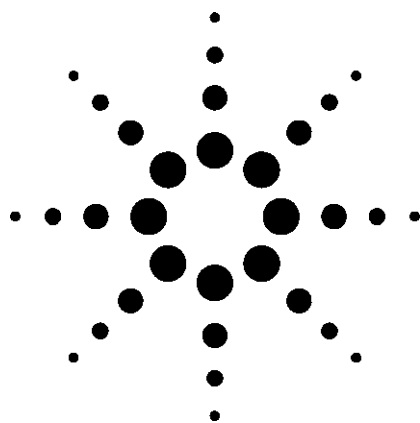




Analysis of Post-Harvest Fungicides and Their Metabolites in Citrus Fruits and Juices by Time-of-Flight and Ion Trap LC/MS

Application

Food Safety

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Abstract

This application note applies both time-of-flight and ion trap liquid chromatography/mass spectrometry (LC/TOFMS and LC/ITMS) to determine two important post-harvest fungicides (imazalil and thiabendazole) and a metabolite of imazalil in oranges and in their juice. Included is both a detailed rapid procedure for sample preparation of citrus fruits and a solid-phase extraction for the isolation of these fungicides and metabolite from juice. Identification of these fungicides and metabolite is carried out using both accurate mass for elemental composition and fragmentation pathways with LC/ITMS to help elucidate pathways of degradation. The results of the analysis of actual samples from the marketplace for fruit and juice are included.

Introduction

Thiabendazole and imazalil are widely used for post-harvest treatment of citrus fruits (Figure 1) in order to preserve the fruit during the transport process, which may take from several days to several weeks. The fungicide treatment is of the fruit skin only. The maximum residue limits (MRLs) for imazalil and thiabendazole in Europe and the United States require that these compounds be monitored before consumption of the fruit [1, 2]. Since these compounds are used ubiquitously, they are frequently detected in imported citrus fruits [3]. Thus, many commercial samples must be inspected to ensure food safety. Additionally, imazalil is easily metabolized to 1-(2,4-dichlorophenyl)-2-(1H-imidazole-1-yl)-1-ethanol, which is sometimes detected in citrus fruits [4]. In the United States, the sum of imazalil and its metabolite is regulated, so a method for the parent compound should also include its metabolite [5].

While papers have reported analytical LC/MS procedures for imazalil and thiabendazole, less is known about the metabolite of imazalil, and there are, as yet, no published reports using LC/MS accurate-mass analysis [6]. This application note describes a rapid and simultaneous method using electrospray liquid chromatography/time-of-flight-mass spectrometry and liquid chromatography/ion trap mass spectrometry (LC/TOFMS and LC/ITMS) for the analysis of these two important post-harvest fungicides in citrus. An earlier application note reported on the analysis of carbendazim and thiabendazole in fruits and vegetables and may be of interest as well [7].



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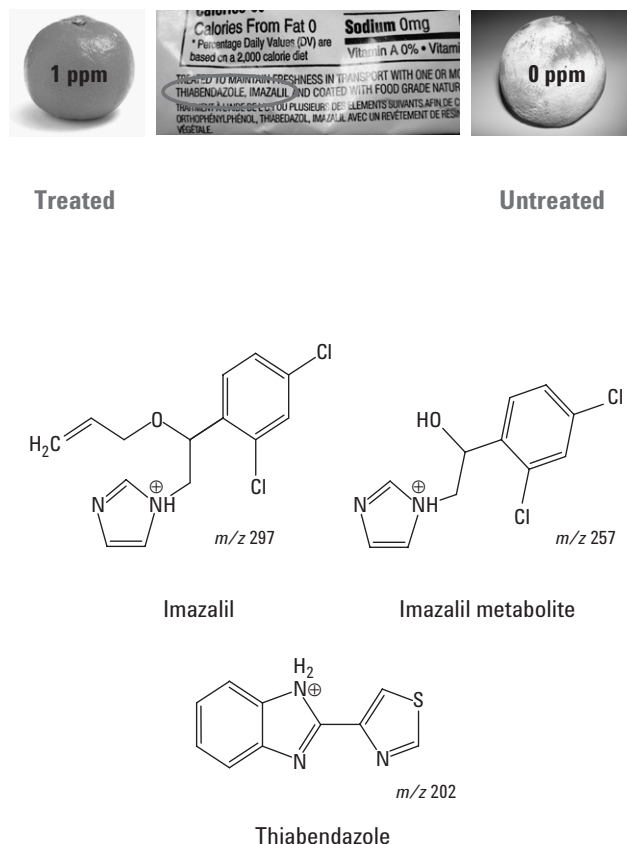


Figure 1. Structure of imazalil, thiabendazole, and an imazalil metabolite, which are post-harvest fungicides used on citrus (oranges and lemons), showing the $[M+H]^+$ ion. Top panel compares treated and untreated oranges.

Experimental Methods

Fruit Extraction: QuEChERS

QuEChERS (quick, easy, cheap, effective, rugged, and safe) is a method that is receiving wide acceptance for rapid extraction of pesticides in food [8].

1. Weigh 15 g of a previously homogenized sample into a 40-mL Teflon centrifuge tube.
2. Add 15 mL of acetonitrile (containing 1% acetic acid).
3. Add 6 g of anhydrous $MgSO_4$.
4. Add 2.5 g of $NaAc \cdot 3H_2O$ (sodium acetate trihydrate).

5. Shake the sample vigorously for 1 minute by using a Vortex mixer at maximum speed or hand shaking.
6. Centrifuge for 3 minutes at 3700 rpm.
7. Add 5 mL of supernate into a 15-mL tube.
8. Add 250 mg of PSA adsorbent and 750 mg of $MgSO_4$.
9. Vortex and shake for 20 s.
10. Centrifuge again for 3 minutes at 3700 rpm.
11. Transfer 1.0 mL into a LC/MS vial.
12. Evaporate the 1.0 mL supernate to dryness and bring back up in 8%/92% methanol/water for LC/TOFMS analysis and ion trap analysis.

LC/TOFMS analysis of fruit and vegetable extracts is performed by injecting 50 μ L. Nonfortified samples are analyzed directly at this same point by either LC/TOFMS or LC/ITMS.

Fruit-Juice Extraction

Solid-phase extraction (SPE) is used for the extraction of post-harvest fungicides from fruit juices and sodas based on juices. The cartridge of choice is the Accubond C-18 SPE cartridge (part number 188-1356).

1. Prepare the 5-mL SPE cartridge by taking 5 mL of methanol to wet the cartridge followed by 5 mL of deionized water to remove the methanol for good recovery of the fungicides.
2. Allow juice to stand, equilibrate to room temperature and degas prior to SPE, especially if bubbles are present.
3. Centrifuge and filter (0.2-micron TFPE filter to remove remaining solids) 5 mL of juice prior to dilution with 5 mL of deionized water.
4. Pass the juice through the cartridge and rinse with 2 mL of deionized water.
5. Elute with 5 mL of methanol.
6. Evaporate the methanol to 200 μ L under nitrogen.
7. Dilute 1:3 with deionized water before injection into the LC/TOFMS or LC/ITMS.

LC/TOFMS Method

LC Pumps	Agilent 1100 binary pumps, injection volume 50 μ L with standard Agilent 1100 ALS
Column	ZORBAX® Eclipse XDB-C8, 4.6 mm $\times$ 150 mm, 5 μ m, product number 993967-906
Mobile Phases	A = acetonitrile and B = 0.1% formic acid in water
Gradient	5 minutes isocratic at 10% A, followed by a linear gradient to 100% A in 30 minutes at a flow rate of 0.6 mL/min
Instrument	Agilent LC/MSD TOF time-of-flight mass spectrometer with dual electrospray source, positive ESI, capillary 4000 V
Nebulizer	40 psig
Drying gas	9 L/min, 300 °C
Voltages	Fragmentor 190 V, skimmer 60 V, Oct DC1 37.5 V, OCT RF V 250 V
Reference masses	m/z 121.0509 and 922.0098
Resolution	9500 $\pm$ 500 at m/z 922.0098
Mass range	50–1000 m/z
Flow rate	Reference A Sprayer 2 is at a constant flow rate during the run

LC/ITMS Method

Chromatography	Identical to LC/MSD TOF methods for direct comparison of peaks
LC Pumps	Agilent 1100, injection volume 50 μ L
Column	ZORBAX Eclipse XDB-C8, 4.6 mm $\times$ 150 mm, 5 μ m, product number 993967-906
Mobile phases	A = acetonitrile and B = 0.1% formic acid in water
Gradient	5 minutes isocratic at 10% A, followed by a linear gradient to 100% A in 30 minutes at a flow rate of 0.6 mL/min
Instrument	Agilent 1100 Series LC/MSD Trap. Positive ESI, Capillary 3200 V, Nebulizer 40 psig, drying gas 9 L/min, gas temp 300 °C. Capillary exit 70 V, Skimmer 60 V
Detection	Trap accumulation, 50,000 counts with maximum accumulation time of 200 ms

Results and Discussion

Imazalil in Oranges

Figure 1 shows two oranges, one with a treatment of the post-harvest fungicides (imazalil and thiabendazole) and the other orange without treatment. Note the intense growth of mold when no treatment of post-harvest fungicides is used. The purpose of the post-harvest fungicides is to prevent serious mold formation during transport and sale of citrus fruits. This treatment is directed at the orange peel itself, rather than the entire fruit; therefore, it poses a lesser risk to the consumer.

Figure 2 shows the LC separation and chromatogram of the orange-peel extract that contains two large peaks, which were suspected to be the post-harvest fungicides, imazalil and thiabendazole. They were identified using the following

technique. The accurate mass spectrum of each peak was taken and A+2 isotopes were examined (Figures 2 and 3). For example, the peak at 17.9 minutes was suspected to be a chlorine-containing species based on the mass spectrum (Figure 3). In fact, the presence and number of chlorine atoms in the compound can be easily attained taking into account both the relative intensity of the $^{37}\text{Cl}/^{35}\text{Cl}$ signals and the accurate mass differences between the two masses. As can be seen in Figure 3, the accurate mass of the m/z 297 peak was 297.0556 with a ^{37}Cl isotope signal of 299.0527, with a relative intensity of about two-thirds of the main peak. The mass difference between both signals is 1.9971, which is very near to the exact mass difference between ^{35}Cl (34.9689) and ^{37}Cl (36.9659) (1.9970). This evidence combined with peak area shows that the unknown compound unequivocally contains chlorine atoms.

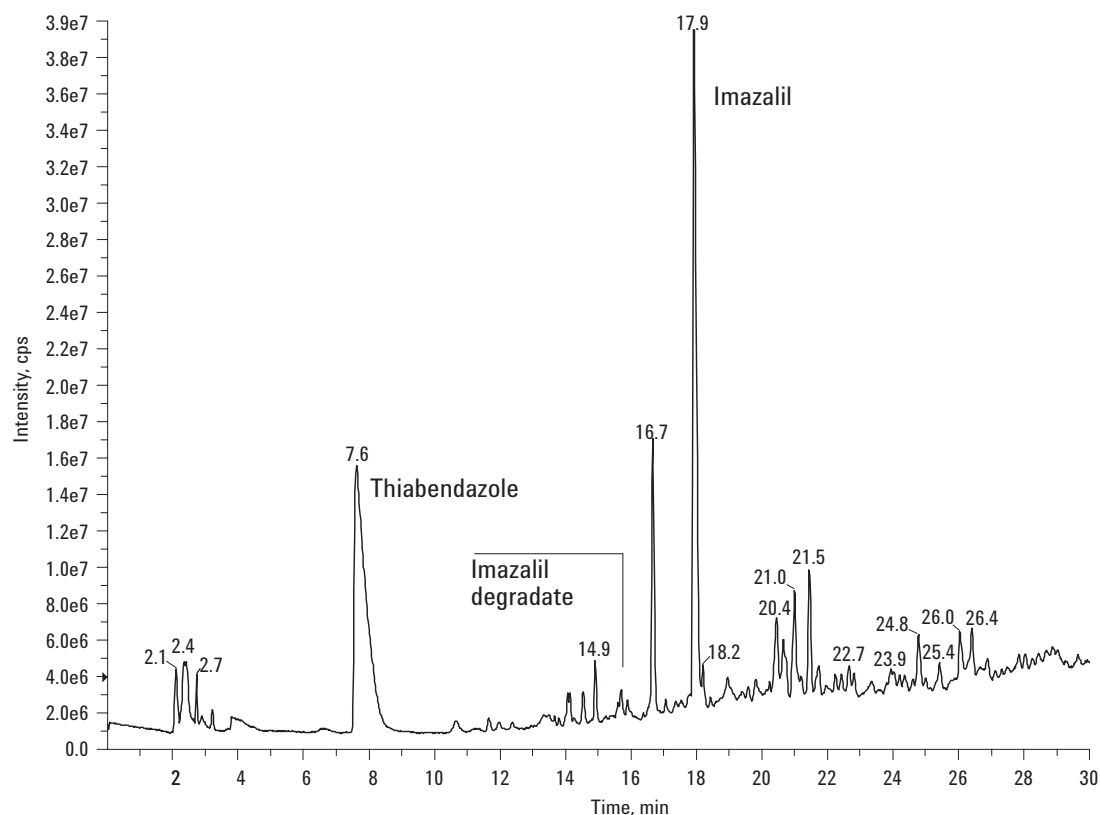


Figure 2. LC/MSD TOF total ion chromatogram (TIC) of orange peel, note the two large peaks in the chromatogram are imazalil and thiabendazole.

Furthermore, the relative abundance of the isotopic signal indicates that the chlorine isotope is present with two atoms (the natural distribution of ^{35}Cl . ^{37}Cl is nearly 3:1 (75.77% ^{35}Cl ; 24.23 % ^{37}Cl), and two chlorine atoms give the characteristic pattern shown in Figure 3. This fact makes much easier the assignment of an elemental composition for the suspected species. Using the calculator tool of the TOF software, we set, as a stringent criterion, the number of chlorine atoms to two. Using an accuracy error threshold of 3 ppm (the maximum error of the LC/MSD TOF), only one formula

was found ($\text{C}_{14}\text{H}_{15}\text{N}_2\text{OC}_{12}$, 0.3 ppm error). This formula was searched against the “The Merck Index” database. The use of the Merck Index is explained in an application note [9]. It has to be taken into consideration that the additional hydrogen atom present in the measured ions due to the positive ionization mode must be subtracted from the elemental compositions provided by the calculator tool before entering the formula into the database. The Merck Index gave a unique match with the formula for imazalil.

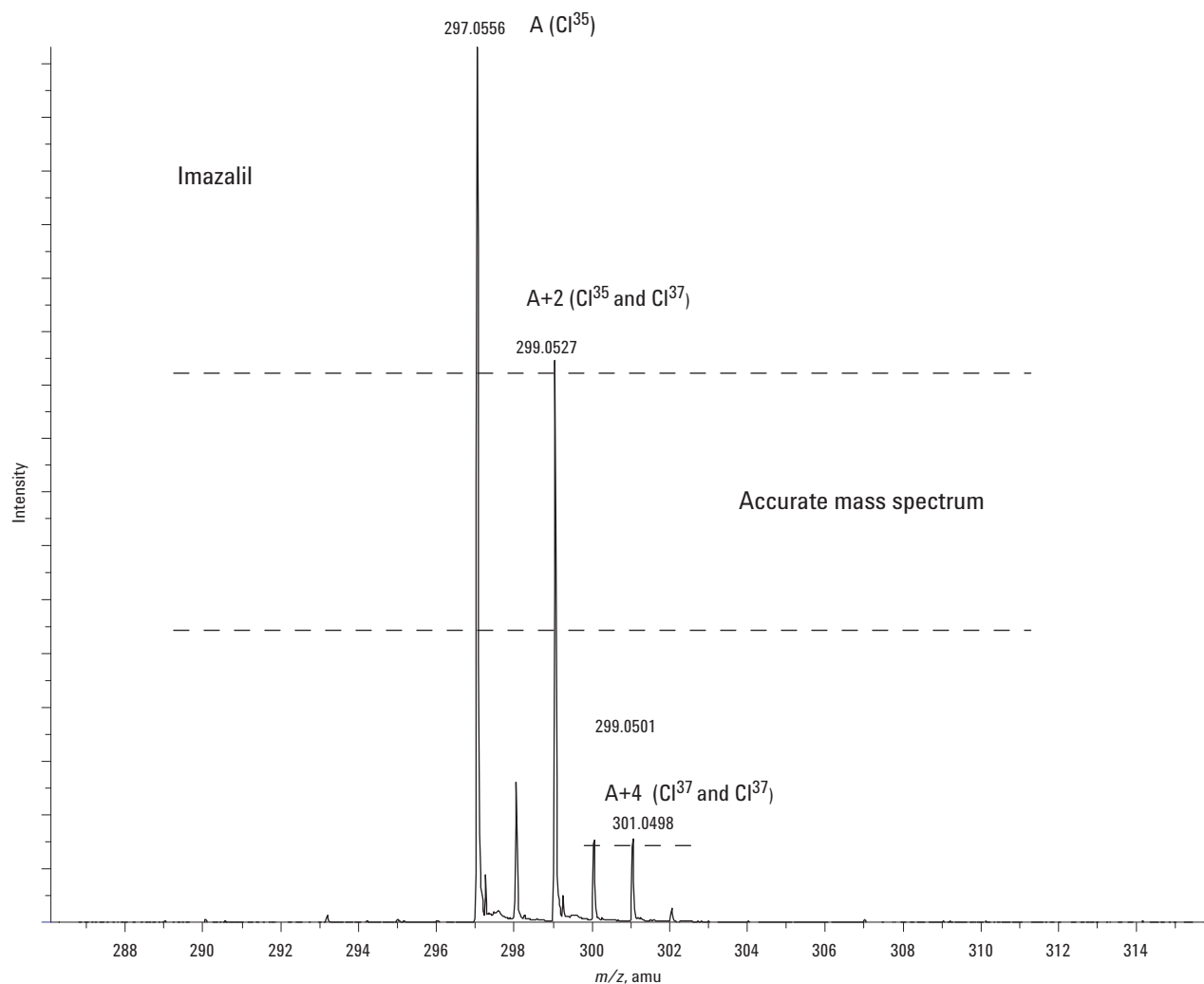


Figure 3. Imazalil detection in orange peel measured accurate mass, $m/z = 297.0556$ theoretical mass = 297.0556, accuracy 0.3 ppm, formula is $C_{14}H_{15}N_2OCl_2$.

Likewise the mass spectrum was taken for the peak at 7.6 minutes in Figure 2 and the spectrum is shown in Figure 4, m/z 202.0434. The relative abundance of the isotopic signal indicates that sulfur may be present with one atom. The relative intensity of the A+2 peak is 4.2% with a mass depletion of 1.996 daltons when going from ^{32}S (31.9721) to ^{34}S (33.9679). The mass spectrum shown in Figure 4 has a mass difference of 1.997 daltons and a peak height of 4.7%, which is

quite close to the expected values. Thus, these data indicate the presence of 1 sulfur atom. Entering a mass of m/z 202.0434 gives the unique formula of $C_{10}H_8N_3S$, which is the formula for a protonated ion of thiabendazole, based on the Merck Index search. The next step is to examine the fragmentation of the molecules using the LC/MS ion trap for further structural confirmation.

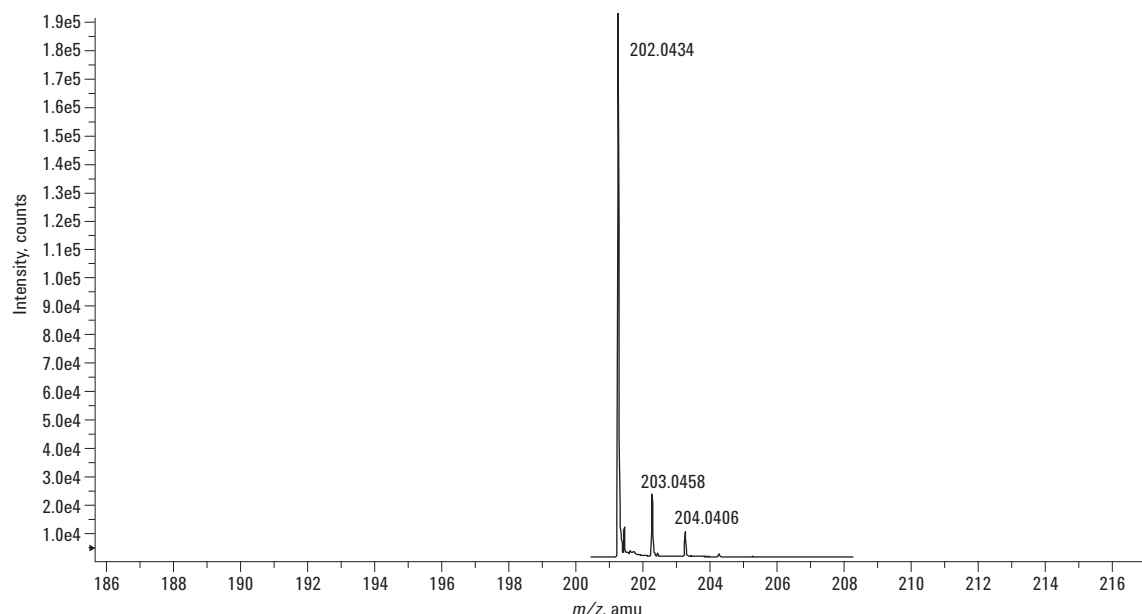


Figure 4. LC/MSD TOF mass spectrum of thiabendazole.

Fragmentation of Imazalil

The next step in the discovery process was to search for characteristic fragment ions of the pesticide to confirm (or refute) its identity as imazalil. The fragmentation pathway of the large peak at 17.9 minutes was determined with LC/ITMS at MS³. The pathway is examined to see if reasonable structures could be drawn for the imazalil fragments based on the detection by LC/TOFMS. Figure 5 shows the structure for the proposed ions from the fragmentation of the unknown peak. The m/z 255 ion results from the loss of 42. MS/MS of the m/z 255 ion gives the m/z 187 and 159 ions, whose proposed structures are shown in Figure 5.

The m/z 159 ion is proposed as a doubly chlorinated tropylium ion. All the structures are consistent with the proposed identification as imazalil.

Next is to examine the accurate mass spectrum from the LC/TOFMS. Here we found two fragment ions (m/z 255 and 159) with a relative abundance of respectively, 5% and 10% of that of the protonated molecule. The accurate mass of fragment 1 was 255.0084 (Table 1), and a ³⁷Cl signal m/z of 257.0055 (data not shown). From both the relative intensity of these signals and the difference between the two masses, it can be deduced that the two chlorine atoms present in imazalil remain in this fragment.

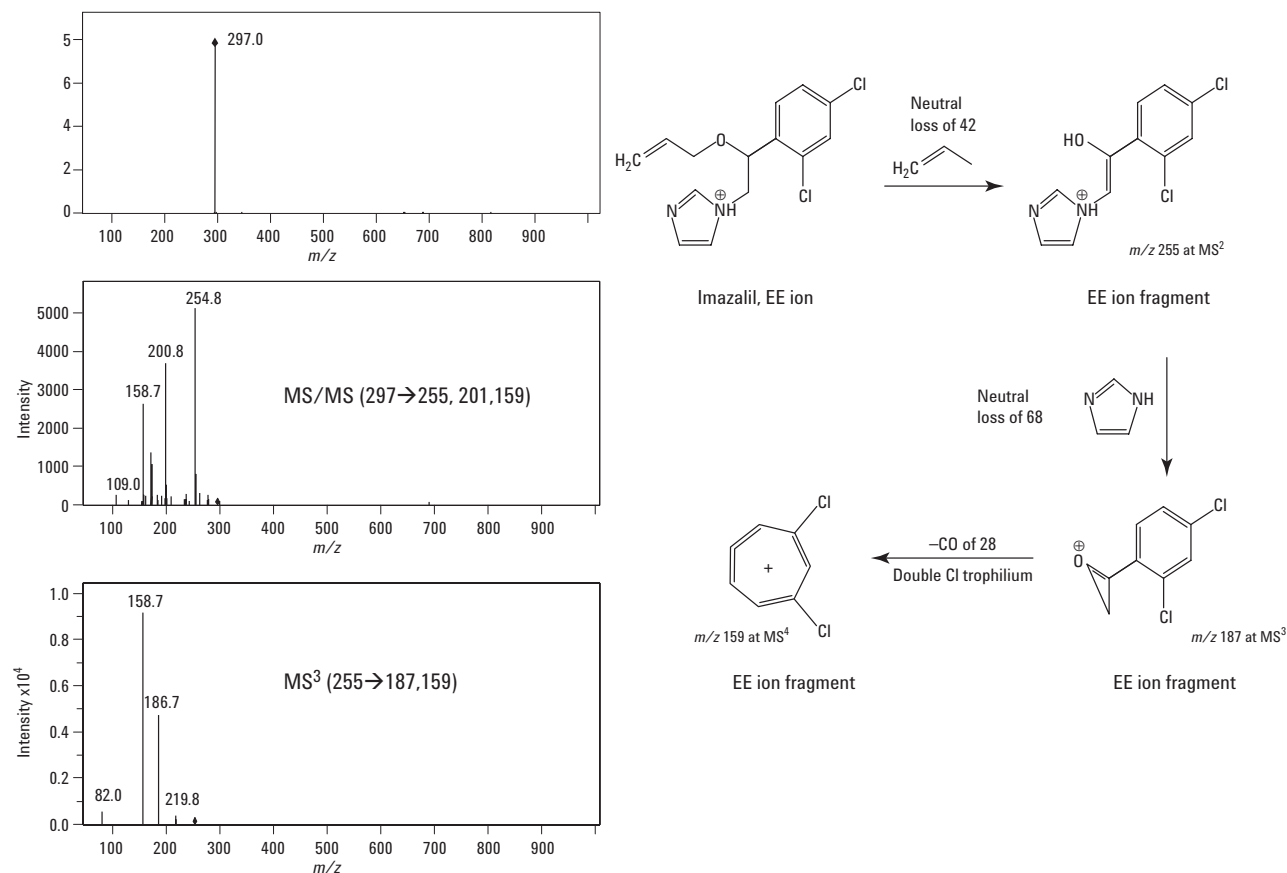


Figure 5. Ion trap MS² and MS³ to show fragmentation pathway of imazalil.

As can be seen in Table 1, the accurate mass of this fragment gave also three possible elemental compositions in the calculator tool. The first formula (C₁₁H₉N₂OC₁₂), (-0.8 ppm error) fitted with the proposed structure, involving a loss of C₃H₆ (accurate mass loss of 42.0469 versus 42.0465 u) in relation with the proposed parent species. Moreover, the accurate mass spectrum (relative intensity and mass differences) evidenced the presence of two chlorine atoms on fragment 2. The measured mass (*m/z* 158.9762) gave a unique elemental composition (C₇H₅Cl₂), that corresponds to the formation of a doubly chlorinated tropylium fragment-ion. At this point, we combined the fragmentation information from the LC/MSD Trap, as shown in Figure 5 for the proposed structure of

imazalil, with the accurate mass information, that all show a perfect match for the detection of imazalil.

These two lines of evidence (LC/TOFMS and LC/ITMS) provide fragment ions and information to confirm the identity of the proposed species based on fragmentation of the parent structure as imazalil, without the use of standards! Likewise, thiabendazole was examined by ion trap and gives a *m/z* 175 fragment ion, that fits with the structure of thiabendazole. The data are not shown here but were shown in a previous application note for thiabendazole [9]. We verified these determinations by standards for final confirmation.

Table 1. LC/MSD TOF Fragment Ions of Imazalil

<i>m/z</i> experimental	Elemental compositions	<i>m/z</i> calculated	Error, ppm	Comments
297.0556	C₁₄H₁₅N₂OCl₂	297.0556	<0.1	Imazalil
	C ₃ H ₁₅ N ₈ O ₄ Cl ₂	297.0588	-8.3	-
	C ₁₁ H ₁₉ N ₂ OSCl ₂	297.0590	-9.0	-
255.0084	C₁₁H₉N₂OCl₂	255.0086	-0.8	Fragment 1
	C ₉ H ₇ N ₅ Cl ₂	255.0073	4.3	-
	C ₈ H ₁₁ NO ₄ Cl ₂	255.0060	9.4	-
158.9762	C₇H₅Cl₂	158.9763	-0.6	Fragment 2

Imazalil Metabolite

In the orange extract, we found a peak (at a retention time of 14.9 minutes, see Figure 2) with an ion with the same isotopic pattern as imazalil (two chlorine atoms). Taking into account that it had the same number of chlorine atoms, and it also appeared before imazalil, we considered that it could be related to imazalil, perhaps a metabolite. The accurate mass of the ion was 257.0245 with a ³⁷Cl signal of 259.0216 (see Figure 6). It gave a unique elemental composition in the calculator tool: C₁₁H₁₀N₂OCl₂ (0.8 ppm error, also see Table 2). For confirmation purposes, we searched for additional fragments but we did not find any characteristic fragment ion of that compound by TOF LC/TOFMS. The obtained elemental composition was then searched against two databases (The Merck Index and ChemIndex) with no positive

results. Then, we search the elemental composition against the Sigma-Aldrich commercial electronic catalogue, and we found a unique match: “1-(2,4-dichloro-phenyl)-2-imidazol-1-yl-ethanol”. The structure of this compound, shown in Figure 1, is “compatible” with the degradation of imazalil (see Figure 5 the *m/z* 255 ion). This suggests that the degradation product is the “neutral” species corresponding to the degradation of imazalil at the same site that cleaves to yield the *m/z* 255 fragment.

A literature search on imazalil’s degradation products produced data and reports that agreed with our results (Imazalil-metabolite, R14832) [4]. In fact, in the United States, the sum of imazalil and imazalil metabolite is regulated, so the survey of residual imazalil metabolite is also required [4]. Finally, we confirmed the identity of the degradate by standard matching.

Table 2. Accurate Mass and Elemental Composition of Imazalil, Imazalil Degradate, and Thiabendazole in Orange Peel Extracted with Methanol

Compound	Formula	Selected ion	<i>m/z</i> experimental	<i>m/z</i> calculated	Error	
					mDa	ppm
Imazalil	C ₁₄ H ₁₄ Cl ₂ N ₂ O	[M+H] ⁺	297.0556	297.0556	+0.1	0.3
Imazalil Degradate	C ₁₁ H ₁₀ Cl ₂ N ₂ O	[M+H] ⁺	257.0245	257.0243	+0.2	0.8
Thiabendazole	C ₁₀ H ₇ N ₃ S	[M+H] ⁺	202.0434	202.0434	+0.2	<0.1

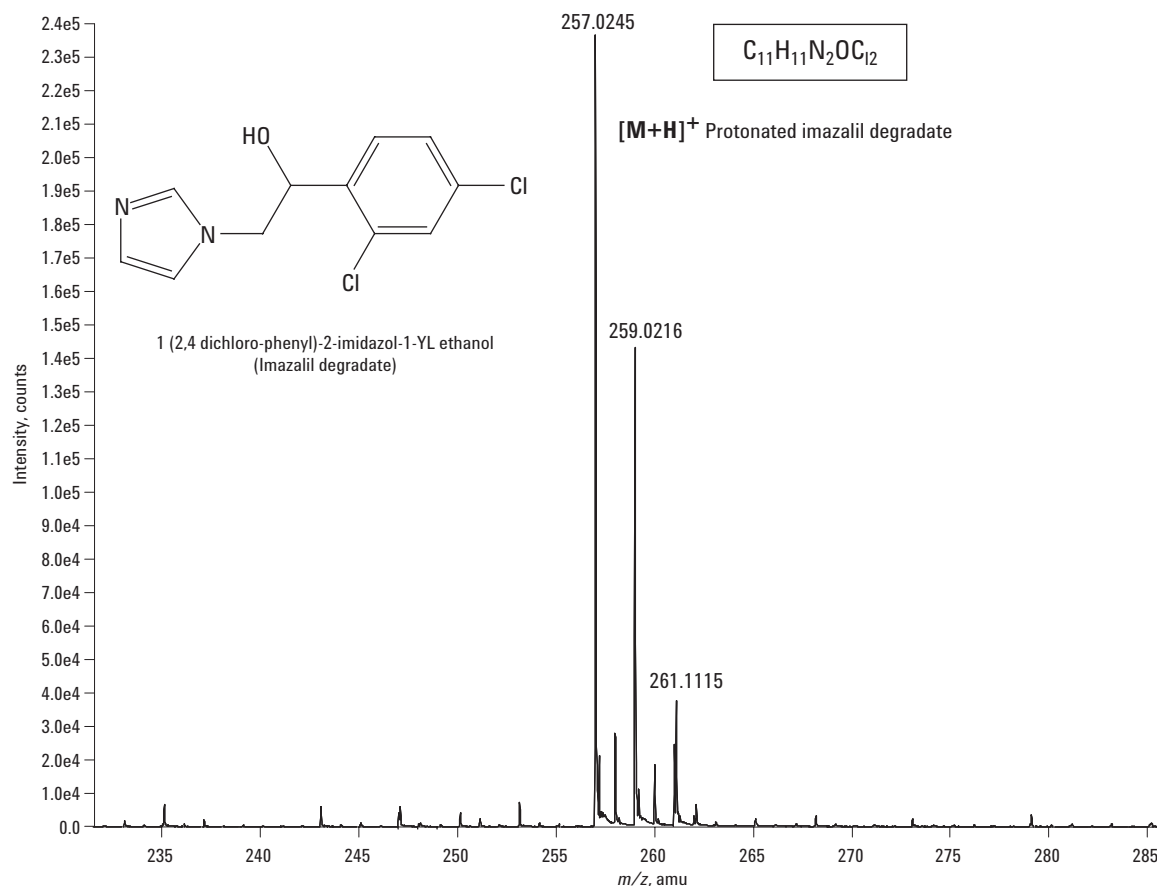


Figure 6. LC/MSD TOF spectrum of imazalil degradate.

Identification of Imazalil in Orange Juice

Figures 7 and 8 show the LC/TOFMS chromatogram (see peak at 18.0 minutes) and identification of imazalil (m/z 297.0556) in a juice product made from oranges. Several samples were analyzed and only this sample contained imazalil. No identifications of thiabendazole were observed. It is assumed that the production of juice usually does not involve the squeezing of the peel. However, in some types of “juice machines” the entire orange is squeezed without removing the peel. In this case, the opportunity for including fungicides in the juice may occur. Note that the LC/TOFMS analysis gave good results in spite of the complex matrix and complex chromatogram of the juice extract.

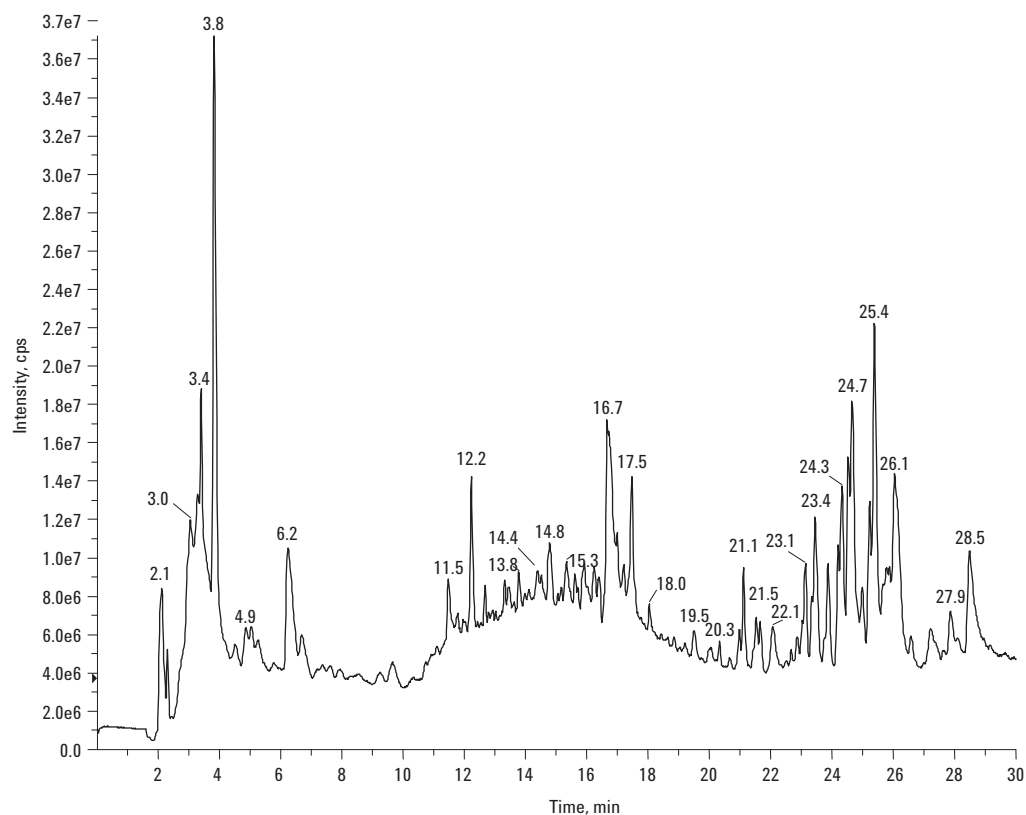


Figure 7. LC/MSD TOF TIC of orange juice extract.

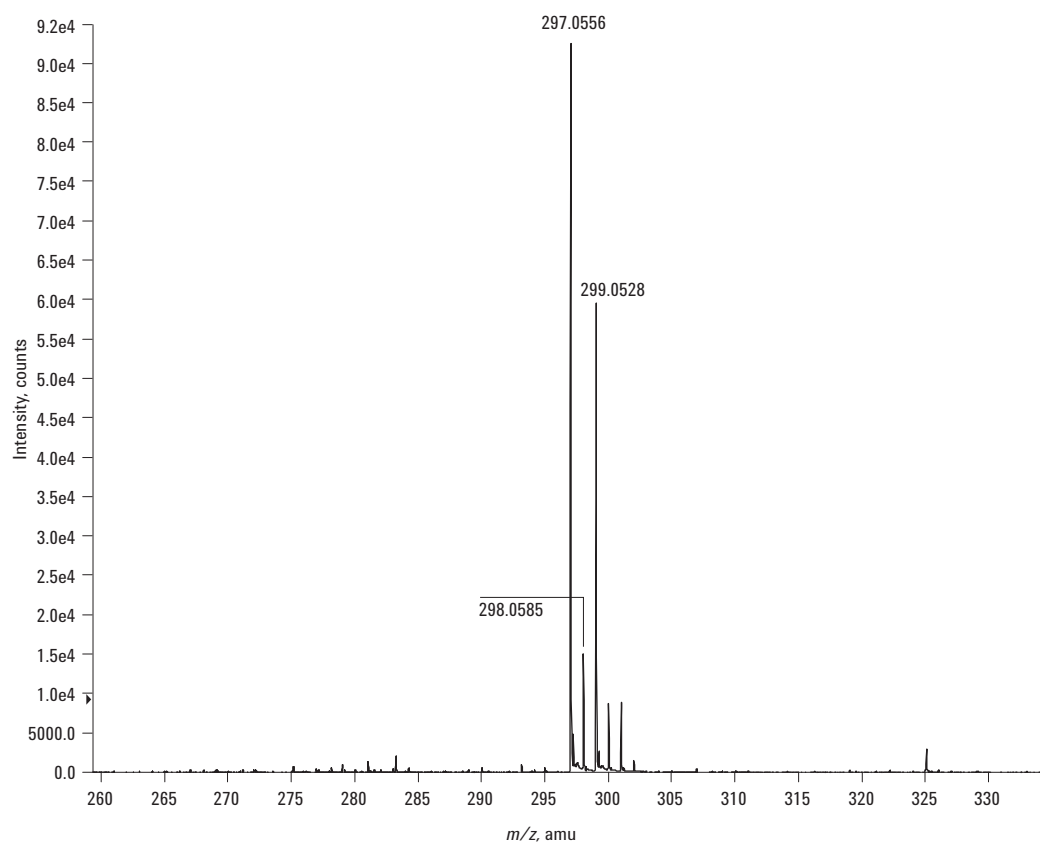


Figure 8. LC/MSD TOF mass spectrum of imazalil in orange juice made from peel.

Conclusions

- LC/TOFMS analysis is a powerful tool for identification of fungicides in fruits and juices and their metabolites and is a new tool for environmental food chemistry.
- Elemental composition and structural fragmentation of standards and fragment ions are possible, especially with ion trap MS/MS confirmation.
- The identification of metabolites is possible, in the case of imazalil, without standards, when both LC/TOFMS and LC/ITMS are used. These concentrations are equal to or lower than the EU directives for controlled fungicides in fruits and juices.

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