Both High-Resolution Chromatography and Accurate Mass Spectrometry are Essential for Analysis of Isobaric Pesticides in Complex Food Matrices

Application Note

Food Safety

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Abstract

To ensure detection and quantitation of isobaric pesticides in a complex food matrix such as pepper, both high-resolution liquid chromatography and Q-TOF accurate mass spectrometry are required, as demonstrated here using the Agilent 1290 Infinity LC System coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS.

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Introduction

With the increasing globalization of the food industry, there is greater scrutiny on food safety, particularly pesticide contamination. Food testing laboratories require the ability to detect and quantify hundreds of pesticides in a myriad of foodstuffs at very low levels of contamination. Mass spectrometry is a key tool for pesticide detection due to its sensitivity and specificity.

A major issue for pesticide detection in food is the possibility of missing important pesticides in food (false negatives), due to masking by complex matrices. Given the common occurrence of fungicides and insecticides in foods, such as lettuce, tomato, and pepper, the prospect of false negatives is daunting. However, both high-resolution chromatography and high-resolution mass spectrometry are powerful tools for preventing false negatives [1,2].

The ability to detect all pesticides in food matrices is made more difficult by the existence of isobaric pesticide compounds. Isobars are compounds with the same nominal mass but different molecular formulae, while an isomer has the same molecular formula. Isobars present unique separation challenges, and it is necessary that both high-resolution chromatography and high-resolution mass spectrometry are required to fully resolve them, particularly in complex food matrices.

This application note describes a published study [3] designed to determine the role of chromatography and mass spectrometry in resolving and identifying five isobaric pesticides of mass *m/z* 314 MH^+, spiked into red pepper (*Capsicum annuum*), a complex matrix containing more than 2,000 molecular species.

Experimental

Reagents and standards

Depending on the solubility of each compound, individual pesticide stock solutions (approximately 1,000 µg/mL) were prepared in pure acetonitrile or methanol, and stored at –18 °C. Reagents were used and obtained as described [3].

The five pesticides were chosen for spiking based on their isobaric protonated masses (all had a nominal mass of *m/z* 314), relatively common usage, and the similar chromatographic characteristics for two of the compounds [3]. The five pesticides used were: hexaconazole, isazophos, isoxathion, kresoxim-methyl, and triazophos (Table 1). All pesticide standards were purchased from AccuStandard.

Table 1. The Five Isobaric Pesticides: Accurate Masses and Intensities of Their Main Adduct Ions

			$[M+H]^+$		$[M+Na]^+$		Isotope presence	
Pesticide	**Formula**	**RT*(min)**	**Mass**	**% Intensity**	**Mass**	**% Intensity**	**^{37}Cl**	**^{34}S**
Hexaconazole	$C_{14}H_{17}Cl_2N_3O$	23.4	314.0821	100	–	0	Yes	No
Triazophos	$C_{12}H_{16}N_3O_3PS$	23.9	314.0723	100	336.0542	10.5	No	Yes
Isazophos	$C_9H_{17}ClN_3O_3PS$	25.0	314.0490	100	336.0309	2.3	Yes	Yes
Kresoxim-methyl	$C_{18}H_{19}NO_4$	25.0	314.1387	2.2	336.1206	100	No	No
Isoxathion	$C_{13}H_{16}NO_4PS$	26.5	314.0723	100	336.0542	13.8	No	Yes

Instruments

This study was conducted using an Agilent 1290 Infinity LC System coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate-Mass Q-TOF LC/MS System equipped with electrospray Jet Stream Technology. Three columns were used:

- Agilent ZORBAX XDB C-8 reverse phase (4.6 mm × 150 mm, 3.5 µm)
- Agilent ZORBAX C-18 reverse phase column (2.1 mm × 100 mm, 1.8 µm)
- Agilent Phenyl column (2.1 mm × 50 mm, 1.8 µm).

The instrument run conditions are shown in Table 2.

Table 2. LC and Q-TOF MS Run Conditions

LC run conditions	
Column	Agilent ZORBAX XDB-C8 Reversed-Phase, 4.6 × 150 mm, 3.5 µm (963967-906) Agilent ZORBAX Eclipse XDB-C18, 2.1 × 100 mm, 1.8 µm (928700-902) Agilent SB-Phenyl, 2.1 × 50 mm, 1.8 µm (827700-912)
Column temperature	250 °C
Injection volume	20 µL
Mobile phase	A) 0.1% formic acid in water v/v B) Acetonitrile
Linear gradient	10% B for 5 minutes, then 10% B to 100% B over 25 minutes at constant flow, hold at 100% B for 10 minutes
Flow rate	0.6 mL/min
Q-TOF MS conditions	
Ion mode	ESI, positive ion mode
Nebulizer pressure	45 psig
Drying gas flow rate	10 L/min
Drying gas temperature	250 °C
Sheath gas flow rate	11 L/min
Sheath gas temperature	350 °C
Nozzle voltage	0 V in positive ion mode
Fragmentor voltage	190 V
Capillary voltage	3,500 V
Skimmer voltage	65 V
Octopole RF	750 V
Mass range	50–1,000 *m/z*
Detector rate	2 GHz
Resolving power	35,000 ± 500
Accuracy	≤ 2 ppm

Sample preparation

A methanol/water extraction (80:20) of 3 g of *C. annuum* was performed as described [3]. All extracts were shown to be free of pesticide before spiking experiments, using the method described herein.

Results and Discussion

Ion characteristics of the five pesticides

Individual standards of each of the five pesticides in solvent were injected to determine their ion response factors and relative intensities (Table 1). The MH^+ ion was the most intense ion for hexaconazole, isazophos, isoxathion, and triazophos, with a 100% relative response factor. Kresoxim-methyl gave only a weak response for the MH^+ ion (2.2%).

Sodium adducts can complicate pesticide identification by electrospray and LC/MS/MS because of lack of fragmentation. For example, the sodium adduct for kresoxim-methyl (with a methylimino acetate moiety) gave a sodium adduct at a 100% relative response. The three organophosphates gave low sodium adduct intensities (Table 1), and only hexaconazole, which is not an organophosphate, did not form a sodium adduct. Therefore, the differences in ion formation for the five pesticides provide a means of differentiating them using both *m/z* 314 (MH^+) and 336 [$M+Na^+$] ions.

Separation by high-resolution chromatography

The pepper matrix is a challenging example for high-resolution chromatography because it is known to contain thousands of natural compounds that easily extract using methanol. Figure 1 shows a portion of an extracted ion chromatogram (EIC) at *m/z* 314 and 336 of all five compounds spiked into the pepper matrix, using a C-8 (3.5-µm particle size) column. The *m/z* 314 ion (upper trace) reveals four large peaks, and the *m/z* 336 ion (lower trace) shows three peaks. This pattern may be the result of coelution of two of the isobaric peaks, or the failure of one of the five pesticides to ionize. However, based on the accurate masses and adduct formation of these compounds (Table 1) as well as the injection of individual standards, it appears that kresoxim-methyl coelutes with isazophos at 25 minutes on the chromatogram. Since it essentially does not form the MH^+ ion (Table 1), kresoxim-methyl appears only in the lower *m/z* 336 trace for the sodium adduct.

Separation by high-resolution mass spectrometry

Because none of the compounds are isomeric, separation by Q-TOF MS is theoretically possible, based on the slight differences in their exact masses. Using the standard theoretical calculation for the minimum resolving power needed to differentiate isobars, it was determined that a minimum mass spectrometric resolving power of ~26,000 would be required to completely separate isazophos and kresoxim-methyl by accurate mass. The 6540 UHD Accurate-Mass Q-TOF LC/MS instrument used in this study was operated at a mass resolving power of 26,500 at *m/z* 300.

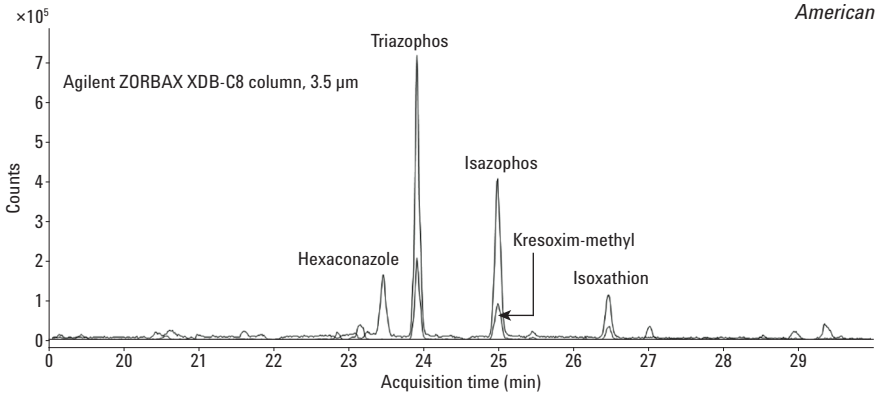

Figure 1. Extracted ion chromatogram (EIC) of five pesticides spiked into a pepper matrix, at m/z 314 (upper trace) and 336 (lower trace). Four of the five pesticides were separated using this Agilent ZORBAX XDB-C8 column with 3.5 µm packing (Reprinted with permission from J. Agric. Food Chem. 61, 2340-2347 (2013). Copyright 2014 American Chemical Society).

Closely examining the EIC for the coeluting compounds, isazophos and kresoxim-methyl, at 25 minutes reveals two *m/z* 336 ions at *m/z* 336.0312 and 336.1210 ($M+Na^+$) with isotopic signatures at *m/z* 337.0339 and 337.1243 and *m/z* 338.0284 and 338.1274 (Figure 2). These isotopic signatures at A+1 and A+2 reveal the presence of chlorine in the first [$M+Na^+$] ion at *m/z* 336.0312 (corresponding to isazophos, Table 1) , and the absence of chlorine in the [$M+Na^+$] ion at *m/z* 336.1210 (corresponding to kresoxim-methyl, Table 1), which fits the chromatographic evidence for coelution for isazophos and kresoxim-methyl. These data provide evidence that it is possible to distinguish two coeluting isobars using high resolution mass spectrometry with the 6540 UHD Accurate-Mass Q-TOF LC/MS instrument operated at a mass resolving power of 26,500 at *m/z* 300.

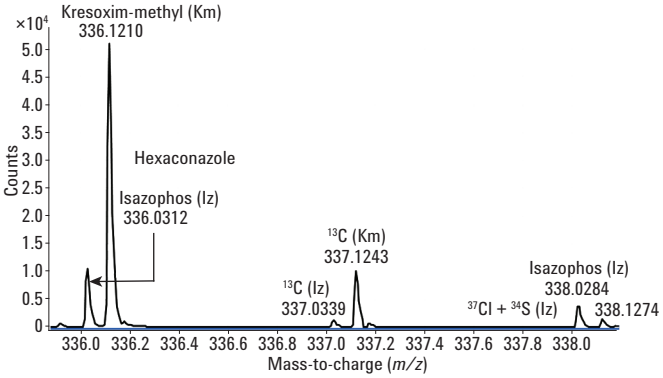

Figure 2. Expanded mass spectrum of the nominal m/z 336 ions of kresoxim-methyl and isazophos, which coelute at 25.0 minutes in the chromatogram shown in Figure 1 (Reprinted with permission from J. Agric. Food Chem. 61, 2340-2347 (2013). Copyright 2014 American Chemical Society).

High-resolution MS/MS analysis of coeluting pesticides

Using MS/MS to confirm the identity of the isazophos ion at *m/z* 314 resulted in the appropriate fragment ions for this structure, and they matched the retention time and mass spectrum for a pure standard.

However, MS/MS analysis of the *m/z* 336 ion, the sodium adduct for both isazophos and kresoxim-methyl, may generate interferences if fragmentation occurs for both compounds.

The results of such an analysis are shown in Figure 3. The two major fragment ions are *m/z* 246.0885, fitting the putative structure for a fragment of kresoxim-methyl, and *m/z* 184.0240, which fits the putative structure for a fragment of isazophos. It is not possible to separate the MS/MS accurate mass fragments of the two compounds because the collision cell isolates the nominal mass of *m/z* 336 for both compounds. As a result, MS/MS analysis for confirmation of the *m/z* 336 sodium adduct of isazophos and kresoxim-methyl requires high-resolution chromatography.

Separation by high-resolution chromatography with MS/MS

After experimenting with various chromatographic gradients and the ZORBAX Eclipse C18 column, which should provide greater retention and efficiency, the two coeluting compounds could not be separated. However, a phenyl column was used with the same initial gradient to successfully separate isazophos and kresoxim-methyl by more than a minute with aromatic interaction, rather than hydrophobic interaction. Performing MS/MS experiments on these resolved ions can then provide the correct spectra for each of the five pesticides in the pepper extract. Changing the selectivity of the HPLC column to augment the separation power of the mass spectrometer can thus provide optimal separation and identification of isobaric and/or isomeric compounds.

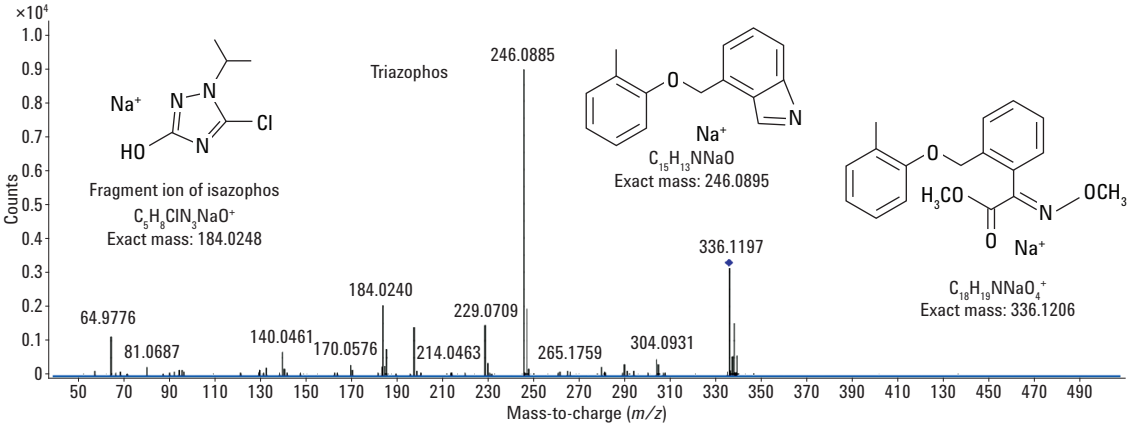

Figure 3. Accurate mass MS/MS spectrum of the m/z 336 ion at a retention time of 25 minutes, from the EIC in Figure 1. The mass spectrum shows masses consistent with both isazophos and kresoxim-methyl (Reprinted with permission from J Agric Food Chem. 61, 2340-2347 (2013). Copyright 2014 American Chemical Society).

High-resolution analysis of pepper matrix

To determine the complexity of the pepper matrix, an accurate mass extraction of all ions above the baseline of 10,000 counts was carried out. This analysis of the pesticide-spiked pepper matrix used an Agilent software feature called Molecular Feature Extractor. All related adducts (proton, sodium, and ammonium) and their related isotopic patterns were grouped into individual extracted ions and displayed as chromatographic peaks. When the pepper matrix was extracted using this tool, 4,235 individual molecular features with ion intensities of 10,000 counts or more were found.

The complexity of a matrix such as pepper can seem overwhelming. However, the high resolution of the Q-TOF mass spectrometer provides the ability to sort through and clearly distinguish the five isobaric pesticides from this matrix. Using a mass window as narrow as ± 0.005 mass units makes this possible. The mass defect, which is the difference between the accurate mass and the nominal mass of an ion, is shifted closer to the nominal mass for the pesticides than it is for the matrix. This is due to the presence of elements such as sulfur and chlorine in the pesticides, whereas most matrix compounds are rich in hydrogen. Using a mass window of ± 0.005 to search for the *m/z* 336 ion characteristic of the sodium adduct for isazophos and kresoxim-methyl, for example, eliminates all of the 4,235 molecular features except for the one specific to these pesticides (Figure 4). This ability to separate the two diagnostic ions for these pesticides (*m/z* 314 and 336) from the more than 4,000 molecular features in pepper is the true value of high-resolution mass spectrometry for the analysis of isobaric pesticides in such complex matrices.

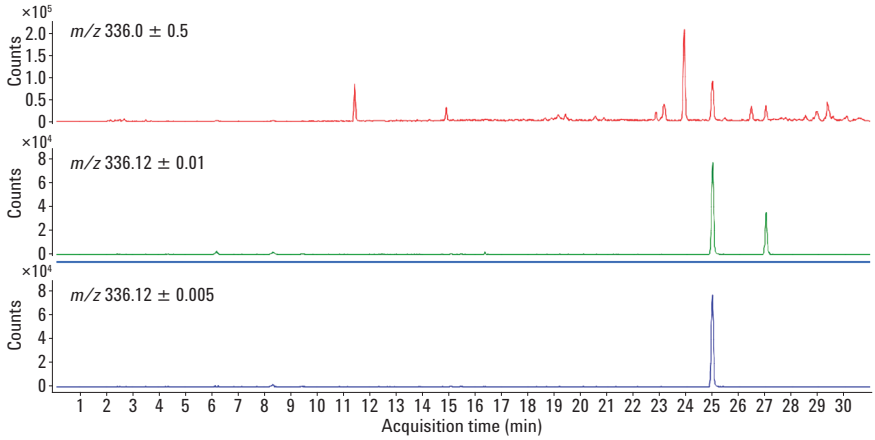

Figure 4. Extracted ion chromatogram at m/z 336 with windows of extraction of ± 0.5, ± 0.01, and ± 0.005 mass units. The extracted ions decrease from approximately 10 features (± 0.5) to one feature (± 0.005) by narrowing the window of extraction, because the mass defect of the compounds in red pepper is larger than 0.12 ± 0.005 (Reprinted with permission from J. Agric. Food Chem. 61, 2340-2347 (2013). Copyright 2014 American Chemical Society).

Conclusions

High-resolution chromatography is valuable for the separation of isobaric pesticides and possible isomers for MS/MS analysis, particularly when the isobars may have interfering precursor and fragment ions. While high-resolution mass spectrometry has the ability to separate closely related isobars, it is also valuable for distinguishing the isobaric compounds from the thousands of compounds present in a complex matrix such as pepper. These two techniques are therefore truly complementary and essential for the analysis of isobaric pesticides in complex matrices.

References

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2. I. Ferrer and E.M. Thurman "Multi-residue method for the analysis of 101 pesticides and their degradates in food and water samples by liquid chromatography/time-of-flight mass spectrometry" *J. Chromatogr. A*. **1175**, 24-37 (2007).
3. E. M. Thurman, *et al*. "Analysis of isobaric pesticides in pepper with high-resolution liquid chromatography and mass spectrometry: complementary or redundant?" *J. Agric Food Chem*. **61**, 2340-2347 (2013).

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