,4-dimethylphenyl formamide.

Finally, we also identified a less abundant fragment ion with m/z 253 at 18.13 min (see Figure 5). This transformation product was partially overlapped with transformation product with m/z 150.

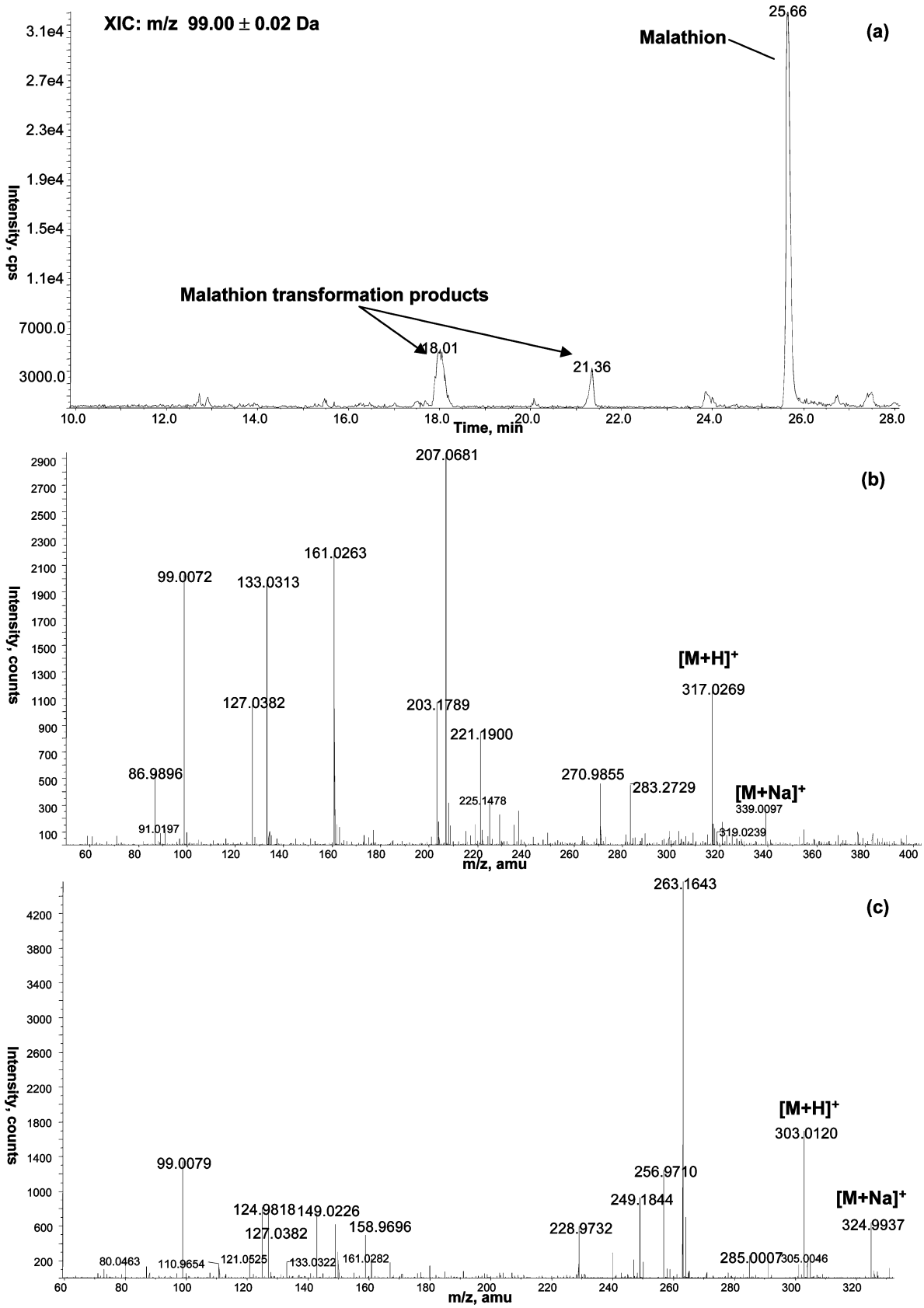

Figure 8. (a) Extracted ion chromatograms obtained from the LC/TOFMS analysis of the studied pepper extract (*m*/*z* 99.00 ± 0.02 Da); (b) accurate mass spectrum of proposed malathion transformation product with *m*/*z* 317 ($[M + H]^+$) at 18.01 min; (c) accurate mass spectrum of proposed malathion transformation product with *m*/*z* 303 ($[M + H]^+$) at 21.36 min.

This is the reason for the peak shape of *m*/*z* 150 as commented on above, and this is because both species contain common fragment ions that are not resolved by the chromatographic separation. The extracted ion chromatogram of *m*/*z* 253.17 ± 0.02

Table 4. Accurate Mass Analysis of ESI-(+)-TOFMS Mass Spectrum of Peaks at 18.0 (*m/z* 317), Corresponding to Malathion TP-1, and its Fragment Ions Detected in the Studied Pepper Extract

m/z_{exptl}	elemtl compstns[a]	m/z_{calcd}	error, mDa	error, ppm	DBE[b]
malathion TP-1 (*m/z* 317) $[M + H]^+$ 317.0271	$C_4H_5N_{12}O_4S$	317.027 19	−0.10	−0.30	8.5
	$C_8H_{14}O_{11}P$	317.026 82	0.27	0.86	2.5
	$C_{12}H_9N_6OS_2$	317.027 37	−0.28	−0.88	11.5
	$C_6H_{12}N_{14}OP$	317.026 81	0.28	0.90	13.5
	$C_{19}H_9O_3S$	317.02669	0.41	1.28	15.5
	$C_{11}H_5N_6O_6$	317.0265	0.59	1.87	12.5
	$C_9H_{18}O_6PS_2$	317.027 69	−0.60	−1.88	1.5
	$C_{12}HN_{10}O_2$	317.027 84	−0.74	−2.35	17.5
	$C_6H_{10}N_{10}PS_2$	317.026 34	0.75	2.37	7.5
	$C_{11}H_{13}N_2O_5S_2$	317.026 04	1.05	3.34	6.5
	$C_9H_{10}N_4O_7P$	317.028 16	−1.06	−3.35	7.5
	$C_3H_9N_8O_8S$	317.025 85	1.24	3.92	3.5
	$C_5HN_{16}S$	317.028 53	−1.43	−4.52	13.5
	$C_{13}H_{10}N_4O_2PS$	317.025 66	1.44	4.53	11.5
	$C_7H_{13}N_2O_{10}S_2$	317.028 54	−1.44	−4.55	2.5
$[M + Na]^+$ (*m/z* 339) 339.0097	$C_9H_{17}O_6PNaS_2$	339.009 64	0.058	0.17	1.5
fragment 1 (*m/z* 270.9) 270.9855	$C_7H_{12}O_5PS_2$	270.985 83	−0.33	−1.22	2.5
fragment 2 (*m/z* 207) 207.0681	$C_8H_{15}O_4S$	207.06855	−0.45	−2.20	1.5
fragment 3 (*m/z* 161) 161.0263	$C_6H_9O_3S$	161.026 69	−0.39	−2.44	2.5
fragment 4 (*m/z* 133) 133.0315	$C_5H_9O_2S$	133.031 77	−0.28	−2.10	1.5

[a] Elemental composition calculator tool (minimum and maximum number of atoms): C [0−50]; H [0−100]; O [0−25]; N [0−20]; S [0−2]; P [0−1]; C1 [0−0]. [b] Double bond and ring equivalent.

is shown in Figure 5; two peaks can be observed, that corresponding to amitraz at retention time 28.78 min and also the peak corresponding to amitraz transformation product *m/z* 253 (zoomed), with the proposed structure, which is the same as the structure proposed for the fragment ion of amitraz. The final transformation pathway of amitraz in this pear extract, revealed from the data collected by LC/TOFMS is outlined in Figure 6. After we identified these four compounds, we made a literature search on amitraz and its transformation products and we found data and reports that agreed with our results.[16] The unique and more remarkable feature of the proposed strategy is that no standards were required to accomplish the successful identification of both amitraz and its main transformation products in real market-purchased samples, in contrast to other studies where large concentrations of the target compound are used in a remarkably different ambient (laboratory simulated degradation experiments) in comparison with real conditions where the degradation products should be detected.

Identification of Malathion and Two Transformation Products from Its Hydrolysis in Pepper. The proposed strategy was also applied to malathion, an insecticide that is also a chemical warfare agent derivate. In this other example, the transformation products does not match exactly with the structures of the fragment ions of the parent pesticide as was the case of amitraz detected in pears. However, the presence of common fragment ions, corresponding to characteristic moieties of the parent species, fosters the elucidation of possible transformation products. In this case, we used two small fragment ions, as diagnostic ions, which resulted to be common to both malathion and the detected TPs. First, malathion was identified by accurate mass measurements and confirmed by accurate mass analysis of in-source fragment ions and matching the retention time with standards. The total ion chromatogram of the extract, in which malathion was detected, is shown in Figure 7a, and the accurate mass spectrum for this peak at 25.6 min is shown in Figure 7b. In addition to the protonated molecule (*m/z* 331.0437), and its corresponding sodium adduct, several fragment ions characteristic of malathion were detected and elucidated. Structures for six fragments ions were proposed according to accurate mass analysis performed on malathion (see Table 3) and are shown in Figure 7c. The presence of malathion is, thus, confirmed. Both *m/z* 99.0074 and 127.0387 were used as diagnostic ions (they can be defined as fragment ions found in all members of a family). These ions enabled the elucidation of two transformation products of malathion, which is described in detail.

As an example, the extracted ion chromatogram of *m/z* 99.00, using a narrow mass window (40 mDa), is shown in Figure 8a. Three main peaks appear in this chromatogram: the peak corresponding to malathion at retention time 25.66 min and the two transformation products with *m/z* 317 ($[M + H]^+$) and 303 ($[M + H]^+$), detected at 18.01 and 21.36 min, respectively. The accurate mass spectrum for the proposed transformation products is shown in Figure 8b,c.

As can be seen, the accurate mass spectrum in both cases is quite complex, including several coeluting ions from the pepper matrix. In a preliminary step, the ions that correspond to malathion TP-1 are confirmed using the chromatographic data. In our study with malathion transformation products, the chromatographic shapes allow us the confirmation of the main fragment ions from both malathion metabolites. This is a drawback of this procedure inherent to LC/electrospray TOFMS; it is not possible to isolate the precursor ion to obtain a clean MS/MS spectrum; only fragmentation could be enhanced by using heavier "fragmentor" voltages. In fact, the unique fragmentation available in single TOF instruments is in-source CID fragmentation to perform "pseudo MS/MS" experiments. When these experiments are accomplished at low concentration levels in complex matrixes with several coeluting species, the assignment of low-abundant fragment ions became a hard task, being difficult to ensure whether the ions correspond to the studied species. This is circumvented if using complementary mass spectrometric techniques with "real" MS/MS capabilities, because a selective isolation and efficient fragmentation (a fragmentation in a collision cell is usually more effective than that performed "in-source") can be performed, obtaining satisfactory MS/MS spectra even at very low concentrations. In contrast, these MS/MS instruments (i.e., Q-TOF) do not offer the same performance of single TOF in terms of mass accuracy of fragment ions (in MS/MS mode), because continuous dynamic accurate mass calibration cannot be performed over the

(16) Pierpoint, A. C.; Hapeman, C. J.; Torrents, A. *J. Agric. Food Chem.* **1997**, *45*, 1937−1939.

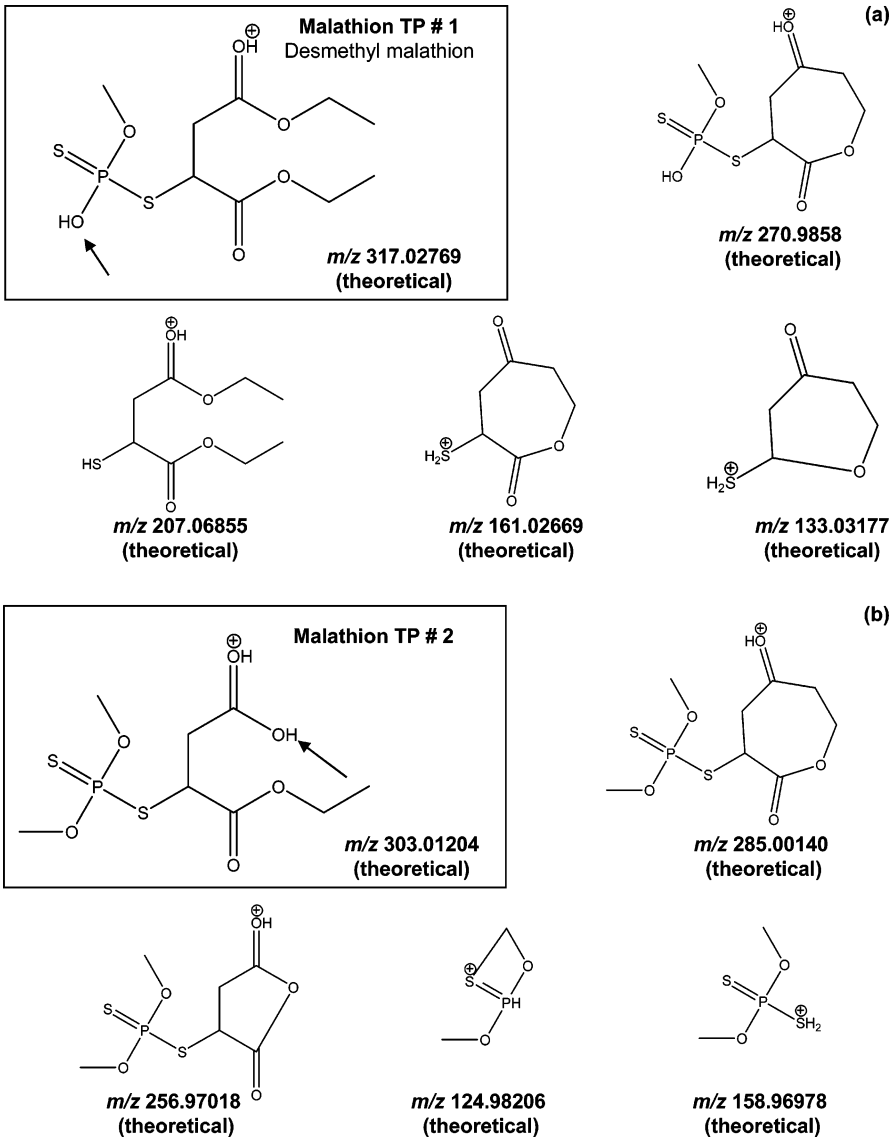

Figure 9. (a) Inset: Proposed structure for the detected malathion degradation product (retention time. 18.01 min) corresponding to a ester hydrolysis of a methyl group from the dimethyl-phosphorothioic acid moiety. Proposed structures for characteristic fragment ions of proposed malathion TP-1. (b) Inset: Proposed structure for the detected malathion transformation product (retention time. 21.36 min) corresponding to an ester hydrolysis of a ethyl group from the "succinic acid" moiety. Proposed structures for characteristic fragment ions of proposed malathion TP-2.

course of an analysis, yielding mass accuracy errors typically in the 5−10-mDa range. For the later, probably Orbitrap or FT-ICR analyzers offer unsurpassed accurate mass measurement capabilities in MS/MS (MS^n) experiments for small-molecule research. Of course, the more the data you collect with different MS instruments the better, but it is also a balance between money and time, not only analytical performance. Our conclusion is that state-of-the-art LC/TOFMS instruments are very well suited to this kind of research, providing fully satisfactory results. However, there is no perfect method, and maybe there could be cases where LC/TOFMS is unable to resolve a problem, and then the use of complementary (and usually more expensive) techniques (i.e., Q-TOFMS, Orbitrap, etc.) would offer new possibilities and additional information to solve the problem.

The accurate mass analysis performed on this malathion TP-1 (m/z 317) is shown in Table 4. Taking into account the elemental composition of malathion, the assignment of the elemental composition (see Table 4) of malathion transformation product is quite intuitive ($[M + H]^+$ 317.0271 experimental; elemental composition $C_9H_{18}O_6PS_2$, −1.9 ppm error).

Accurate mass analysis on the sodium adduct was used for further confirmation of the proposed elemental composition ($[M + Na]^+$, experimental m/z 339.0097; elemental composition $NaC_9H_{17}O_6PS_2$, 0.2 ppm error). The loss of methylene (CH_2) rapidly suggests an ester hydrolysis and subsequent elimination of a methyl group. This hypothesis is confirmed after drawing the structure for the fragment ions, including the loss of a methyl group from the original malathion molecule. In this sense, in ions where the organophosphorus part remained, the mass defect, which involves the presence of the P atom, was helpful to confirm that the fragment belong to the studied species and also to help the assignment of its elemental composition. In this case, the fragment ion with m/z 270.9855 is the key for the confirmation of the proposed structure, being consistent with the proposed

Table 5. Accurate Mass Analysis of ESI-(+)-TOFMS Mass Spectrum of Peaks at 21.3 min (*m/z* 303), Corresponding to TP-2 and Its Fragment Ions, Detected in the Studied Pepper Extract

m/z_{exptl}	elemtl compstns[a]	m/z_{calcd}	error, mDa	error, ppm	DBE[b]
malathion TP-2 (*m/z* 303) $[M + H]^+$ 303.0120	**$C_8H_{16}O_6PS_2$**	303.012 04	−0.047	−0.15	1.5
	$C_{11}H_7N_6OS_2$	303.011 72	0.27	0.89	11.5
	$C_3H_3N_{12}O_4S$	303.011 54	0.45	1.50	8.5
	$C_8H_8N_4O_7P$	303.012 51	−0.51	−1.69	7.5
	$C_7H_{12}O_{11}P$	303.011 17	0.82	2.72	2.5
	$C_5N_{14}O_6P$	303.011 16	0.83	2.75	13.5
	$C_6H_{11}N_2O_{10}S$	303.012 89	−0.89	−2.95	2.5
	$C_{18}H_7O_3S$	303.011 04	0.96	3.16	15.5
	$C_{10}H_3N_6O_6$	303.010 85	1.14	3.77	12.5
	$CH_8N_{10}O_5PS$	303.013 2	−1.20	−3.96	3.5
	$C_5H_8N_{10}O_7PS_2$	303.010 69	1.30	4.29	7.5
	$C_9H_{12}N_4O_2PS_2$	303.013 38	−1.38	−4.57	6.5
$[M+Na]^+$ (*m/z* 325) 324.9937	$C_8H_{15}O_6PNaS_2$	324.993 99	−0.29	−0.90	1.5
fragment 1 (*m/z* 285) 285.0007	$C_8H_{14}O_5PS_2$	285.001 4	−0.78	−2.75	2.5
fragment 2 (*m/z* 256.9) 256.9710	$C_6H_{10}O_5PS_2$	256.970 18	0.81	3.18	2.5
fragment 3 (*m/z* 158.9) 158.9696	$C_2H_8O_2PS_2$	158.969 78	−0.19	−1.18	−0.5
fragment 4 (*m/z* 124.9) 124.9818	$C_2H_6O_2PS$	124.982 06	−0.27	−2.12	0.5

[a] Elemental composition calculator tool (minimum and maximum number of atoms): C [0−50]; H [0−100]; O [0−25]; N [0−20]; S [0−2]; P [0−1]; C1 [0−0]. [b] Double bond and ring equivalent.

structure for malathion derivate. In addition, the absence of the fragment ions with *m/z* 124.9818 and 158.9696, which corresponds to the organophosphorus moiety (dimethylphosphorodithioic acid), and present in both malathion and TP-2 (*m/z* 303), is consistent with the structure proposed as well. The proposed metabolite is desmethyl malathion.[17] This is another example of the application of the proposed strategy based on fragmentation−degradation relationships, in order to find out pesticide transformation products, without using standards a priori. The accurate mass analysis performed on desmethyl malathion and its main fragment ions is included in Table 4. The structures proposed for this metabolite and its characteristic fragment ions are shown in Figure 9a.

The accurate mass spectrum of the second metabolite of malathion (retention time 21.3 min) is shown in Figure 8c, and the accurate mass analysis performed on this malathion TP (*m/z* 303) is shown in Table 5. The first elemental composition was the one that fitted best ($[M + H]^+$ 303.0120 experimental; elemental composition $C_8H_{16}O_6PS_2$, −0.15 ppm error), especially taking into account the elemental composition of malathion. Accurate mass analysis on the sodium adduct was also used for further confirmation of the proposed elemental composition ($[M + Na]^+$, experimental *m/z* 324.9937; elemental composition $NaC_8H_{15}O_6PS_2$, 0.9 ppm error). The mass defect due to phosphorus atom is especially helpful to assign all the fragment ions and their elemental compositions. The accurate mass analysis evidence the loss of C_2H_4 also suggests an ester hydrolysis with ethyl group elimination. This hypothesis is consistent with the proposed structures of its fragment ions, which are shown in Figure 9b. The presence of fragment ions (*m/z* 124.9818 and 158.9696) evidence that loss of C_2H_4 is due to an elimination of a ethyl group in the other part of the molecule rather than two methyl groups in the organophosphorus moiety. This is another example of the identification of pesticide transformation products by fragmentation−degradation relationships, without using standards a priori. To provide a safer and comprehensive elucidation of the structure of these two degradation products, probably additional confirmatory data, including MS/MS or MS^n data would be required, but the proposed methodology enabled the identification of two transformation products.

In both cases (malathion and amitraz), the concentration levels were not very high with regard to the parent species. It is difficult to provide quantitative data since no standards are available for most of the identified TPs. From the data available and assuming a similar response and sensitivity of parents and degradates, in both cases, the total amount of the degradation products represents over 15% of the concentration of the parent species.

Concluding Remarks. Degradation of pesticides in food is a very important field to work in depth. Together with the structural elucidation, toxicity evaluation of the identified compounds must be accomplished. For the first, state-of-the-art of LC/TOFMS instruments offers unique features of TOF (high full-scan sensitivity and accurate mass measurements), enabling high-sensitivity, full-scan spectrum acquisition of any peak in a chromatogram and providing accurate masses of ions for the identification of unknowns compounds, since we get information on both accurate mass and possible elemental compositions of most the ions generated by a specific compound. As has been shown, this makes possible the identification of unknown degradation products of pesticides a priori (without using standards), based on the use of fragmentation−degradation relationships. The fragmentation pathways of the parent species can be used to predict possible degradation products, since the bonds that are easily cleaved are those that might be broken in reaction to ambient conditions.

There can be also cases where the transformation involves major changes or arrangements in the structure of the degradate with regard to the fragment ion of the parent, but it is always feasible to keep diagnostic ions that reveal part of the structure of possible unknown degradation products. In such cases, information from isotope signatures (especially if Cl, Br, or S is present), from mass defects of ions (the presence of P atoms on organophosphorus pesticides), elemental compositions, DBE values, and their compatibility between parents and suspected/proposed transformation products, chromatographic data (transformation products are usually more polar than parents) are required along with MS/MS experiments to elucidate and understand the way the parent pesticide is degraded and the subsequent identification of the transformation product. This single strategy proposed in this work to be applied for pesticide residue research in foodstuffs, could be further extended to other fields of application including the study of the metabolism of

(17) Bavcon, M.; Trebse, P.; Zupancic-Kralj, L. *Chemosphere* **2003**, *50*, 595−601.

pharmaceuticals, the detection of new metabolites in biological fluids, impurity profiling studies, or even differential metabolomics.

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SUPPORTING INFORMATION AVAILABLE

Toxicity data available for the identified compounds is included in Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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