

Metabolomics of Hermaphroditic *C. elegans* via Isotopic Ratio Outlier Analysis using High-Resolution Accurate Mass LC/MS/MS

Kevin J. McHale¹, Mark Szewc¹, Gregory S. Stupp², Chaevien Clendinen², Ramadan Ajredini², Arthur S. Edison², Chris Beecher³

¹Thermo Fisher Scientific, Somerset, NJ, ²University of Florida, Gainesville, FL, ³IROA Technologies, Ann Arbor, MI

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Overview

Purpose: To investigate metabolic changes in hermaphroditic *C. elegans* in an untargeted manner using isotopic labeling and high-resolution accurate mass (HRAM).

Methods: IROA[®]-labeled glucose was used in a selective ratio for monitoring changes in experimentally-challenged hermaphroditic *C. elegans*. These changes are measured using HRAM after separation via reverse-phase liquid chromatography (RPLC) and by hydrophilic-interaction liquid chromatography (HILIC). In-house software was used to identify and quantify the ¹³C-enriched components. Identified IROA components were searched against the KEGG database for compound name association. Data-dependent MS/MS spectra were used to confirm compound identifications by library matching, or to assist in structural elucidation.

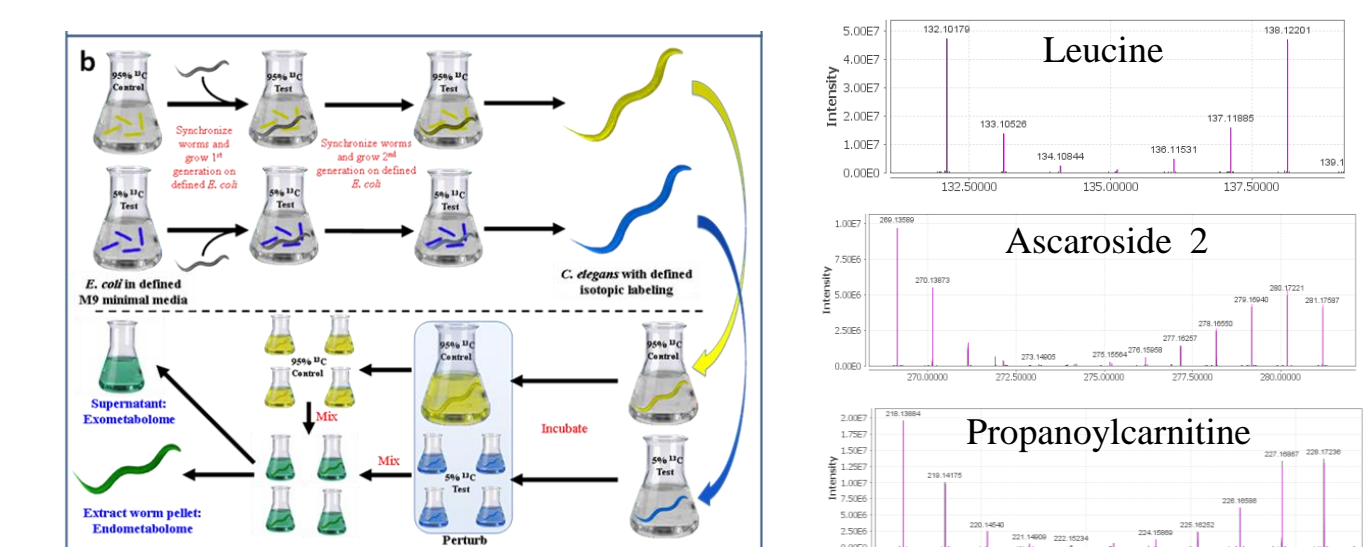
Results: IROA with HRAM identified 1303 and 1409 components by HILIC and RPLC, respectively, that had at least a 2-fold change in challenged hermaphroditic *C. elegans*. The complementary chromatographic techniques of HILIC and RPLC each showed examples where separation of isomers was critical for unambiguous compound identifications. Several ascarosides were observed to be up-regulated, including icas#9, which is a known attractant to hermaphroditic *C. elegans*.

Introduction

Caenorhabditis elegans is one of the best-studied animals in science. Despite this, metabolomic studies in *C. elegans* have only recently become active areas of research. The Isotopic Ratio Outlier Analysis (IROA) protocol uses ¹³C-isotopic signatures to identify and to quantitate metabolites (Figure 1).¹ It reduces error introduced during sample preparation and analysis, including ionization suppression by the use of IROA standards. The marriage of IROA and high-resolution accurate mass (HRAM) LC/MS/MS with *C. elegans* metabolomics allows experiments which assess the biological response to stresses or stimuli. These experiments would conventionally be difficult due to interferences by metabolites of unlabeled organisms. With IROA labeling and HRAM detection, metabolites can be distinguished in an untargeted manner, quantitated and unambiguously identified to their chemical formulas.

FIGURE 1. IROA Experimental design.

The Isotopic Ratio Outlier Analysis (IROA) protocol uses ¹³C-isotopic media to create IROA signatures in all metabolites by allowing the bacterial diet of the worms to grow in either a 5% or a 95% ¹³C enriched media. Once labeled, the worms and all of their metabolites are also fully labeled. The 5% labeled worm metabolites contribute the lower mass (left-hand) portion or the IROA pattern. The 95% labeled metabolites contribute the higher mass (right-hand) side of the IROA pattern. If the 95% is the control and thus constant, the 5% experimental is always measured against a common denominator.



Methods

Sample Preparation

E. Coli grown in minimal media supplemented with 95% or 5% randomly ¹³C-labeled glucose was fed to wild-type *C. elegans* hermaphrodites. Once worms reached young adult, the 5% labeled worm population was split into 4 replicates then incubated with male worms. The 95% labeled worms served as a control and were not challenged. The two worm populations were mixed in a 1:1 ratio and harvested. The recovered supernatant and worm pellets from the biological replicates were dried under nitrogen stream and stored at -80 C. Prior to LC/MS/MS analyses, samples were thawed and reconstituted with 28 µL 50% ACN.

Methods (cont.)

Liquid Chromatography

Samples were separated by injection of 2 µL via reverse-phase LC and HILIC using the Ultimate 3000 RSLC (Thermo Scientific). RPLC was on a Thermo Scientific Gold aQ 2.1 mm x 150 mm, 1.9 µm with a gradient of 0-95% B (A: 0.1% HCOOH; B: ACN + 0.1% HCOOH) in 12 min. HILIC was on a proprietary 2.1 x 150 mm column using a gradient of 10-70% A over 15 min., where A is 10 mM NH₄OAc adjusted to basic pH (B: ACN).

Mass Spectrometry

All data were acquired on the Thermo Scientific Q Exactive using external mass calibration. Sample analyses were conducted in both positive and negative ESI modes as separate LC/MS/MS runs. Full-scan MS were collected at a mass resolution of 70,000 FWHM from m/z 70 – 1000. Data-dependent MS/MS scans were acquired at 17,500 FWHM.

Data Analysis

The LC/MS data was analyzed using in-house MATLAB scripts. The software detects and characterizes IROA peaks which are visible in the mass spectra due to the enriched levels of ¹³C. IROA components were searched against the KEGG database to determine compound identities based on the elemental compositions as established by HRAM.

Results

HILIC versus RPLC

Owing to the widely varying polarities of the compounds in metabolomics experiments, complementary reverse-phase LC and HILIC methods were employed to measure the IROA-labeled samples. Table 1 shows a summary of the results.

TABLE 1. IROA components observed in ¹³C-labeled hermaphroditic *C. elegans* via HILIC & RPLC.

	HILIC	RPLC
# IROA components, 3 of 4 Samples	4021	5090
# IROA components, Named	668	674
# IROA components, 2-fold change	1303	1409
# IROA components, 2-fold change, Named	81	100
# IROA components, unique to LC method	20	39

While the number of observed IROA components in RPLC is about 25% higher than with HILIC, the total number of named components, as determined with a KEGG database search, was comparable. A statistical t-test evaluation was conducted to ascertain the IROA components that showed at least a 2-fold change (p-value ≤ 0.05) in the challenged hermaphroditic *C. elegans* samples. The RPLC method again shows a slight advantage in the number of IROA components displaying a 2-fold change.

Inspection of the named IROA components with a statistical change reveals two interesting findings. First, many of the components unique to RPLC are lipophilic in nature (e.g., fatty acids, ascarosides). Owing to the fact that a greater number of named and unique components were observed by RPLC suggests that these lipophilic compounds play a significant role in the interactions between hermaphroditic and male nematodes. Indeed, the ascarosides are a known class of signaling compounds that regulate behavior in *C. elegans*.² Second, less than 10% of the IROA components showing a 2-fold change were identified in the KEGG database, but for all of these the most likely formula was determined. This indicates that many of the statistically relevant compounds in these experiments have yet to be positively identified. Interpretation of the HRAM spectra and MS/MS data are ongoing.

FIGURE 2. XICs for Carnitine and 2-Amino adipic Acid, RPLC. Chromatograms are ±5 ppm.

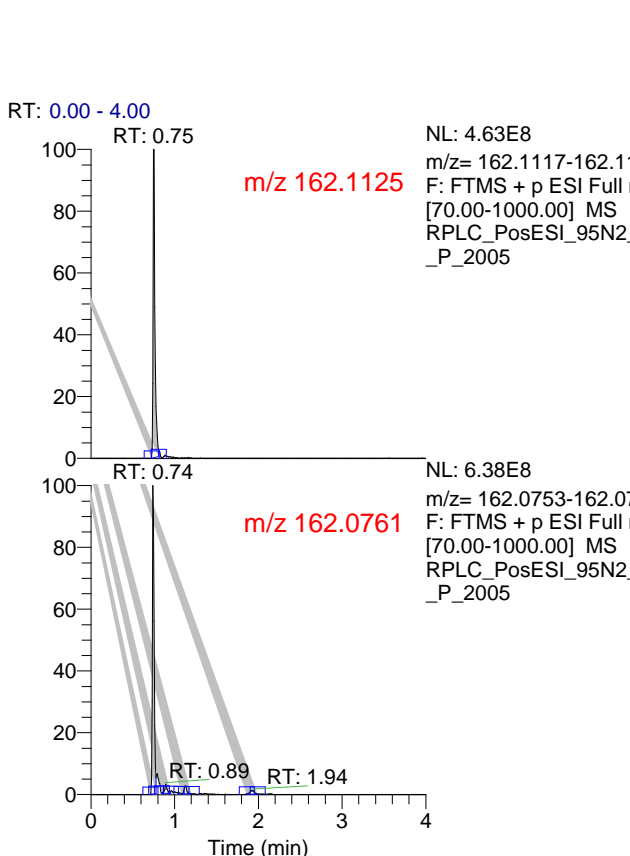
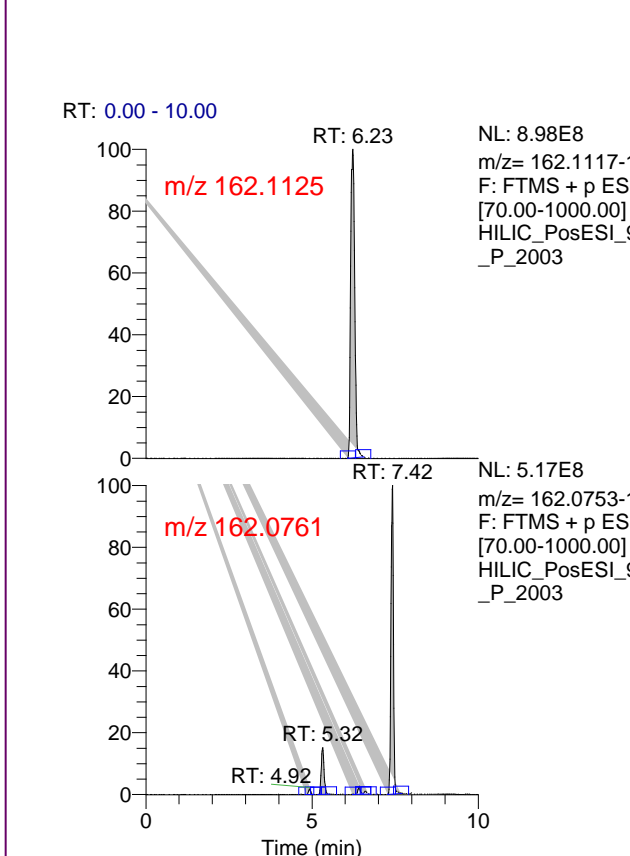


FIGURE 3. XICs for Carnitine and 2-Amino adipic Acid, HILIC. Chromatograms are ±5 ppm.



Figures 2 and 3 display the extracted ion chromatograms (XICs) for Carnitine and 2-Amino adipic Acid by RPLC and HILIC, respectively. These two compounds were determined to be statistically changing by IROA measurements. There are several interesting LC/MS experimental findings regarding these two components. First, these isobaric compounds coelute with RPLC (near the void volume). Nevertheless, the ultrahigh resolution of the Q Exactive easily mass resolves these two isobaric ions at 70,000 FWHM (Figure 4). Second, these components are chromatographically separated by HILIC. The identities of the major HILIC peaks were confirmed by HRAM MS/MS with library matching (data not shown). Third, close inspection of the HILIC data show at least two other isomeric species of 2-Amino adipic Acid. Data-dependent HRAM MS/MS of m/z 162.0760 at 4.92 min. and 5.32 min. are presented in Figures 5 and 6, respectively. Neither the Thermo Scientific Metabolomics MS/MS library nor the on-line METLIN MS/MS library had a match for these fragment ion spectra. Manual interpretation has putatively identified them as O-Acetylhomoserine and Glutamate Methyl Ester.

FIGURE 4. HRAM of Carnitine & 2-Amino adipic Acid at 70,000 FWHM by RPLC.

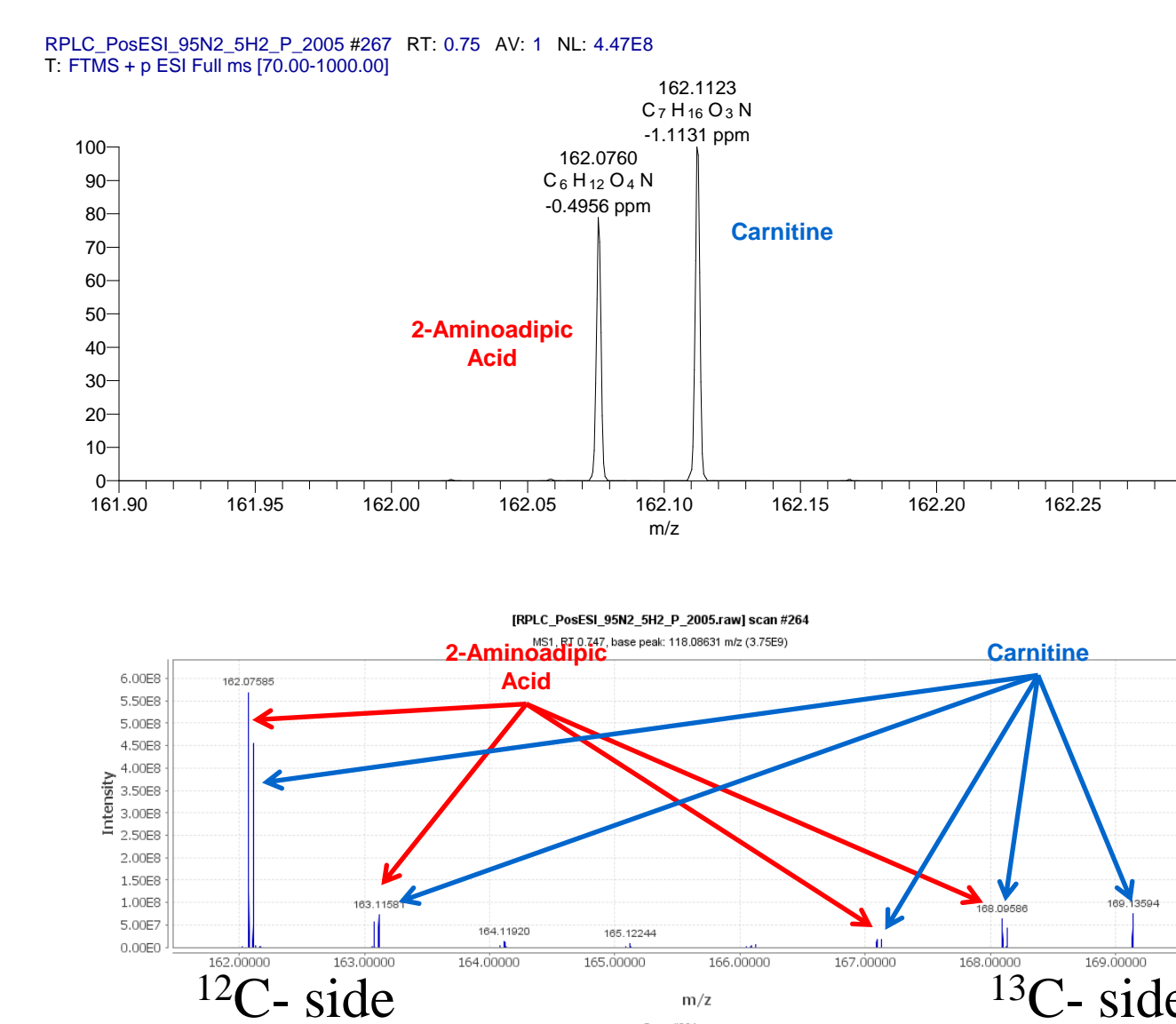


FIGURE 5. HRAM MS/MS of 2-Amino adipic Acid Isomer at 4.92 min by HILIC. Spectrum is consistent with O-Acetylhomoserine.

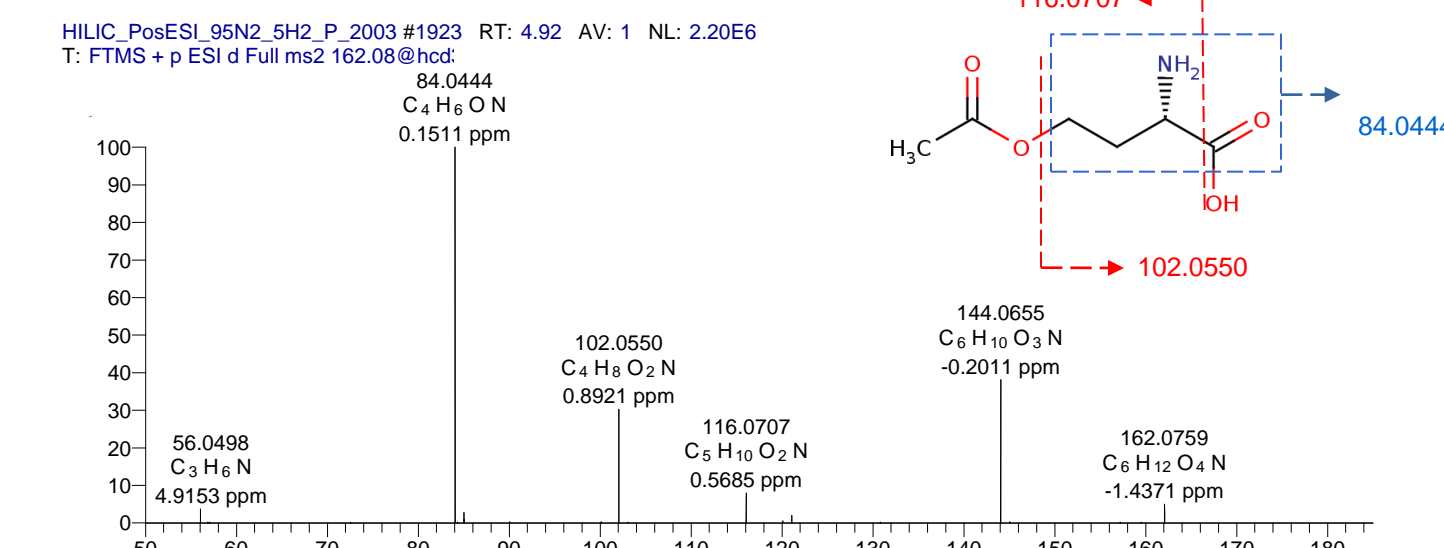
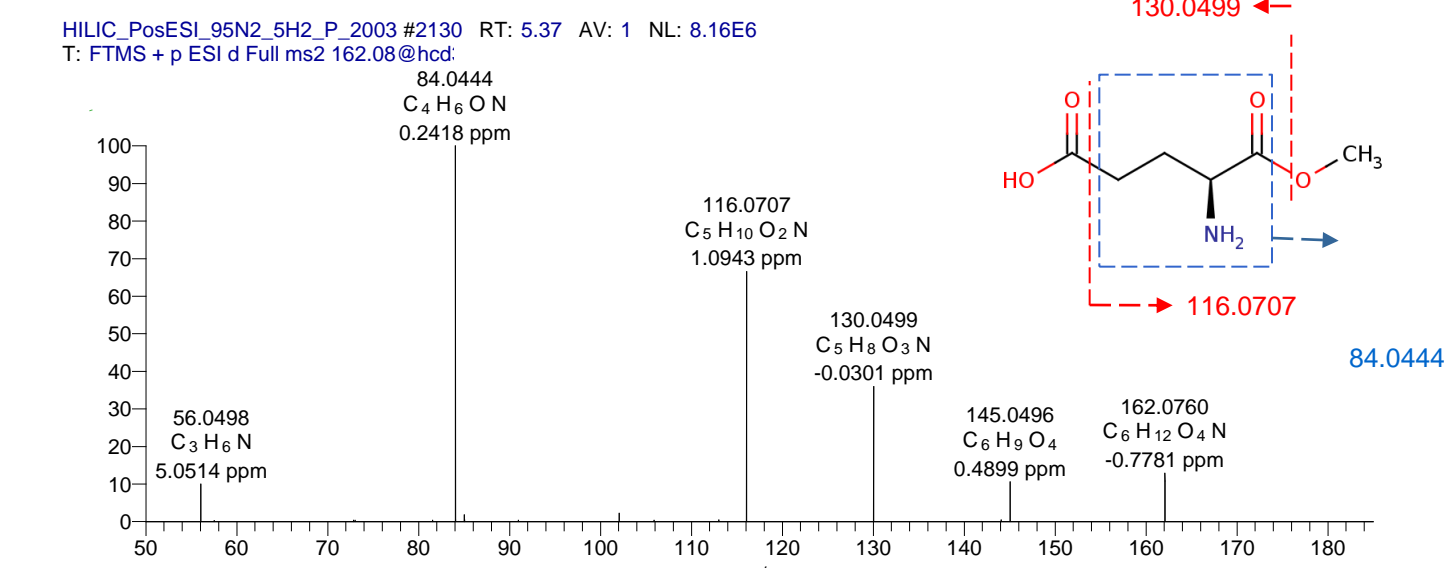


FIGURE 6. HRAM MS/MS of 2-Amino adipic Acid Isomer at 5.32 min by HILIC. Spectrum is consistent with Glutamate Methyl Ester.



Ascarosides

In recent years, ascarosides have been identified as signaling pheromones in *C. elegans*.² These pheromones are responsible for various social interactions, including sexual attraction and repulsion in hermaphroditic nematodes. Figure 7 shows the RPLC chromatograms for several ascarosides that are up-regulated in the pellet of the 5% ¹³C-labeled hermaphrodite samples. Note that RPLC is the preferred method for the ascarosides, as HILIC does not separate structural isomers observed via RPLC (data not shown). One of the ascarosides, icas#9, is an indole ascaroside, whose structure was confirmed by HRAM MS/MS (Figure 8). The icas#9 ascaroside has been shown to promote attraction and aggregation in hermaphroditic *C. elegans*.³ The same research group also showed that ascr#3, a strongly repulsive ascaroside, serves to balance the attractive effect of icas#3 at high concentrations. The presence of increased concentrations of ascr#9 in these samples may serve a similar purpose, but this supposition warrants further investigation.

FIGURE 7. XICs of Ascarosides, RPLC. Data from *C. elegans* pellet. Chromatograms are ±5 ppm.

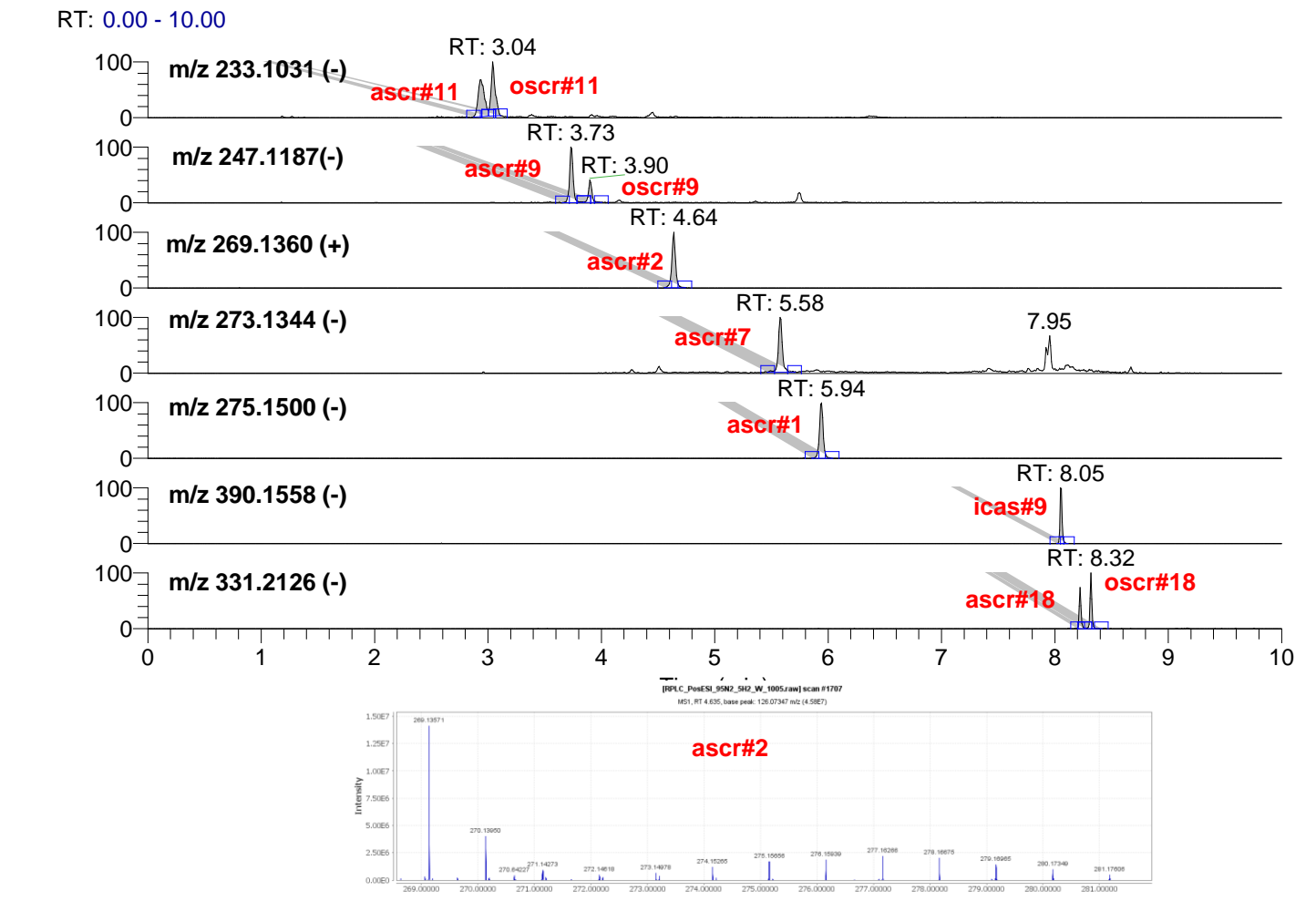
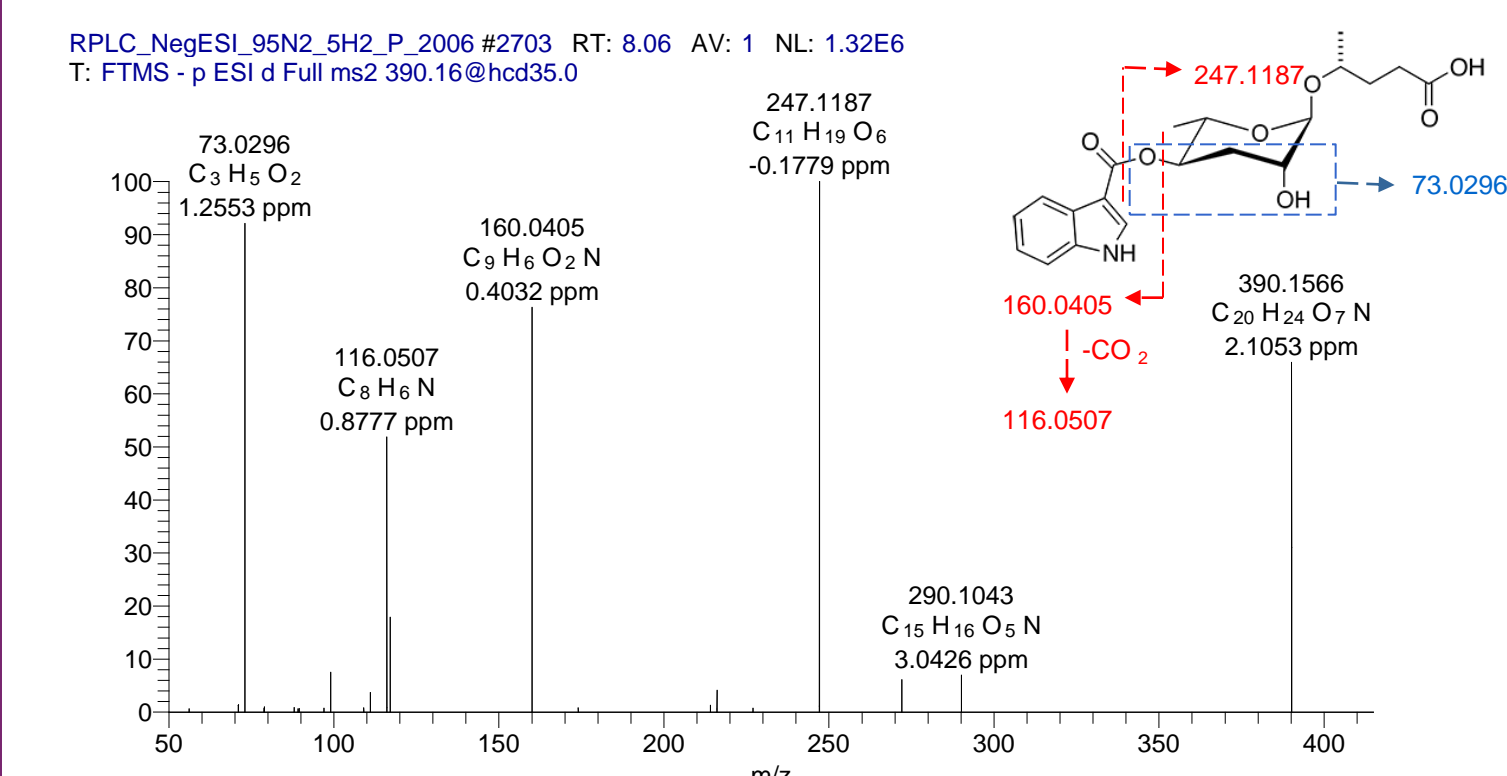


FIGURE 8. HRAM MS/MS, icas#9. m/z 73.0296 is a diagnostic negative ion fragment for all ascarosides.



Conclusion

- IROA with HRAM on the Q Exactive identified >1300 components that are statistically changing by at least 2-fold in hermaphroditic *C. elegans*.
- RPLC and HILIC are complementary LC methods that are necessary in providing a complete metabolomics picture for hydrophilic and lipophilic compounds.
- HRAM MS/MS data provide confirmations for IROA component identifications, and structural information for unknown compound identifications.
- Ascarosides were a key class of signaling compounds observed to be statistically changing in hermaphroditic *C. elegans*. Future work will examine the specific and synergistic nature of target ascarosides on *C. elegans*' behavior.

References

- de Jong FA, Beecher C: Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis (IROA[®]), an isotopic-labeling technique for accurate biochemical profiling. *Bioanalysis*; 2012 Sep;4(18):2303-14. PMID: 23046270
- Ludwig AH and Schroeder FC. Ascaroside signaling in *C. elegans* (Jan. 18, 2013). *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.155.1, http://www.wormbook.org
- Srinivasan J, von Reuss SH, Bose N, Zaslaver A, Mahanti P, Ho MC, O'Doherty OG, Edison AS, Sternberg PW and Schroeder FC. A Modular Library of Small Molecule Signals Regulates Social Behaviors in *Caenorhabditis elegans*. *PLoS Biol.* 2012 January; 10(1), doi: 10.1371/journal.pbio.1001237.

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