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RESEARCH ARTICLE

The Chromatographic Role in High Resolution Mass Spectrometry for Non-Targeted Analysis

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Abstract

Resolution improvements in time-of-flight instrumentation and the emergence of the Orbitrap mass spectrometer have researchers using high resolution mass spectrometry to determine elemental compositions and performing screening methods based on the full-scan data from these instruments. This work is focused on examining instrument performance of both a QTOF and a bench-top Orbitrap. In this study, the impact of chromatographic resolution on mass measurement accuracy, mass measurement precision, and ion suppression is examined at a fundamental level. This work was extended to a mixture of over 200 pesticides to determine how well two different software algorithms componentized and correctly identified these compounds under different sets of chromatographic conditions, where co-elution was expected to vary markedly.

Key words: Chromatographic separation, Peak coalescence, Co-elution, Mass accuracy

Introduction

High resolution mass spectrometry coupled to liquid chromatography (LC/MS) affords the ability to screen for large numbers of chemicals in a single full-scan LC-MS analysis. To this point, most studies have focused on the targeted screening for compounds based on the accuracy of the mass measurement and resultant chemical formulae. This type of approach has been applied in screening of food [1–4], environmental applications [5–7], and forensic applications [8–10], where large numbers of compounds are assessed. An extension of this work is the non-targeted workflow where compounds outside of a target list are searched (i.e., data mining) and chemical formulae are generated from the accurate mass information [11]. In both these cases, database searching can be utilized to reduce the list of possibilities, and the application of formula generation criteria [1, 4, 6, 8] increases the chances of proper identification.

Although a principal advantage of high-resolution mass spectrometry is chemical formula generation [12–15], even under the best mass spectrometric conditions, formula generation can only be limited to the number of possibilities within the error tolerance of the measurement. For example, Kind and Fiehn have shown that for accurate mass measurements at m/z<400, even a mass error of <1 ppm results in the generation of a number of potential elemental formulae; this number increases with mass, making compound identification even more difficult [14]. It should be noted that two recent publications from Little et al. report that in practice most chemicals are in the range of m/z 200–600 [16, 17].

Kind and Fiehn have formulated seven rules for optimizing the use of high accuracy mass measurements for formula generation from LC-MS data, including: restricting the number of elements, application of LEWIS and SENIOR rules, examination of the isotopic pattern, scrutiny of the ratios of H/C, and heteroatoms, and the probability of the elements to account for multiple elements [12]. Furthermore,

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Erve et al. noted that mass accuracy decreases with increasing mass resolution in the Orbitrap, which further increases the uncertainty in formula generation [15]. Blake et al. have reported that manual modifications to the Orbitrap deflector voltage, the injection level of the central electrode, and both lens and pulse voltages improved the mass accuracy of the LTQ-Orbitrap XL [18]. However, in the Exactive Orbitrap, these settings are not available to the user for optimization. Other factors that can affect the accuracy of mass measurements and resultant formulae generation include ion suppression (which can decrease the signal-to-noise ratio and the accuracy of mass measurements [19, 20]) and, in the case of the Orbitrap, mass peak coalescence [21–25].

The goal of this work is to address the factors that affect mass resolution and mass measurement accuracy in LC-MS, which in turn influences the development of automated data analysis strategies to screen LC-MS data from samples in which there may be wide variations in matrix background [7, 23]. As part of this work, data limiting factors such as co-elution and ion suppression were examined as they relate to the instrument performance. Co-elution and ion suppression significantly hinder the ability to make an accurate mass measurement and, in some cases, become more important than the instrument mass resolution and mass measurement accuracy. The use of ambient ionization techniques such as DART [25, 26] and DESI [27–29], where chromatographic separation is not used, may further exacerbate the effects of some of these factors. Consequently, it is important to understand the limitations the data, and the way it is generated, impose on the accuracy of software algorithms used to automatically identify the components of complex mixtures.

In this study, we have developed a model system to address the effects of co-elution and ion suppression on accurate mass measurements, in which we systematically examine six nominally isobaric compounds on two different types of high resolution mass spectrometers, an Exactive Orbitrap and an Agilent 6538 QTOF (Santa Clara, CA, USA). The measurements were made using a controlled set of experiments designed to mimic co-elution and to evaluate some pitfalls associated with dependence on high resolution mass measurements alone. The effect of mass, mass accuracy, and resolving power on the ability of the instruments to separate these compounds was examined. Ultimately, these experiments defined both the instrument capabilities and some of the basic criteria for performing non-targeted analyses, and the resultant parameters were incorporated into a subsequent examination of a complex pesticide mixture. Here, a mixture of 247 pesticides was analyzed with three different chromatographic conditions on both instruments, and the data were processed using software (both vendor and third party) designed to automatically process these data in both a targeted and non-targeted manner. Fundamentally, knowledge of the limitations in this approach will allow researchers to perform better analysis and will help improve future search algorithms for this type of work.

Experimental

All solvents used in this work were Optima Grade (ThermoFisher Scientific, Pittsburg, PA, USA). Six nominally isobaric pesticides (Figure 1) were obtained from an in-house stock. The pesticides were diluted to 50 pg/μL in a 90:10 mixture of H_2O (0.1 % formic acid):ACN (0.1 % formic acid). The mixture of 247 pesticides was also obtained from an in-house stock solution and diluted to 50 pg/μL in a 90:10 mixture of H_2O (0.1 % formic acid):ACN (0.1 % formic acid).

Samples were analyzed on two instruments: (1) Acquity UPLC (Waters, Milford, MA, USA) connected to an Exactive Orbitrap (ThermoFisher Scientific, San Jose, CA, USA), and (2) Agilent 1290 UHPLC connected to an Agilent 6538 QTOF (Santa Clara, CA, USA). The Exactive data presented was measured at 100 K resolution; however, data was evaluated at the other resolution settings (12.5, 25, and 50 K). In addition, lock mass was evaluated and there was no significant difference between data acquired with or without using lock mass (not shown). All Agilent data were acquired with two reference masses (*m/z* 121.050873 and *m/z* 922.009798) in 4 GHz mode (40 K resolution at mass 1521.97). It should be noted that the Agilent QTOF utilizes an 8-bit analog-to-digital, ADC, detector.

Chromatographic separations were performed on a BEH C18 2.1 mm×100 mm, 1.7 μ column (Waters) under different conditions. For loop injections, a 50:50 mixture of H_2O (0.1 % formic acid):ACN (0.1 % formic acid) (A:B) was introduced into the mass spectrometer at 400 μL/min isocratic, whereas samples were injected through a 10 μL loop (Rheodyne, Oak Harbor, WA, USA). Three chromatographic approaches were used to evaluate the data:

1. One column, 30 min gradient
2. One column, long gradient (70 min)
3. Four columns, long gradient (70 min)

Gradient elution for these approaches was performed using the aforementioned mobile phases and a linear gradient using the following configuration:

1. 90 % A (hold for 1 min)
2. 10 % A (linear), hold
3. Re-equilibrate

These mobile phase conditions did not allow for all 247 compounds to chromatograph (e.g., ammonia adducts), and those compounds that did not elute were used to determine false positives.

Exactive data were processed using IntelliTarget/IntelliXtract (Advanced Chemistry Development; ACD Labs, Toronto, ON, Canada). Agilent files were processed using MassHunter B.04.00 (Agilent), these files were too large

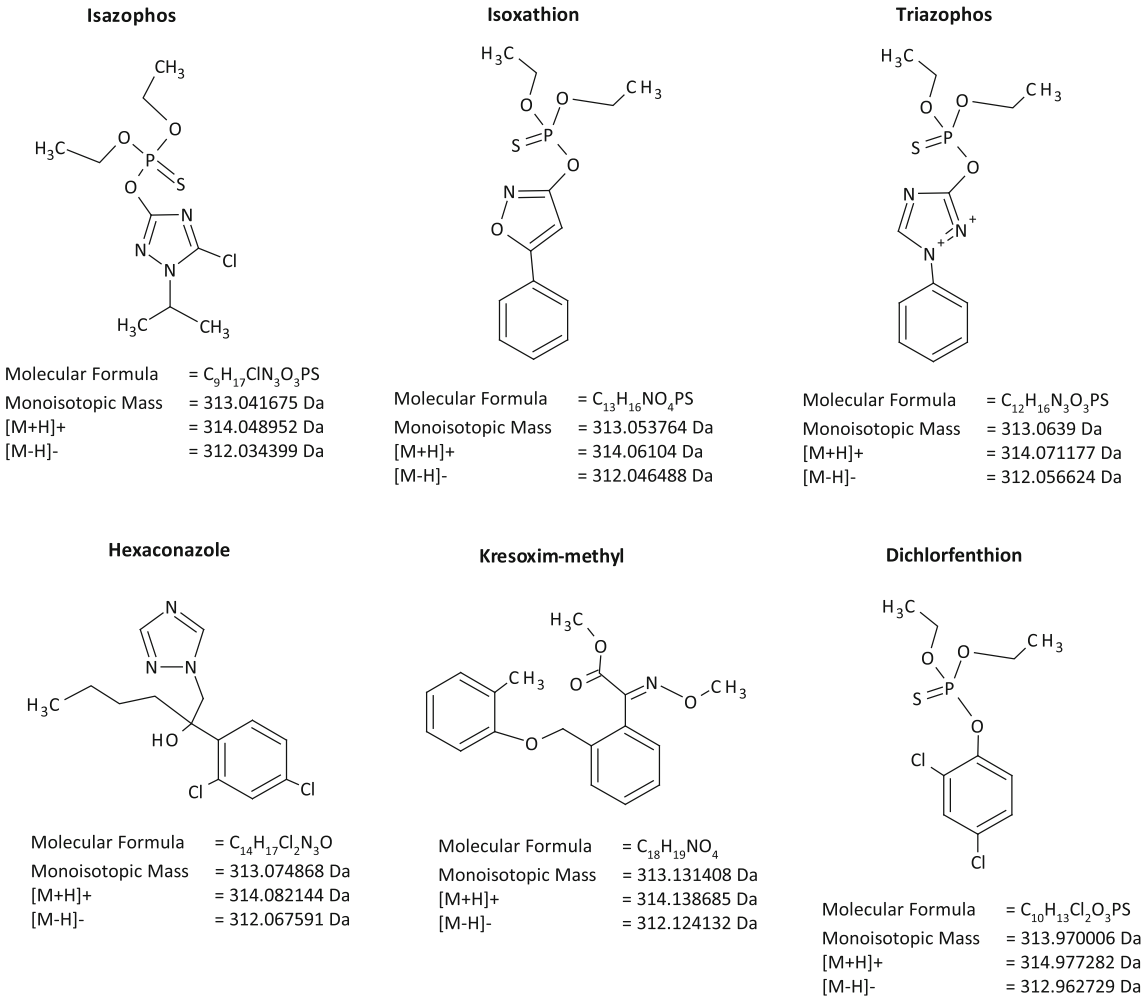

Figure 1. Structures of the six isobars used in the study

(>3 GB) to be processed using IntelliTarget. For targeted analysis, a list of compounds containing the name and elemental formula were used with IntelliTarget (ACD Labs) and Find by Formula (Agilent) processing. For the non-targeted approach IntelliXtract (ACD Labs) and Molecular Feature Extraction (Agilent) processing was used in the absence of any compound list, which allowed the software to identify any potential components that the software regarded to be LC-MS data.

Results and Discussion

Peak Coalescence

The mixture of six (nominal) isobars was initially analyzed with UPLC separation on the Exactive in profile mode, to determine the mass resolution required for discrimination. A 30 min gradient showed chromatographic separation for all six, with two of the compounds (hexaconazole and triazophos) displaying partial co-elution. For the compounds that did not co-elute, the spectra observed yielded the expected results with *m/z* values falling within the expected mass measurement error (<2 ppm). However, when evaluating the co-eluting compounds, the mass spectra for each chromatographic peak were extracted, and a large mass error was observed. A more narrow extraction window showed a valley between the two chromatographic peaks (Figure 2). The representative mass spectra for the resolved chromatographic peaks were less than 2 ppm from the theoretical mass; however, in the valley between the two compounds a different mass spectrum was observed, with the ratio of isotopic masses close to those of hexaconazole. However, the measured *m/z* corresponded to neither compound; $[M+H]^+$ was −13 ppm off from the theoretical for hexaconazole and +18 ppm off from triazophos. It is believed that the spectrum observed is the result of coalescence between the secular frequencies of the molecular ions, producing a new mass spectrum. To explain, these two ions, which are close in mass, are simultaneously introduced into the ion trap for the period of time, where their chromatographic peaks overlap. During this time interval, the near coincidence of their secular frequencies results in ion cloud overlap and a distortion of the measured frequency, producing a mass measurement error. These results were reproducible and

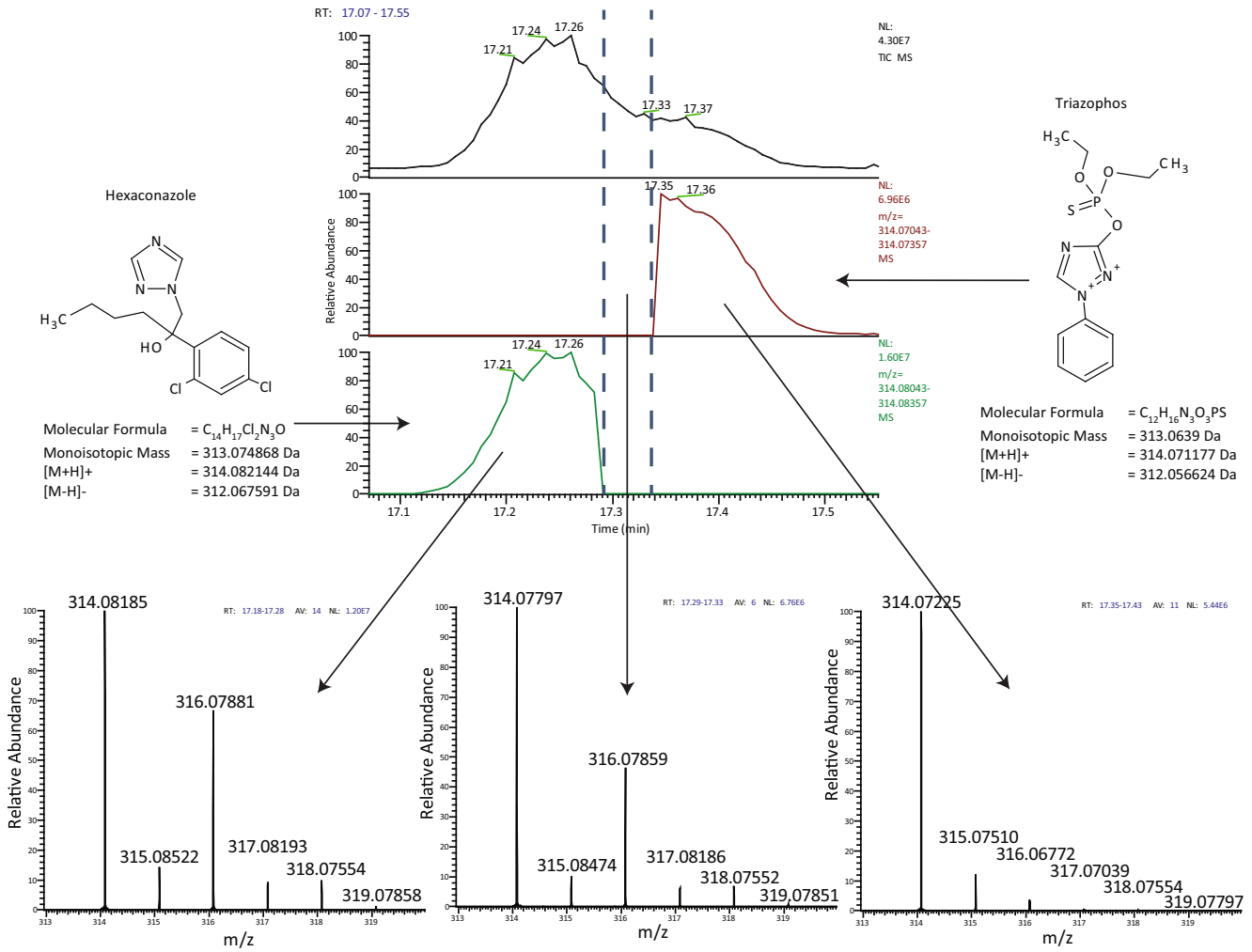

Figure 2. When hexaconazole and triazophos co-elute, there is a region within the chromatographic peak where a mass shift occurs in the Exactive. This region of peak coalescence resulted in a mass measurement that was 15 ppm lower than expected for hexaconazole and 16 ppm higher than expected for triazophos

independent of concentration. This is the first known report of this phenomenon in the Orbitrap for singly-charged ions during a chromatographic time-frame.

Results obtained with the QTOF instrument were also characterized by instrument-induced limitations. For the QTOF, the instrument was unable to separate the molecular ions for the two co-eluting compounds in the region of chromatographic overlap—the estimated resolution for this instrument at the *m/z* range of the model compounds is 31 K—and, in addition, displayed/reported the area of co-elution as saturated. The extracted ion chromatogram displayed a valley; however, only one mass spectrum was observed and because the ions were labeled as saturated (in the Agilent software they have an asterisk) the ion intensities were questionable. Dilution of the sample did not alleviate this observation but did make observation of both compounds in the chromatogram more difficult as the limit of detection was approached.

To further study the impact of co-elution, such as might occur when matrix ions co-elute with analytes of interest in complex samples, the mixture of these six isobars was analyzed by loop injection. A 50:50 mixture of ACN (0.1 % formic acid):H_2O (0.1 % formic acid) at 400 µL/min was introduced into the mass spectrometer and a 10 µL loop was filled with a 50 pg/µL solution of the isobars (Figure 3). In the first case, all six compounds were injected into the instrument (Figure 3a). As a result of ion suppression, only two compounds are readily visible: isazophos (≈6 ppm error) and triazophos (≈21 ppm error). In addition, the mass spectra for kresoxim-methyl and dichlorfenthion were not observed without magnifying their respective mass regions. Only upon close inspection of the peak at *m/z* 314.07786 would one conclude that more than one compound is present. Not unexpectedly, similar results were observed in the data acquired with the QTOF, indicating that in such cases, automated data extraction/processing would be challenged to

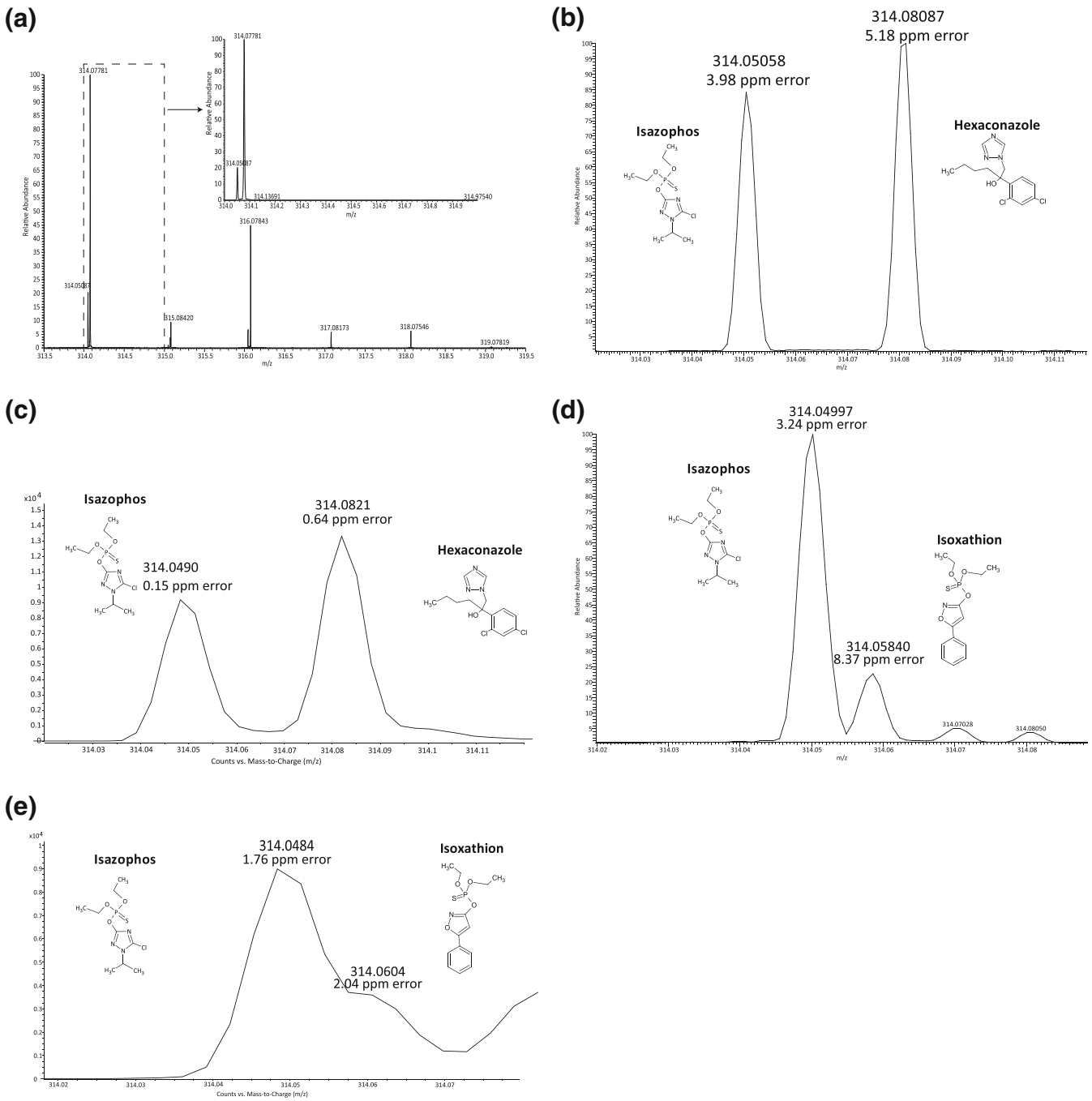

Figure 3. **(a)** To evaluate the mass resolution and mass accuracy of each instrument a mixture of all six compounds was injected into the instrument through loop injection. Because of ion suppression, only two of the compounds were visible: triazophos and isazophos. **(b), (c)** A mixture of two isobars was injected into each instrument to determine the impact of co-elution on mass error and mass accuracy. Hexaconazole and isazophos (0.03 Da apart) were compared on the Orbitrap **(b)** and the QTOF **(c)**. **(d)**, **(e)** Isazophos and isoxathion (0.01 Da apart) were compared on the Orbitrap **(d)** and QTOF **(e)**

discern the number of components. Subsequent formula generation would also be greatly hindered and would probably result in false negatives. To lessen the effect of suppression and further examine the interplay between mass resolution and mass accuracy, these experiments were continued by pairing the components and performing loop injections (Figure 3b, c, d, and e). Exactive experiments were performed at 100 K resolution, and QTOF experiments were performed at 4 GHz (40 K nominal resolution, approximately 31 K at the nominal *m/z* of the mixture). Figure 3b and c compare isazophos and hexaconazole, which differ in mass by 0.03 Da. As expected, both

instruments are able to resolve these compounds; however, the mass accuracy with the Exactive suffers. For example, while the greater mass resolution of the Exactive allows the identification of compounds that are less than 0.01 Da apart, mass shifts degrade accuracy as the mass difference between compounds decreases (the lower mass of the pair shifts to a higher measured value and the high mass of the pair shifts to a lower value), and these data also indicate that suppression also impacts the mass accuracy (Figure 3d). As the mass difference decreases, the resolution of the QTOF becomes more of an issue and it is more difficult to discern two compounds (Figure 3e); the mass accuracy begins to degrade as well. These data show that for the nominal isobars in these pseudo-mixtures, the overall mass accuracy of the QTOF is better than that of the Exactive; however, the mass resolution observed with the Exactive is much better. In addition, the QTOF displayed saturation of these compounds at 500 pg injected, and the samples had to be diluted to obtain signal within the limit of the detector; this results from the limited dynamic range of this instrument when operated in the 4 GHz (high resolution) mode. The Exactive did not display this problem.

It should be noted that mass accuracy and precision, as well as mass resolution, have an impact on the application of automated data processing algorithms for component identification. When generating formulae, the more accurate and reproducible the mass measurement, the better the results (i.e., a smaller number of possible structures and a higher likelihood that the correct result is among them). Consequently, mass shifts, particularly those that cannot be produced in a predictable fashion, as might occur from co-elution of matrix components, can have a significant effect on automated data processing results. Similarly, where resolution is concerned, the software must be able to distinguish two compounds in the mass spectral peak (i.e., componentize the data to identify more than one component). If these ions are not discernible, it will lead to a false negative. Furthermore, suppression of one ion in a co-elution scenario will lead to false negatives. The results of this model study of nominal isobars are summarized in Tables 1 and 2, where the error was calculated using:

$$Error(ppm) = \frac{mass_{observed} - mass_{theoretical}}{mass_{theoretical}} \times 10^6 \qquad (1)$$

and show that all three factors have an impact on the results. Considering the mass accuracy of both instruments is between 1 and 2 ppm, these results lead to the conclusion that there is a significant need for some chromatographic separation to minimize the effects of co-elution, decreasing the effect of mass shifts in the ion trap and the influence of unresolved peaks in the QTOF. Chromatographic resolution would also reduce ion suppression, which is a characteristic of electrospray sources on both instruments, which can either reduce signal or enhance signal and hinder quantitative analyses.

Using this information, a mixture of 247 pesticides was analyzed under three different chromatographic conditions on each instrument in order to vary the amount of co-elution and show its effects on automated data processing: a short gradient on one column (column length=10 cm); a long gradient on one column; and a long gradient on four columns (40 cm). The purpose of this was not to optimize the chromatography, but to vary the probability of co-elution and provide a basis for comparison of the effects of mass accuracy, resolution, and ion suppression on the results obtained from each instrument. The mobile phase conditions used were not conducive to observation of all compounds; however, these compounds were included in the processing lists as a test of the number of false positives resulting from incorrect assignments. The resulting data were processed using four software algorithms designed for performing targeted and non-targeted analysis: IntelliTarget/IntelliXtract was used for the Exactive data and MassHunter Molecular Feature Extraction/Find by Formula was used for the QTOF data. All data was processed in two ways. First, the non-targeted approach utilized IntelliXtract (ACD Labs) and Molecular Feature Extraction (Agilent) in the absence of a target list allowing the software to componentize any data that the software considered a chromatographic peak. The resulting list of components was sorted and compared with the list of analytes in the sample by mass. For IntelliXtract, the major parameters were the mass accuracy (0.5 Da), number of scans across the peak (3), and the 13 C/12 C ratio. For Molecular Feature Extraction, the software was set to small molecule analysis, H+species were only selected, and the peak height was restricted to >5000 counts. Second, a list of the compounds (with their chemical formula) was

Table 1. Exactive Mass Errors Associated with Ion Suppression and/or Peak Coalescence

Compound	Expected *m/z*	Isazophos error (ppm)	Isoxathion error (ppm)	Triazophos error (ppm)	Kresoxim-methyl error (ppm)	Dichlorfenthion error (ppm)	Hexaconazole error (ppm)
Hexaconazole	314.08212	−3.98	−1.91	−5.00	−0.99	−0.99	
Isazophos	314.04895		+3.24	+2.76	+0.82	+1.27	+5.18
Isoxathion	314.06104	−8.38		+11.18	+0.29	−0.19	+5.19
Triazophos	314.07118	+3.61	+0.61		+3.64	+3.77	+9.66
Kresoxim-methyl	314.13869	−3.20	−1.32	−2.63		+0.21	+11.73
Dichlorfenthion	314.97728	+0.22	−0.86	−0.16	+0.22		−5.02

Table 2. Agilent QTOF Mass Errors Associated with Ion Suppression and/or Peak Coalescence

Compound	Expected *m/z*	Isazophos error (ppm)	Isoxathion error (ppm)	Triazophos error (ppm)	Kresoxim-methyl error (ppm)	Dichlorfenthion error (ppm)	Hexaconazole error (ppm)
Hexaconazole	314.08212	−0.06	+0.25	−8.02	+0.89	−0.38	
Isazophos	314.04895		−0.48	+1.11	+1.11	+0.15	+0.47
Isoxathion	314.06104	−1.08		+36.8	+1.46	+0.51	−0.76
Triazophos	314.07118	+4.21	+4.53		+19.50	+7.71	+26.82
Kresoxim-methyl	314.13869	+2.91	−8.23	−6.00		+2.59	−0.27
Dichlorfenthion	314.97728	+0.69	−5.34	−2.48	+1.64		+0.37

provided in the software and the data extracted—a targeted approach. IntelliTarget analysis requires at minimum a list of compounds with either their formulae or their mass. Retention time information can be included and, like IntelliXtract, the mass accuracy and other filters are available. Find by Formula searching allows either a target list similar to IntelliTarget or use of a database, and the search criteria (e.g., mass accuracy and retention time) can be also utilized. These data were reviewed for the number of correctly identified, number missed (false negatives), and any situation where the software identified a component (within 5 ppm) that was not in the mixture and/or the retention time for an identified compound was not within one min of the manually-extracted (correct) retention time (false positives). In both cases, only the identification of $[M+H]^+$ was included in the search criteria, ignoring any other potential adduct.

As seen in Figure 4a (30 min gradient, one column) the majority of compounds elute very early in the chromatogram (note: LC-MS data for the QTOF is not shown; however, the chromatograms are similar to those acquired on the Exactive). It is readily apparent that there is co-elution and ion suppression present in these data. Using IntelliXtract, in the non-targeted approach, 621 components were generated. Eighty-one compounds were correctly "identified" from these components. Eighty-six compounds were not componentized (missed), and there were 41 false positives. A closer inspection of these data shows that the majority of missed identifications occurred because the signal was very low, largely due to ion suppression. In the case of the false positives, co-elution played a role by making componentization difficult, and in other cases the software componentized a compound that was within 5 ppm, or simply applied a label to a peak that provided a stronger signal than the actual compound, especially where ion suppression was observed. For the QTOF data, MassHunter's Molecular Feature Extraction (non-targeted analysis) and Find by Formula (targeted analysis) were used to process the data. Molecular Feature Extraction, identified 270 components. Of these, 103 correlated correctly to a mass (and retention time) of the target list. The software failed to componentize 80 compounds (false negatives) and had 16 false positives. Here the misses were due to low signal and the false positives were attributed to incorrect components within the 5 ppm range, or extraction of components that were isotopes of a compound with a high electrospray response (e.g., misidentification of 13 C as 12 C in some cases).

For targeted analysis, IntelliTarget found 166 compounds. Fifty-three of these identifications were correct, 129 were false positives, and 47 compounds were not identified. In the case of the misses, the software was unable to componentize the low intensity and/or suppressed compounds. The large number of the false positives was attributed to incorrect labeling of peaks due to co-elution or the software choosing a different chromatographic peak/mass spectrum that was within the range. MassHunter's Find by Formula operation extracted 230 components. There were only two missed compounds in this data set: one was due to low signal and the other was simply not labeled. In most cases, the 13 false positives were caused by the software extracting components that were within the mass tolerance range but at an incorrect retention time.

To test the effect of increased separation, a 70 min gradient (10 min hold time) was employed on one column (Figure 4b). IntelliXtract processing returned 791 components in the LC-MS data with 95 correct identifications, 104 misses, and 11 false positives. Molecular Feature Extraction processing resulted in 189 components with 77 correct, 127 misses and 5 false positives. In this case, the increased chromatographic resolution provided more componentization; however, the amount of misses increased. In the case of the QTOF data, this could have been caused by saturation of the detector. In these cases, the chromatographic peak has an area where it appears no compound is present and the software must combine the front and back of the peak to recreate the chromatographic peak (and component). This presents a problem, since the software must determine whether this is one component or two. It stands to reason that the signal intensity variation in the chromatographic peak will also play a role in the componentization. In other cases, missed compounds were low in intensity, and sometimes a compound was simply not detected. Another consideration is that both software platforms had a difficult time processing these large files (>3 GB). Both software packages from ACD Labs were unable to process the Agilent files and the MassHunter algorithm had to be

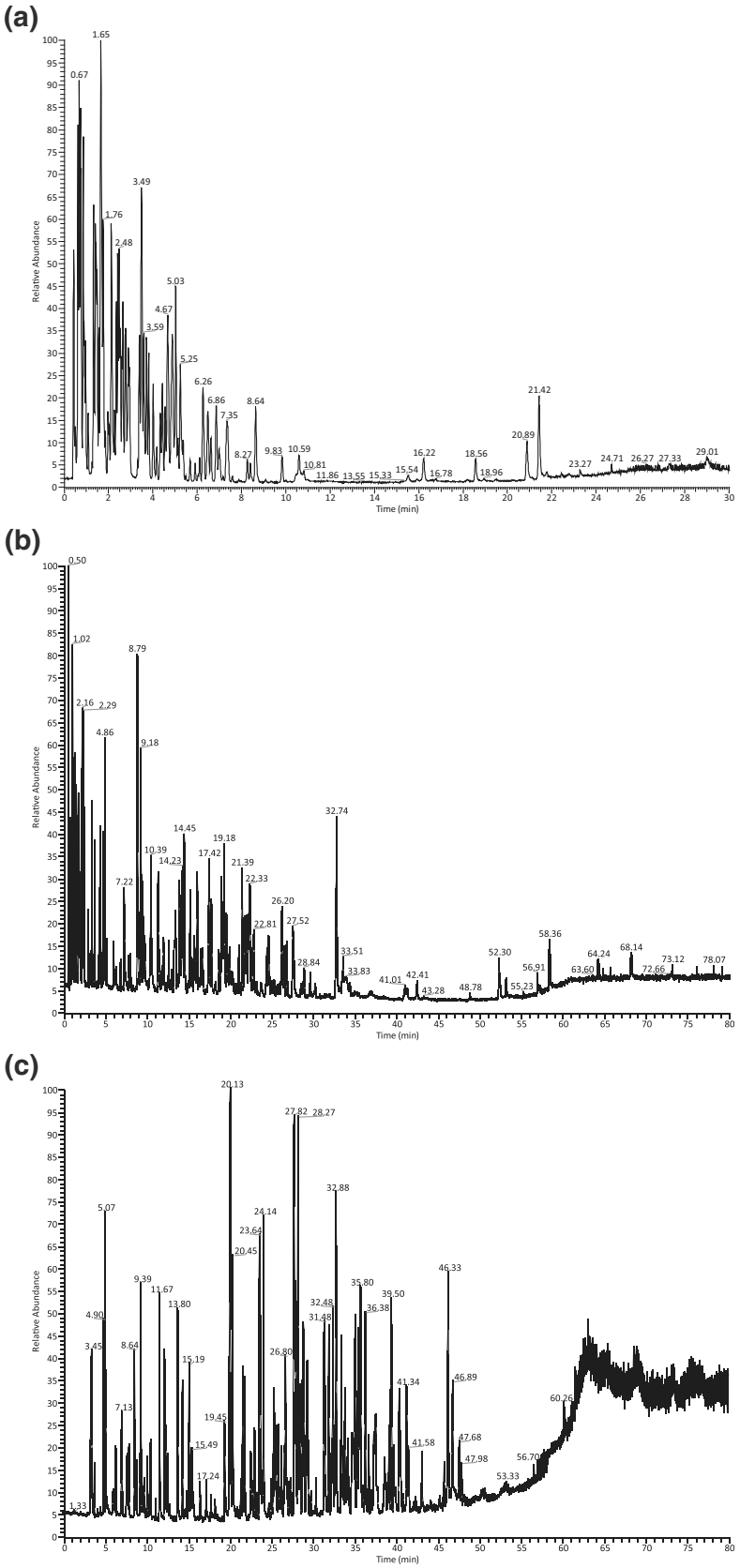

Fig. 4. Different chromatographic conditions were used to test the overall impact of reduced co-elution for data processing algorithms. A short (30 min) gradient was used on one column **(a)**, a 70 min gradient on one column **(b)**, and a 70 min gradient on four columns (40 cm) to achieve higher chromatographic resolution **(c)**

Table 3. Summary of Automated Data Processing for each Software Platform and each Algorithm

		Chromatographic conditions	Found	Not found	False (+)
Agilent MassHunter	MFE	10 cm Short gradient	103	80	16
		10 cm Long gradient	77	127	5
		40 cm	94	102	12
	FBF	10 cm Short gradient	230	2	13
		10 cm Long gradient	186	3	43
		40 cm	178	2	60
ACD Labs	IntelliTarget	10 cm Short gradient	53	129	47
		10 cm Long gradient	166	53	58
		40 cm	153	18	57
	IntelliXtract	10 cm Short gradient	81	86	41
		10 cm Long gradient	95	104	11
		40 cm	93	87	45

MFE=Molecular Feature Extraction; FBF=Find by Formula

modified in order to complete the processing. As a result, some low intensity compounds were likely lost. When IntelliTarget processing was used, the software correctly identified 166 compounds with 53 misses and 58 false positives, which was an improvement from the lower resolution chromatography. Find by Formula processing this software found 186 of the compounds with only three misses and 43 false positives. Here, in both instances, the results improved with the improved chromatography.

Finally, an extreme case of high chromatographic resolution was applied to determine if there was a limit to the correlation between separation and componentization; four columns were connected (40 cm total) and a 70 min gradient with a 10 min hold time was utilized to analyze this sample. Figure 4c shows that there is an improvement in the overall quality of the chromatographic data over that observed with one column using the same gradient. The chromatographic peaks are well-defined and ion suppression and potential peak coalescence should be reduced compared with the previous scenarios. IntelliXtract processing identified 752 components with 93 correct, 87 misses, and 45 false positives. The Find by Formula processing resulted in 307 components with 94 correct, 12 false positives and 102 misses. IntelliTarget searching was better with the longer chromatographic run on four columns where 153 correct identifications were made with 18 misses and 57 false positives. Find by Formula processing correctly identified 178 compounds with only two misses and 60 false positives. A summary of these results are presented in Table 3.

Overall, the results improved when better chromatography was utilized. In the case of non-targeted searching, it appears that more chromatographic separation allows both software packages to better componentize the data; however, this results in more data that must be reduced to correlate to elemental compositions. The relatively small improvement obtained when using four columns, as opposed to a single column with a long gradient, does not justify the need for this experiment. The IntelliTarget software performed better with increased chromatographic resolution, while the MassHunter software showed no significant differences. Of most concern are the numbers of false negatives, and both software programs would need to reduce these for practical usage in a non-targeted scenario; however, the trade-off would be a large number of false positives, which would require a substantial amount of time to verify their presence/absence.

Conclusions

The impact of chromatographic separation is often overlooked when performing non-targeted analysis. Reliance on the resolving power of the mass spectrometer alone to differentiate compounds can have detrimental consequences, particularly as the mixture becomes more complex. Ion suppression due to co-elution can impact the mass accuracy, which will impact the number and accuracy of formulae generated. Suppressed compounds could be missed during automated processing due to low signal-to-noise ratio; therefore, minimization of co-eluting compounds is important. On the chromatographic timescale, peak coalescence was observed in the Orbitrap causing mass shifts of up to 16 ppm from the theoretical masses, further supporting the need for good chromatography. Hardware and software improvements should enhance the utility of automated processing. Improvements in mass spectrometer speed, resolution, and mass accuracy should allow for more stringent search criteria, and detection improvements would reduce the impact of saturation.

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