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Analysis of Isobaric Pesticides in Pepper with High-Resolution Liquid Chromatography and Mass Spectrometry: Complementary or Redundant?

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ABSTRACT: Five isobaric pesticides are analyzed in red pepper (*Capsicum annuum*) by high-resolution chromatography (100,000 theoretical plates/meter) and high-resolution mass spectrometry (resolving power > 25000) to test whether these methods are redundant or complementary when using MS/MS analysis. The five compounds are hexaconazole, isazophos, isoxathion, kresoxim-methyl, and triazophos, with an isobaric mass of m/z 314 and 336. Red pepper was chosen as a complex vegetable matrix with more than 4000 adducted ions (MH^+ and MNa^+). High-resolution chromatography was found to be a valuable tool to separate the isobaric pesticides from one another, whereas the high resolution of the mass spectrometer separated the matrix ions of red pepper easily from the pesticides due to differences in their mass defect. The combination of techniques is especially valuable in MS/MS analysis because of interfering precursor and fragment ions of the isobaric pesticides rather than the complex pepper matrix—a nonintuitive result.

KEYWORDS: *high resolution, mass spectrometry, UHPLC, isobaric pesticides, pepper*

INTRODUCTION

The correct analysis of pesticides in food is especially important these days because of the exportation of food on a worldwide basis,[1−3] and mass spectrometry plays an important role because of the reliability of this method of detection.[3−9] The problem of false negatives, or missing important pesticides in food, remains an issue because complex matrices may mask the detection of some pesticides. Both high-resolution chromatography and high-resolution mass spectrometry are tools to help with false negatives.[3,10−12]

This problem is especially important because of the common occurrence of fungicides and insecticides in foods, such as lettuce, tomato, and pepper. For example, Ferrer et al.[10] found that bell pepper contained more than 2500 natural compounds as determined by liquid chromatography−mass spectrometry using time-of-flight and accurate-mass analysis. Furthermore, pesticides, such as carbendazim, imazalil, and malathion, have commonly been reported in lettuce, tomato, and pepper.[10−13] Examples of isobaric mixtures presenting problems for analysis include the works of Nielen et al.,[14] Crowley et al.,[15] and Thurman et al.[16] Isobars refer to a compound with the same nominal mass but not the same molecular formula, whereas an isomer has the same molecular formula. The most challenging of these reports was by Neilen et al.,[14] who showed that a resolving power of 25000 was required to separate the ion fragments of a banned anabolic steroid, stanazolol, in meat and meat products,[17] where its major marker is the hydroxy metabolite.[17−19] This work was carried out using Orbitrap mass spectrometry at a resolving power of 50000. A similar example was completed by LC-Q-TOF/MS showing that even low mass ions, that is, m/z <100, can be accurately determined for banned veterinary medicines.[16] Thus, there are a number of examples that point to the importance of high-resolution mass spectrometry for the analysis of pesticides and other banned substances in foods and food products. In particular, the question was asked,[16] "How much mass spectrometry resolving power is enough?" This paper focused on the use of MS/MS analysis with high-resolution time-of-flight mass spectrometry of small molecules, with molecular weights <500. Thurman et al.[16] found that when the chromatography is not complex, that is, no interfering compounds, a resolving power of 25000 was typically more than sufficient for MS/MS analysis of a banned anabolic steroid, stanazolol.[16]

Another example of the importance of high-resolution chromatography, as well as accurate mass spectrometry, is the work of Ferrer et al.[20] on isobaric and isomeric pharmaceuticals. They noted that even when mass resolving power was >100,000, there are compounds that will not resolve. These are isomeric compounds with the same formula; therefore, they have the same accurate mass. Sixteen sets of isobaric compounds were found, which was 32% of the analytes examined (100 common pharmaceuticals) with a total of 6% with isomeric compounds (identical accurate mass). In these cases, the use of high-resolution chromatography and MS/MS analysis was needed for identification. Marshall[21] has pointed out that, in fact, because of the difference in chemical bond masses, even isomers will have a different accurate mass, but the masses would differ at the eighth decimal point position or beyond, which would require a resolving power of $>10^{10}$. Currently the most powerful Fourier transform mass spectrometers are capable of a resolving power of

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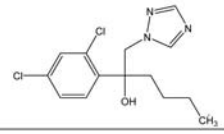
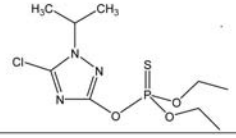
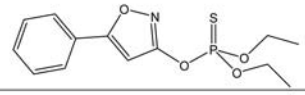
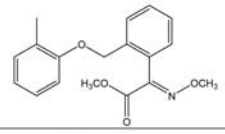
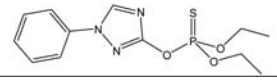
Table 1. Compounds Studied and Accurate Masses of Main Adduct Ions

Name	Elemental Composition	Ret. Time (min.)	$[M+H]^+$	$[M+Na]^+$	Chemical Structure
Hexaconazole	$C_{14}H_{17}Cl_2N_3O$	23.4	314.0821	--	
Isazophos	$C_9H_{17}ClN_3O_3PS$	25.0	314.0490	336.0309	
Isoxathion	$C_{13}H_{16}NO_4PS$	26.5	314.0610	336.0430	
Kresoxim-methyl	$C_{18}H_{19}NO_4$	25.0	314.1387	336.1206	
Triazophos	$C_{12}H_{16}N_3O_3PS$	23.9	314.0723	336.0542	

10^6. Thus, it is not likely that isomers will soon be resolved by accurate mass of the protonated molecule.

Thus, the importance of both MS/MS analysis and the separation ability of chromatography may be needed, which has prompted the research in this paper exploring the redundancy or complementary nature of both high-resolution chromatography and mass spectrometry. However, recent developments in mass spectrometry have favored the use of mass resolving power with little or no discussion of chromatography, which is chiefly noted in the advertisements of mass spectrometry instrumentation. The development of the Orbitrap mass spectrometer in 2005[22] has sparked a series of new instruments with extended flight paths, for both gas and liquid chromatography. The result is that there is now non-Fourier transform mass spectrometric instrumentation with >100,000 resolving power,[23] and several time-of-flight instruments with >50,000 resolving power.[23−25]

Although chromatographic resolution may be taken for granted today, the advances in high-resolution liquid chromatography occurred only 10 years ago. The advances began with ultrahigh-performance chromatography and the development of 1.5 μm packing material and pumps that operate at pressures up to 65000 psi.[26] These two advances allowed the commercial development of a series of sub-2-μm chromatographic columns with >100,000 theoretical plates per meter and ultrahigh-pressure liquid chromatography pumps that run routinely above 10,000 psi.

Finally, we set out to find examples that show when both types of high resolving power, both chromatographic and mass spectrometric, are needed. These questions are addressed with a separation problem involving five isobaric pesticides of mass m/z 314 (MH^+), some of which are known to coelute,[15,27] spiked into a complex red pepper (*Capsicum annuum*) matrix of over 2000 molecular species. The significance of this work will show that ultrahigh-performance liquid chromatography (UHPLC) is most important for the separation of the isobaric pesticides from one another, whereas high-resolution mass spectrometry was most useful for the separation of the matrix compounds from the pesticides when using MS/MS analysis. This distinction, although nonintuitive, is quite important when pesticides are analyzed in vegetable matrices, and distinguishes accurate mass from other mass spectrometry techniques.

MATERIALS AND METHODS

Chemicals and Reagents. Individual pesticide stock solutions (approximately 1000 μg/mL) were prepared in pure acetonitrile or methanol, depending on the solubility of each individual compound, and stored at −18 °C. From these solutions, working standard solutions were prepared by dilution with acetonitrile and water. HPLC grade acetonitrile, methanol, and water were obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Samples and Spiking. Three grams of red pepper (*C. annuum*) from a local grocery was extracted with methanol/water (80:20) after grinding in a mortar and pestle. Ten milliliters of solvent was mixed with the pepper in the mortar, and careful grinding was used to extract the food matrix. The extract was further analyzed to be free of pesticide using the method herein. All extracts were free of pesticide before spiking experiments. Next, the 10 mL of food extract was transferred quantitatively to a screw-top vial and centrifuged for 15 min at 3500 rpm. The liquid layer was carefully transferred to a baked clean vial and filtered through PTFE 0.2 μm filters to use as matrix for all of the experiments. The pepper extracts were a red color.

The five pesticides chosen for spiking were based on their relatively common usage, their isobaric protonated masses (all were nominal mass of m/z 314), and, in some cases, similar chromatographic characteristics as given in the published literature.[15,27] The compounds included hexaconazole, isazophos, isoxathion, kresoxim-methyl, and triazophos (Table 1). All standards were purchased from Accu-Standards (Philadelphia, PA, USA).

UHPLC System. The ultrahigh-performance liquid chromatograph consisted of an Agilent model 1290 pump, autosampler, and column compartment (Agilent Technologies, Inc., Santa Clara, CA, USA) with three columns, a Zorbax C-8 reverse phase column (1.8 and 3.5 μm × 4.6 mm × 150 mm) having 120,000 theoretical plates per meter, a Zorbax C-18 reverse phase column (1.8 μm × 4.6 mm × 150 mm), and a phenyl column (1.8 μm × 4.6 mm × 150 mm) having 110,000 theoretical plates per meter (all columns were Agilent Technologies, Inc., Santa Clara, CA, USA). The mobile phases and gradients for all columns were the same. Beginning with 90% water/0.1% formic acid (A) and 10% acetonitrile (B), the gradient was held for 5 min, and then

over 25 min the mobile phase changed to 100% B at a constant rate and held for 10 min before returning to starting conditions. The flow rate was 0.6 mL/min. This is a gradient that we have used for many applications of pesticides in food, and we have found it to be a useful to reach high peak capacities for large pesticide mixtures.[28]

LC-Q-TOF-MS Analysis. The UHPLC system was connected to an ultrahigh-definition (resolution) quadrupole time-of-flight mass spectrometer model 6540 Agilent (Agilent Technologies, Inc.) equipped with electrospray Jet Stream Technology, operating in positive ion mode, using the following operation parameters: capillary voltage:, 3500 V; nebulizer pressure, 45 psig; drying gas, 10 L/min; gas temperature, 250 °C; sheath gas flow, 11 L/min; sheath gas temperature, 350 C; nozzle voltage, 0 V in positive ion mode; fragmentor voltage, 190 V; skimmer voltage, 65 V; octopole RF, 750 V. LC-MS accurate mass spectra were recorded across the range m/z 50−1000 at 2 GHz. The data recorded were processed with MassHunter software. Accurate mass measurements of each peak from the extracted ion chromatograms were obtained by means of a calibrant solution delivered by an external quaternary pump. This solution contains the internal reference masses (purine ($C_5H_4N_4$ at m/z 121.0509 and HP-921 [hexakis(1*H*,1*H*,3*H*-tetrafluoropentoxy)phosphazene] ($C_{18}H_{18}O_6N_3P_3F_{24}$)) at m/z 922.0098. The instrument provides a mass resolving power of 35000 ± 500 (m/z 1522). Stability of mass accuracy was daily checked, and if values went above 2 ppm error, then the instrument was recalibrated. The instrument was operated in single MS with full spectra, except on those cases when MS-MS was necessary to discriminate isobars. The isolation width was set at medium (m/z ~4), and collision energies of 10, 20, and 40 eV were used for MS/MS experiments.

RESULTS AND DISCUSSION

Separation by High-Resolution Chromatography. Table 2 shows the results of injecting individual standards in solvent of each of the five pesticides to determine their response factors and relative intensities. Hexaconazole, isazophos, isoxathion, and triazophos all gave the MH^+ as the most intense ion with a 100% relative response factor. Kresoxim-methyl gave the sodium adduct as the most intense ion and a very weak response for the MH^+ (2.2%). The sodium adduct formed for four of the five pesticides, with no response for hexaconazole. The structure of hexaconazole does not contain either the carbonyl or the thiocarbonyl structure, which appears to be important in stabilizing the sodium adduct of these pesticides. Organophosphate pesticides are known to form sodium adducts, which can complicate their identification by electrospray and LC-MS/MS because of lack of fragmentation.[28] The data in Table 2 show that the three organophosphates gave sodium adduct intensities of 2.3−13.8%, and only kresoxim-methyl had a large intensity factor of 100%. Thus, both m/z 314 and 336 were used for monitoring the five pesticides in the pepper matrix.

Table 2. Ion Adduct Intensity and Isotope Information for All Compounds Studied

compound	intensity of $[M + H]^+$	intensity of $[M + Na]^+$	presence of Cl-37 isotope	presence of S-34 isotope
hexaconazole	100	0	yes	no
isazophos	100	2.3	yes	yes
isoxathion	100	13.8	no	yes
kresoxim-methyl	2.2	100	no	no
triazophos	100	10.5	no	yes

Figure 1 shows an extracted ion chromatogram at m/z 314 and 336 for a zoomed area of a generic chromatogram using a C-8 and 1.8 μm column with all five compounds spiked into the pepper matrix. Pepper is known to have thousands of natural compounds that extract readily using methanol as an extractant;[10] thus, the pepper matrix is a challenging example for high-resolution chromatography. Figure 1 shows four large peaks for the m/z 314 trace and three peaks for the m/z 336 trace. The upper trace is the m/z 314 ion. These two traces indicate that there may be coelution of two of the isobaric peaks or, alternatively, one of the five pesticides does not ionize. On the basis of the accurate masses and adduct formation shown in Tables 1 and 2 and the injection of individual standards, it appears that kresoxim-methyl is coeluting with isazophos and not forming the MH^+ ion.

To confirm this hypothesis the individual standards were injected in solvent, and the retention time data are shown in Table 1 (see retention times of 25.0 min for isazophos and kresoxim-methyl in solvent). This initial separation was carried out with a 3.5 μm C-8 column with 75000 theoretical plates. The column is a compromise between the high chromatographic resolution with lowest backpressures at reasonable flow rates of 0.6 mL/min. Next, several different gradients were tried without success for separation. Also, a smaller particle size, 1.8 μm packing, was tried with 100,000 theoretical plates per meter. Again, there was no separation of the two isobars, isazophos and kresoxim-methyl. Thus, using the reverse phase C-8 column was not effective to separate these two pesticides, and another chromatographic approach will be tested in a later section.

Separation by High-Resolution Mass Spectrometry. Table 1 shows the structure of the five isobaric compounds that differ by 0.0120−0.0700 mass units. None of the compounds are isomeric (with the same accurate mass); however, they will require a maximum mass spectrometric resolving power of ~26000 to have complete separation by accurate mass with high resolution on the basis of their closest mass differences, as shown in the following calculations. A resolving power of 26000 is based on the smallest mass difference of 0.012 and a nominal mass of 314 (i.e., 314 divided by 0.012 yields resolving power at 50% separation at half-height of 26167 for what is called full width at half-maximum (fwhm)). For complete baseline separation of the two closest masses it requires twice fwhm or ~50000 resolving power.[3] This calculation presumes that the two compounds with the nearest mass difference should coelute and be of equal intensity. The instrument used in this study was operating at a mass resolving power of 26500 at a mass of m/z 300, which should be adequate to separate these five pesticides at fwhm.

A closer look at the mass spectrum for the coelution of isazophos and kresoxim-methyl at 25 min (Figure 2) shows that there are two m/z 336 ions at m/z 336.0312 and 336.1210 (MNa^+) with isotopic signatures at m/z 337.0339 and 337.1243 and m/z 338.0284 and 338.1274. These isotopic signatures at A +1 and A+2 show that chlorine is present in the first MNa^+ ion at m/z 336.0312 (corresponding to isazophos, Table 2) and not present in the MNa^+ ion at m/z 336.1210 (corresponding to kresoxim-methyl, Table 2), which fits the hypothesis of coelution for isazophos and kresoxim-methyl. Thus, it is possible to distinguish isazophos and kresoxim-methyl as their sodium adducts by high-resolution mass spectrometry. If kresoxim-methyl had formed a MH^+ at m/z 314, theoretically, they would have also separated. These calculations apply for accurate mass measurements but, as the next section will attempt to show, there are mass interferences when an MS/MS experiment is performed because nominal mass is used for the isolation of the precursor ion, in this case for either m/z 314 or 336.

High-Resolution MS/MS Analysis of Coeluting Pesticides. To confirm that the protonated molecule of isazophos in

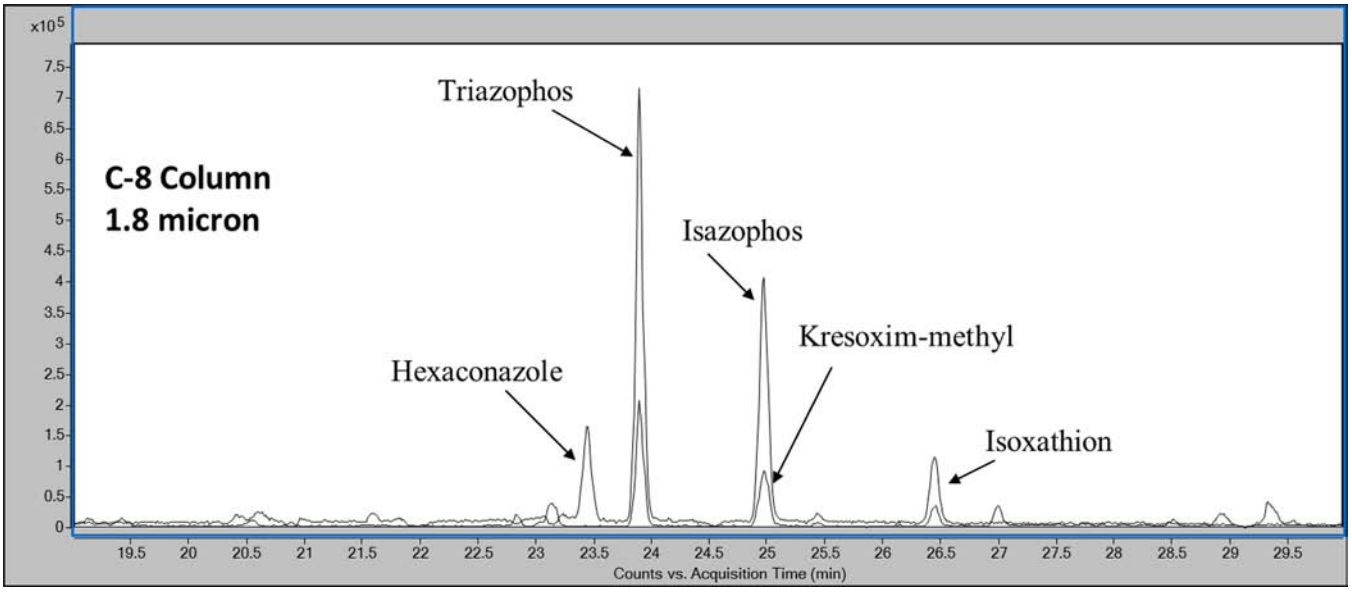


Figure 1. Extracted ion chromatogram, m/z 314 and 336, for spiking of five pesticides into a pepper matrix. Chromatogram shows separation of four of the five pesticides using a C-8 column with 1.8 μm packing.

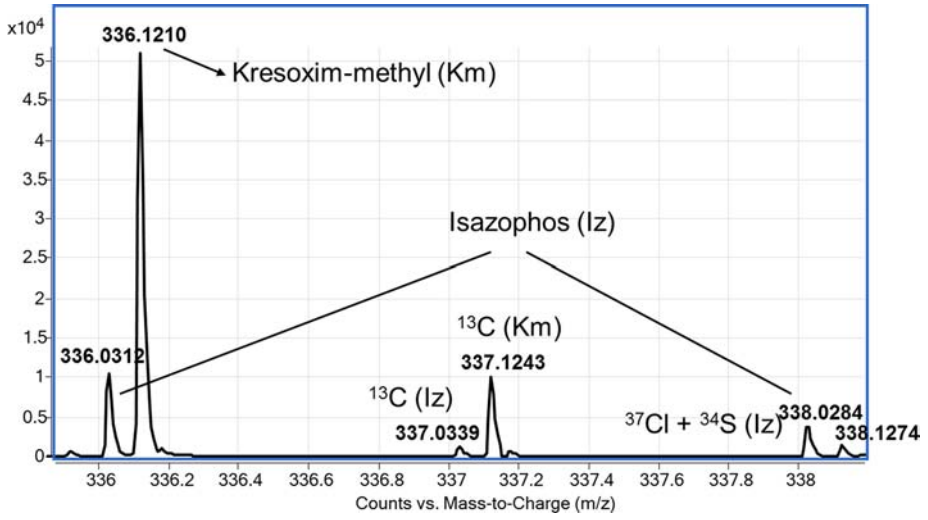


Figure 2. Zoomed mass spectrum of the m/z 336 ions of kresoxim-methyl and isazophos, which coelute at 25.0 min in the chromatogram shown in Figure 1.

pepper matrix is the mass at m/z 314.0490, an MS/MS experiment was carried out, which is shown in Figure 3. There were major fragment ions at m/z 272.0017, 243.9702, 215.9390, 162.0426, 119.9957, 96.9507, and 78.9402. Putative structures are drawn for each of these accurate mass fragment ions. The losses proceed initially by incremental losses of 42, 29, and 28 u, which is consistent with alkyl losses from the structure of isazophos. The base peak ion at m/z 119.9557 is consistent with the putative structure of the chlorinated triazine ring fragment, and the ions at m/z 96.9507 and 78.9402 are consistent with the thiophosphate structure of isazophos. Thus, the ions associated with the fragmentation at nominal mass of m/z 314 fit the correct structure for isazophos and match the correct retention time and mass spectrum for a pure standard (data not shown). Even though kresoxim-methyl coelutes with isazophos, because it does not form a MH^+ ion or is very weak (~2% ion intensity shown in Table 2), it does not interfere with the MS/MS of the isazophos at m/z 314.

However, an MS/MS experiment of the m/z 336, which is the sodium adduct for both izasophos and kresoxim-methyl, may have interferences if fragmentation occurs for both compounds. The MS/MS of m/z 336 is shown in Figure 4. The two major fragment ions are m/z 246.0885, which fits the putative structure for a fragment of kresoxim-methyl, and the ion at m/z 184.0240, which fits the putative structure for a fragment of izasophos.

Thus, despite a resolving power capable of separating the sodium adducts of izasophos and kresoxim-methyl, 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. This problem exists for all MS/MS instruments currently being used with liquid chromatography and MS/MS analysis. Thus, high-resolution chromatography is needed when doing MS/MS analysis for confirmation of the m/z 336 sodium adduct of isazophos and kresoxim-methyl.

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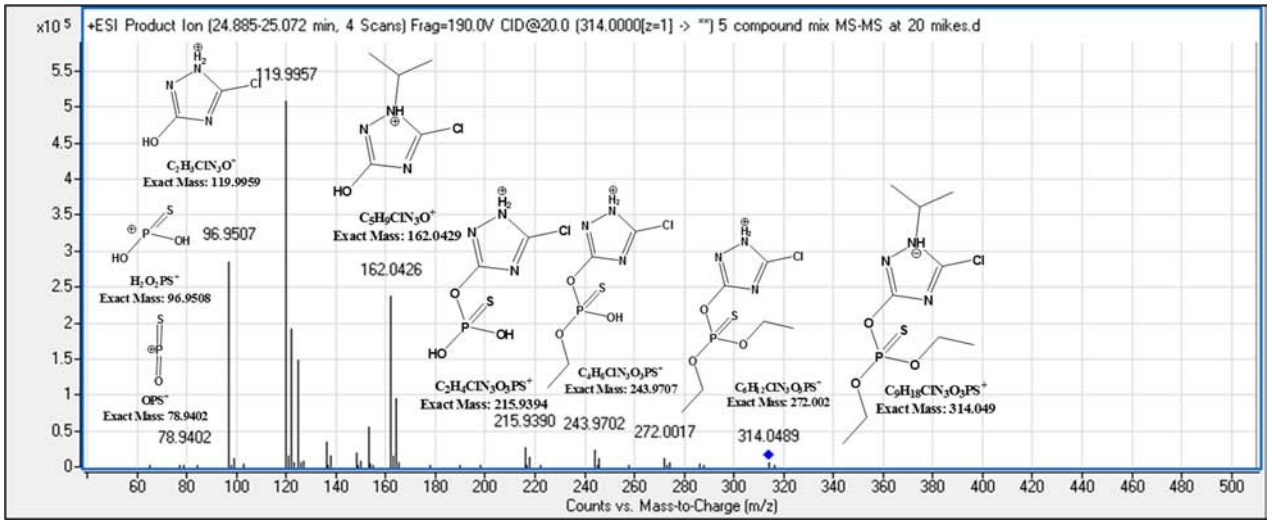

Figure 3. Accurate mass MS/MS spectrum of the m/z 314 ion at a retention time of 25 min from Figure 1. Mass spectrum shows masses consistent with isazophos.

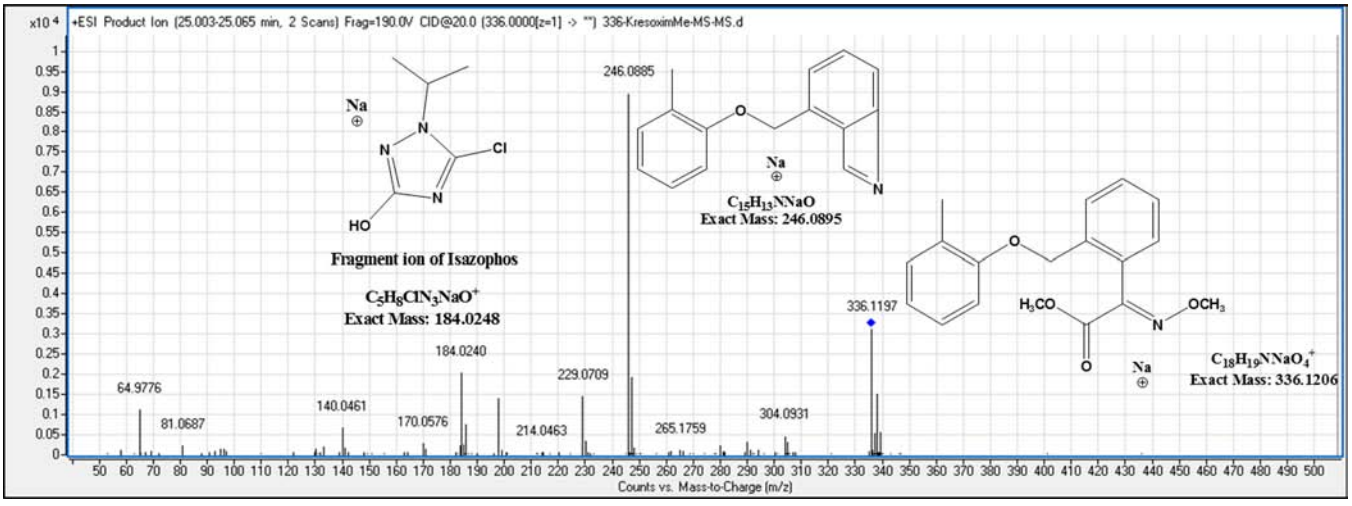

Figure 4. Accurate mass MS/MS spectrum of the m/z 336 ion at a retention time of 25 min from Figure 1. Mass spectrum shows masses consistent with both isazophos and kresoxim-methyl.

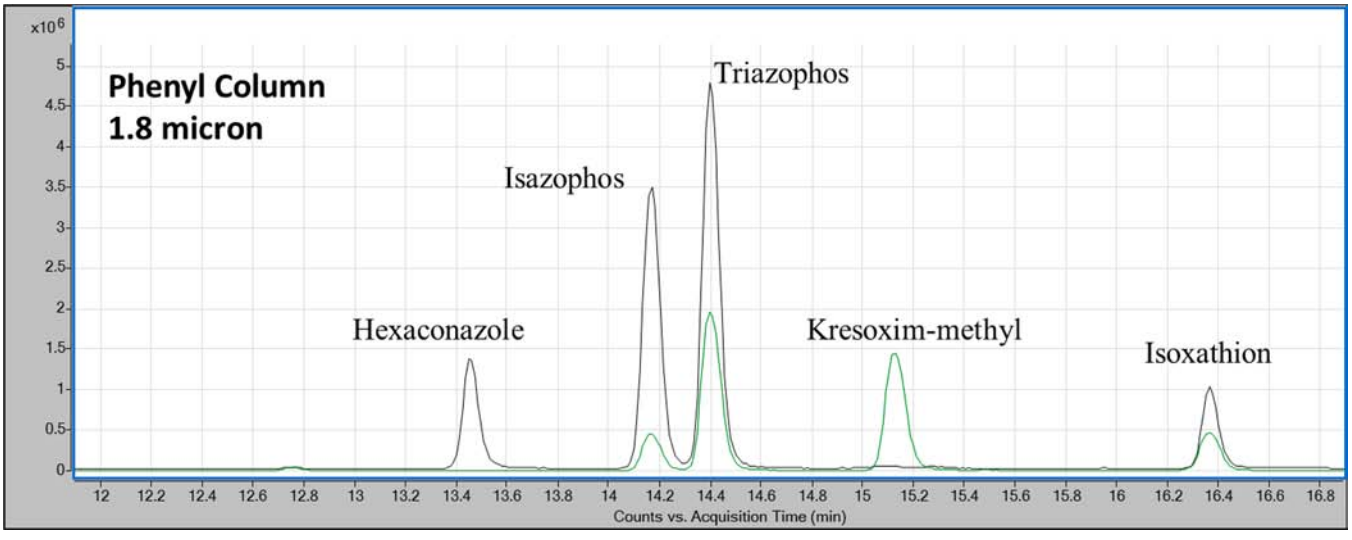

Figure 5. Extracted ion chromatogram, m/z 314 and 336, for spiking of five pesticides into a pepper matrix. Chromatogram shows separation of all five pesticides using a phenyl column with 1.8 μm packing.

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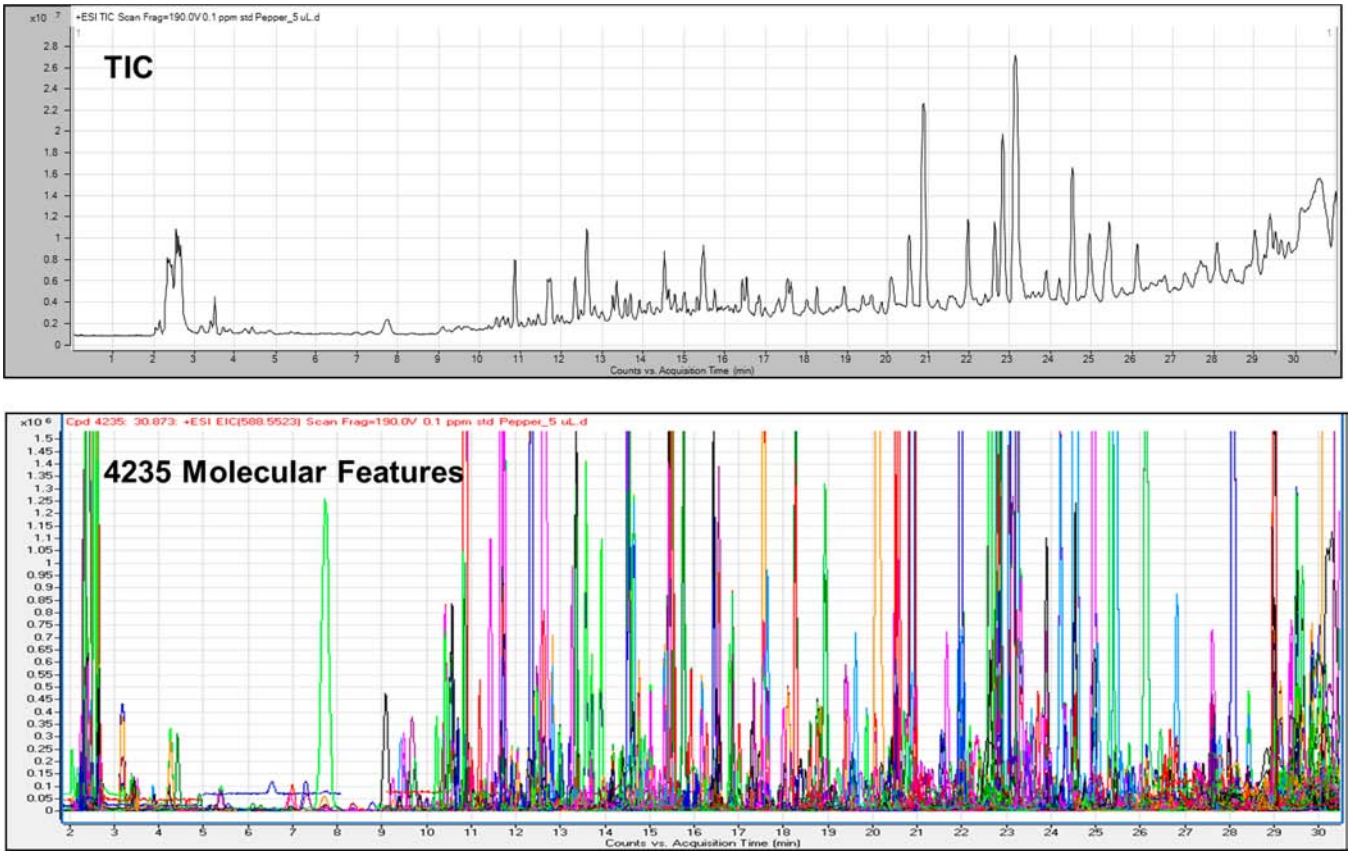

Figure 6. (Top) Total ion chromatogram (TIC) with accurate mass for the pesticide-spiked pepper matrix. (Bottom) Extracted molecular features for the spiked pepper matrix with 4235 molecular features.

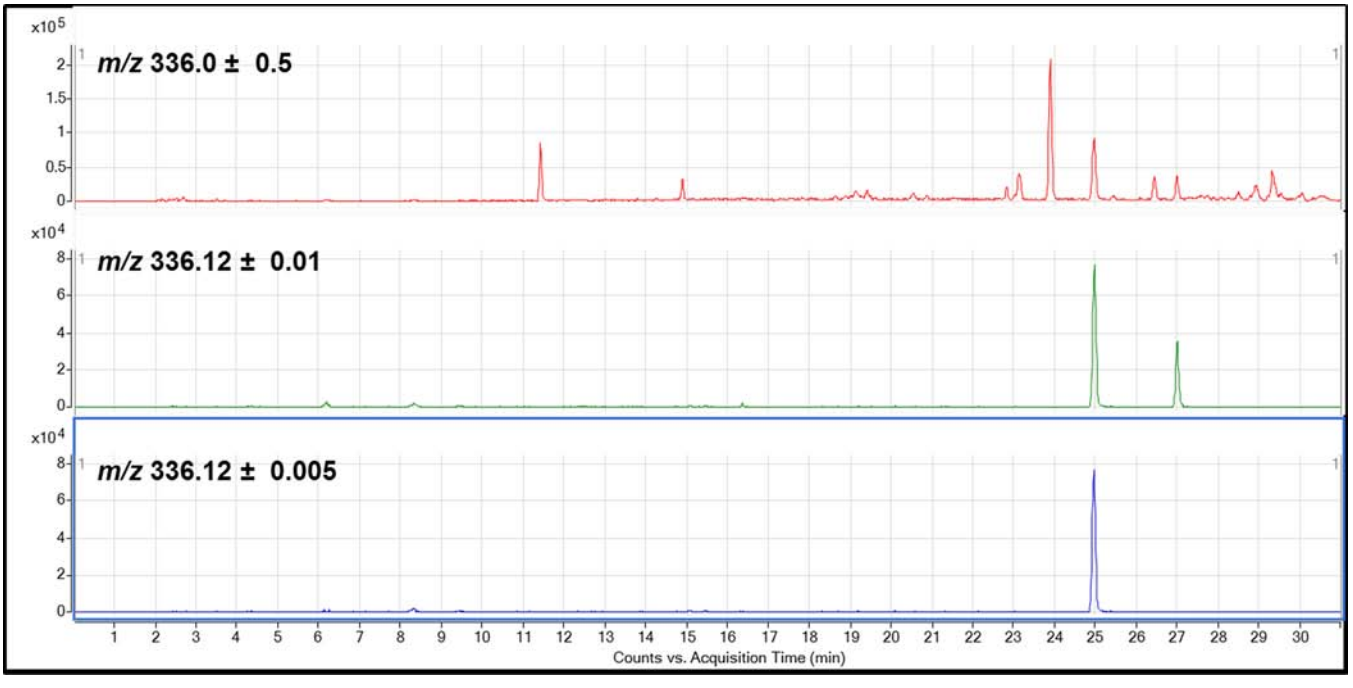

Figure 7. Extracted ion chromatogram at m/z 336 with windows of extraction of ±0.5, ±0.01, and ±0.005. The extracted ions decrease from approximately 10 features to one feature by narrowing of the window of extraction. The features decreased from 10 to 1 because the mass defect of the red pepper is larger than 0.12 ± 0.005.

Separation by High-Resolution Chromatography with MS/MS. To separate kresoxim-methyl from isazophos, several chromatographic gradients were tried. Earlier attempts with various solvent conditions using both C-8 and C-18 columns were unsuccessful, as explained earlier. Thus, selectivity was changed from hydrophobic interaction to a phenyl or aromatic

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interaction. Figure 5 shows the extracted ion chromatogram for the phenyl column at m/z 314 (MH^+, red trace) and 336 (MNa^+, black trace). This time there are indeed five peaks coincident with each of the five pesticides shown in Table 1, but as a combination of their protonated and sodium adducts. Kresoxim-methyl now elutes at 15.15 min (m/z 336) and isazophos at 14.2 min (m/z 314 and 336). The extracted ion of m/z 314 also shows that only four compounds have the MH^+ ion, because kresoxim-methyl (at 15.15 min) does not form the MH^+ ion. Now MS/MS experiments are able to successfully get the correct spectra for each of the five pesticides in the pepper extract. Thus, this example shows the power of changing the selectivity of the HPLC column to augment the separation power of the mass spectrometer. The next and last section examines the effect of the pepper matrix on high-resolution separations.

High-Resolution Analysis of Pepper Matrix. To measure the complexity of the pepper matrix, an accurate mass extraction of all ions above the baseline of 10000 counts was carried out. This analysis of the pesticide-spiked pepper matrix uses a software feature called molecular feature extractor. The program groups all related adducts, proton, sodium, and ammonium, and their related isotopic patterns into individual extracted ions and displays them as chromatographic peaks (Figure 6). When the pepper matrix was extracted, it contained 4235 individual molecular features with ion intensities of 10000 counts or more. The 10000 count rule of thumb is a value that yields a valid accurate mass and isotopic pattern for the A+1 and A+2 isotopes of all ions formed, which is needed for formula generation and testing by accurate mass. Figure 6 shows the overlaying of the 4235 molecular features as well as the total ion chromatogram for the pepper matrix spiked with the five pesticides.

The complexity of the spiked pepper sample reminds one of the proverbial search for "the needle in the haystack." The value of the high resolution of the mass spectrometer and the measurement of accurate mass lies in the ability to search through the "haystack", the pepper matrix, to find the m/z 314 and 336 ions of the five pesticides. In this case, though, the search is done one accurate mass at a time. For example, the sodium adduct of kresoxim-methyl has an accurate mass of m/z 336.1210; thus, the extraction of the ion will focus on using a smaller extraction window of m/z 336. The value of narrowing the extraction window is shown in Figure 7. Here the m/z 336 ion is extracted with three window sizes of ±0.5, ±0.01, and ±0.05 mass units (u). The number of molecular features narrows from 4235 total features to approximately 10, then 2, and finally only 1 at the narrowest extraction window of ±0.05 mass unit, the final single peak being the sodium adduct of kresoxim-methyl.

Thus, the majority of the 4235 molecular features represent the pepper matrix. The exceptions are the five spiked pesticides and their molecular adducts. If we examine the mass defects of the matrix versus the mass defect of the five pesticides, they are easily separated using the high resolving power of the mass spectrometer. The mass defect refers to the difference between the nominal mass and the accurate mass of the compound.[29] Thus, the sodium adduct of isazophos has a mass defect of 0.0309, and kresoxim-methyl has a mass defect of 0.1206. Because of the structure of many pesticides, which contain elements such as sulfur, phosphorus, and the halogens, the mass defect is shifted closer to the nominal mass[29] than the majority of the matrix of most vegetables, which are rich in hydrogen relative to the pesticides. Typically, at a mass of m/z 336, the majority of ions in the pepper matrix have a mass defect from 0.15 to 0.25. This shift is taken advantage of with the high resolution of the mass spectrometer, which results in the reduction of interfering ions as shown in Figure 7. Thus, the value of high-resolution mass spectrometry for the analysis of isobaric pesticides lies in the ability to separate the m/z 336 ion (or the m/z 314 ion, data not shown) from the possible interfering ions of the matrix.

The question posed in the title, of whether high-resolution chromatography and high-resolution mass spectrometry are redundant, has a surprising answer. That is, high-resolution chromatography is valuable for the separation of isobaric pesticides and possible isomers for MS/MS analysis, and high-resolution mass spectrometry has the ability both to separate closely related isobars and to separate the isobaric compounds from the complex unknown compounds of a vegetable matrix, such as pepper.

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Notes

The authors declare no competing financial interest.

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