Metabolomics

Moving beyond ddMS^2: Improving annotation confidence in untargeted metabolomics using higher resolution MS and parallel ion trap experiments

Brandon Bills, Sunandini Yedla, Rahul Deshpande, Bashar Amer, Ralf Tautenhahn, Susan Bird, Vlad Zabrouskov. Thermo Fisher Scientific, San Jose, CA. 95134. USA

Abstract

Purpose: Enhance the annotation of elemental formulas by increasing Orbitrap resolution to resolve isotopic fine patterns. Improve annotation confidence through supplementary MS^n or breakdown curve data via parallel ion trap scans.

Methods: NIST1950 SRM plasma extract was analyzed using a Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer at multiple resolutions, with AcquireX sequences incorporating ddMS^2, ddMS^2 with ddMS^3, and ddMS^2 with breakdown curves, to evaluate the impact on the confidence of compound annotations.

Results: Compounds analyzed at higher resolutions were found to have more detailed isotopic fine patterns which in turn allowed for differentiation of isobaric formulas. This was more pronounced at m/z values above 400 where the number of potential elemental formulas within 1ppm mass accuracy was higher. Annotation confidence was improved through the addition of data collected in the ion trap. Collecting MS^3 data provided additional criteria to compare and match against Thermo Scientific™ mzCloud™ mass spectral library or through FISh scoring and allowed for the differentiation of certain compounds where the MS^2 results were similar but the MS^3 spectra differed. Break down curve data collected within the ion trap provided additional data points that could be used to differentiate similar compounds that produced different fragment intensities at different energy levels.

Introduction

In metabolomics, samples contain both known and unknown compounds needing characterization. Mass spectrometry helps determine elemental formulas via precursor m/z and structural composition through fragmentation data. However, higher m/z values lead to more potential formulas, complicating accuracy. Isomers and isobars further challenge single-spectrum characterization. This work aims to utilize increased Orbitrap resolution for better elemental formula annotation using isotopic fine patterns and boost confidence by collecting additional MS^n or breakdown curve data through parallel ion trap scans.

Materials and methods

Sample Preparation

NIST SRM 1950 plasma from Millipore Sigma (Massachusetts) was extracted and reconstituted in a methanol:water solution.

Test Method(s)

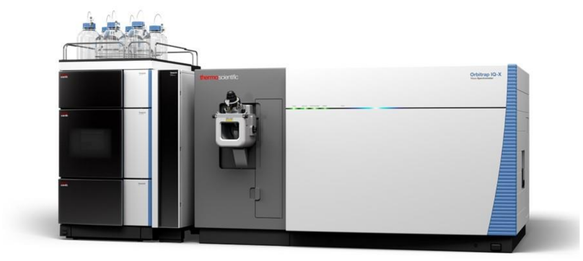
1 µL of plasma extract was injected on a Thermo Scientific™ Hypersil GOLD™ HPLC column using a Thermo Scientific™ Vanquish™ Horizon UHPLC system. Solvent A was water with 0.1% formic acid and solvent B was methanol with 0.1% formic acid. The gradient ranged from 0%B to 98%B.

Analysis was carried out on an Orbitrap IQ-X Tribrid mass spectrometer. The sample was analyzed in MS1 mode at 60K, 120K, 240K, 500K, and 1 million resolution. Fragmentation data was collected using Thermo Scientific™ AcquireX intelligent data acquisition to iteratively analyze the sample for more complete compound coverage. Three sequences incorporating ddMS^2, ddMS^2 with ddMS^3, and ddMS^2 with breakdown curves were collected on the same sample.

Data Analysis

Raw data was processed using Thermo Scientific™ Freestyle™ software to predict theoretical isotopic patterns and isobaric species and Thermo Scientific™ Compound Discoverer™ software along with Thermo Scientific™ Mass Frontier™ spectral interpretation software to evaluate plasma compounds for elemental composition prediction, annotation confidence, and potential fragment structures.

Figure 1. Vanquish Horizon UHPLC system and Orbitrap IQ-X Tribrid mass spectrometer



Results

Resolution and elemental formula prediction

Evaluating Increased resolution

Elemental composition is predicted based on accurate mass and resolution. As m/z increases, the number of isobaric elemental compositions increases, even within 1 ppm mass error. Table 1 shows the number of predicted a2 isotopic peaks for compounds at different resolutions and the number of theoretical isobars.

Table 1. Predicted a2 isotopic peaks and theoretical isobars at different resolutions and mass accuracies

Compound Information			Number of a2 isotopic fine peaks* at resolution					Theoretical isobars⁺ within mass tolerance		
Name	m/z	Formula	60K	120K	240K	500K	1M	1 ppm	3 ppm	5 ppm
Cystamine	153.0515	$C_4H_{13}N_2S_2$	2	3	3	3	3	1	2	3
Phenylalanine	166.0863	$C_9H_{11}NO_2$	1	3	4	5	5	1	2	3
Melatonin	233.1284	$C_{13}H_{16}N_2O_2$	1	2	4	4	5	1	2	4
Biotin	245.0954	$C_{10}H_{16}N_2O_3S$	2	2	4	5	5	3	9	12
Palmitic Acid	255.2330	$C_{16}H_{32}O_2$	1	1	3	3	4	1	1	1
Glucose-1-P	261.0370	$C_6H_{13}O_9P$	1	1	2	2	3	5	11	22
Maltose	341.1089	$C_{12}H_{22}O_{11}$	1	1	2	3	4	6	21	32
3'-AMP	348.0704	$C_{10}H_{14}N_5O_7P$	1	2	3	5	6	9	31	54
Cholic Acid	407.2803	$C_{24}H_{40}O_5$	1	1	1	3	4	4	10	15
Coenzyme A	768.1225	$C_{21}H_{36}N_7O_{16}P_3S$	1	1	2	4	5	86	277	400+

*peaks over 1% relative abundance of the most intense isotope in that cluster resolved at half maximum
⁺Only considering the elements: H, C, N, O, P, S

Isotopic fine peaks, peaks from heteroatoms, can eliminate incorrect elemental composition predictions. For example, figure 2 shows the a2 isotopic peak for 496.3398 at resolutions from 60K to 1M. At 1M four distinct peaks show up.

Figure 2. The a2 isotopic pattern for a peak at 496.3398 measured at increasing resolutions.

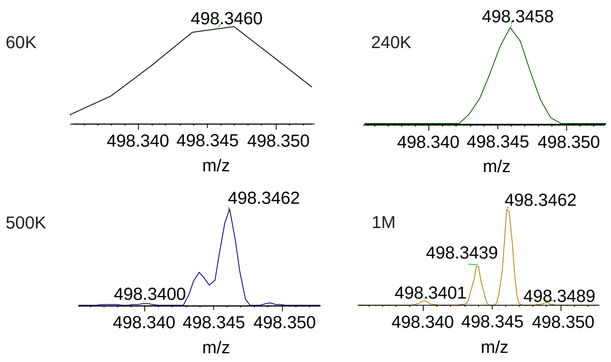
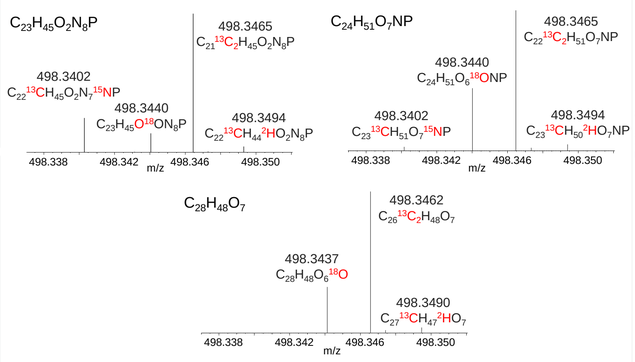


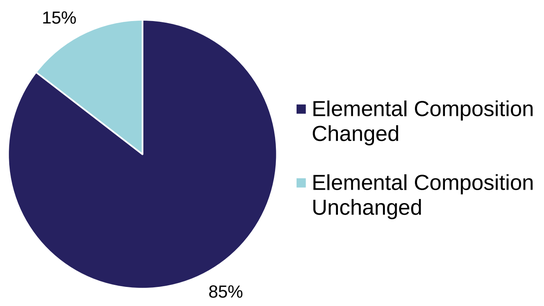
Figure 3 shows a2 patterns for three predicted elemental compositions for 496.3398. $C_{24}H_{51}O_7NP$ has a small (but still present) peak for $^{13}C+^{15}N$, a more intense peak for ^{18}O, and a small peak for $^{13}C+^{2}H$, which is closest to the a2 pattern at 1M.

Figure 3. The a2 patterns for three predicted elemental compositions for 496.3398



To get an understanding of the overall impact of increased resolution, features over m/z 400 were evaluated at 60K resolution and at 1M resolution using Compound Discoverer software. Elemental compositions were checked to see if they changed with the additional isotopic information. When comparing all compounds found in common at 60K and 1M resolution over m/z 400, 85% had their predicted composition change when using 1M resolution (figure 4).

Figure 4. Pie chart of compounds over m/z 400 that had elemental composition prediction change when using 1M resolution relative to 60K



Collecting ddMS^3 in the ion trap

Additional information through parallel scans

When collecting ddMS^2 scans the goal is to collect enough information to be able to confidently annotate the compound during data processing. This entails either comparing experimental spectra to library data to confirm matches to known compounds or using the fragments to predict the structure of an unknown. Issues arise when there are isomers with similar structures and MS^2 spectra

Figure 5. Comparison of the HCD MS^2 CE 40 for Acetaminophen and the isomer 2-Acetamidophenol from mzCloud mass spectral library

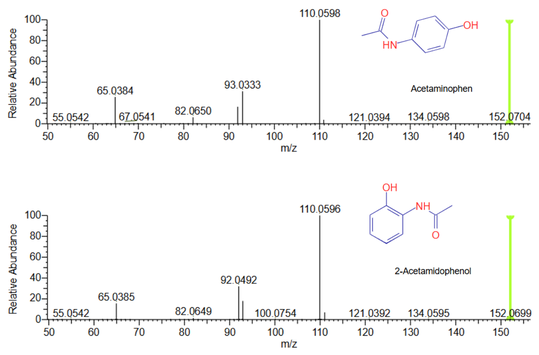
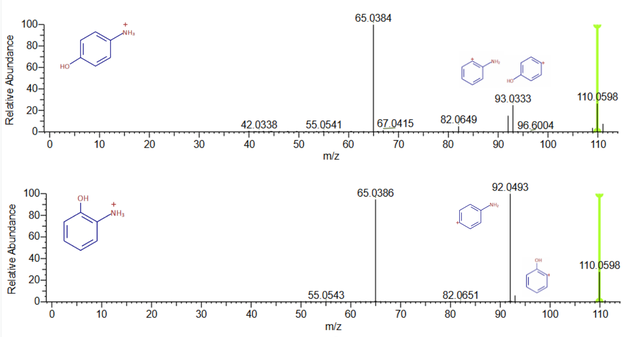


Figure 5 shows two positional isomers, acetaminophen and 2-acetamidophenol, fragmented at the same collision energy. Aside from some slight differences in peak intensities, these spectra are very similar and difficult to distinguish from each other. Additional information is needed to confidently tell these two compounds apart.

Data dependent MS^3 targets MS^2 fragments for further fragmentation providing a deeper characterization of a subsection of the structure. However, this additional scan can take time and reduce experimental throughput. To minimize the impact of collecting this additional information, the scan can be collected in the ion trap during the time frame when the transient is being collected in the Orbitrap.

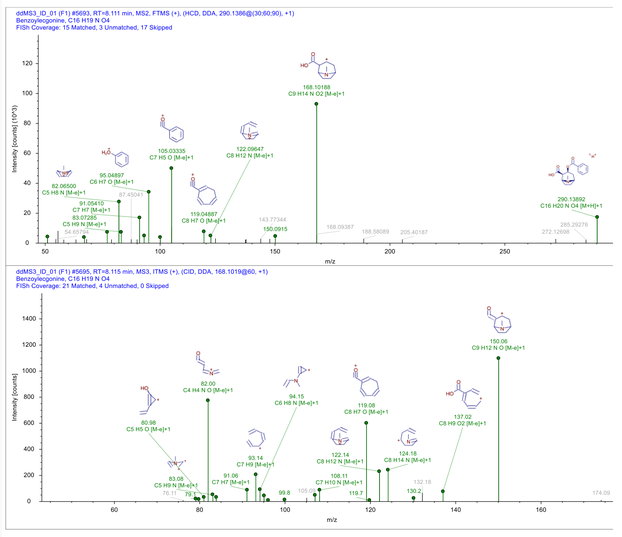
This additional information can be used to help distinguish compounds that are similar in the MS^2. For example, in figure 6 the 110.0600 fragment common to both compounds in figure 5 is fragmented further. In acetaminophen the fragment for 92.0493 is a factor of 5 times less intense compared to the same peak in 2-acetamidophenol making the two more distinguishable during post analysis data processing.

Figure 6. Comparison of the HCD MS^3 transition 152.0698->110.0596 at CE 40 for Acetaminophen and the isomer 2-Acetamidophenol from mzCloud mass spectral library



If there is no compound with MS^3 spectra in the library, the experimental data can be used to check proposed structures. In Compound Discoverer software MS^n trees can be submitted for FISh scoring to compare in silico predicted fragments to experimental data. This allows results from libraries without MS^3 data, such as structures from Chemspider, to be evaluated to determine which structures explain the largest number of MS^2 and MS^3 fragments. For example, in figure 7 the structure for benzoylecgonine was compared against the MS^3 data collected during analysis. Compound Discoverer software was able to explain the MS^2 and MS^3 fragments.

Figure 7. FISh coverage of peak at m/z 290.1386 compared to benzoylecgonine



Collecting a breakdown curve in the ion trap

Advantages of stepped and individual collision energies

A standard approach to unknown analysis is stepped collision energy to acquire fragments from a range of energies. However, some isomers will be less distinguishable using stepped collision energy. For example, in figure 8 the theoretical stepped spectra for theophylline and paraxanthine are shown for the 5 most intense fragments while figure 9 shows the individual CE from 40 to 60 CE.

Figure 8. Theoretical paraxanthine and theophylline spectra at stepped HCD 40,60,80

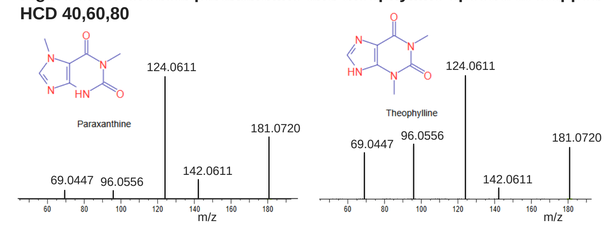
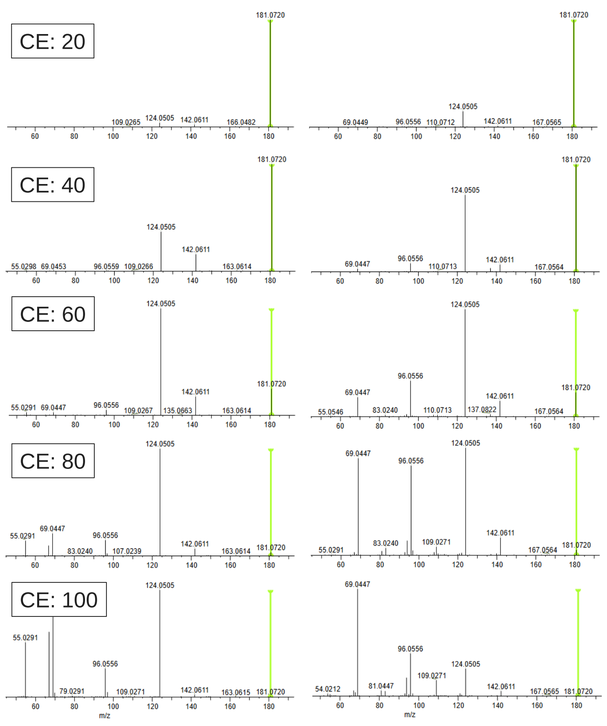


Figure 9. mzCloud mass spectral library spectra for Paraxanthine and Theophyline at different CE



While there are some minor intensity differences present in figure 8, the differences are far more pronounced at CE 80 and 100 in figure 9 making it easier to confidently match experimental spectra to mzCloud mass spectral library. This is another example of a scan that can be collected in parallel with the Orbitrap by utilizing the ion trap.

Conclusions

In this work we showed how utilizing higher resolution and the ion trap data can improve confidence in annotated results

- Higher resolution shows more of the isotopic fine pattern which helps elemental prediction
- MS^3 data provides additional data for library comparisons and unknown characterization
- Break down curve data can provide additional information on individual energy levels

Conflict of Interest

The authors BB, SY, RD, BA, RT, SB, and VZ are employees of Thermo Fisher Scientific whose instrumentation and software were used to acquire and process the data

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