Application Note 592

Metabolomics of Hermaphroditic *C. elegans* via Isotopic Ratio Outlier Analysis (IROA) Using High-Resolution, Accurate-Mass LC-MS/MS

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Key Words

Isotopic Ratio Outlier Analysis (IROA®), Untargeted metabolomics, *Caenorhabditis elegans*, High-Resolution, Accurate-Mass (HR/AM) liquid chromatography-tandem mass spectrometry, Q Exactive, Ascarosides, ClusterFinder™

Goal

The goal of this study was to investigate metabolic changes in hermaphroditic *C. elegans* in an untargeted manner using isotopic labeling and high-resolution, accurate-mass (HR/AM) mass spectrometry.

Introduction

Caenorhabditis elegans is one of the best-studied animals in science and the genetics of this “worm” is especially well defined.[1] Despite this, metabolomic studies in *C. elegans* have only recently become an active area of research. The Isotopic Ratio Outlier Analysis (IROA®) protocol uses highly patterned ^{13}C isotopic signatures to identify and quantitate metabolites (Figure 1).[2]

Based on the IROA pattern seen in mass spectral analysis, both the accurate mass and the number of carbons in any molecule are determined. The combined information can significantly improve the accuracy of formula determination. The inclusion of a biologically similar internal standard, i.e. the control IROA 95% ^{13}C-labeled sample, increases the accuracy of quantitation by making every measurement relative to an appropriate isotopic standard. The use of an isotopic IROA standard also reduces error introduced during sample preparation and analysis including ionization suppression. The marriage of IROA and HR/AM liquid chromatography-tandem mass spectrometry (LC-MS/MS) with *C. elegans* metabolomics allows experiments that assess the biological response to stresses or stimuli. When done conventionally, these experiments would be difficult due to interferences by metabolites of unlabeled organisms. With IROA labeling and HR/AM detection, metabolites can be distinguished in an untargeted manner, quantitated, and identified to their chemical formulas.

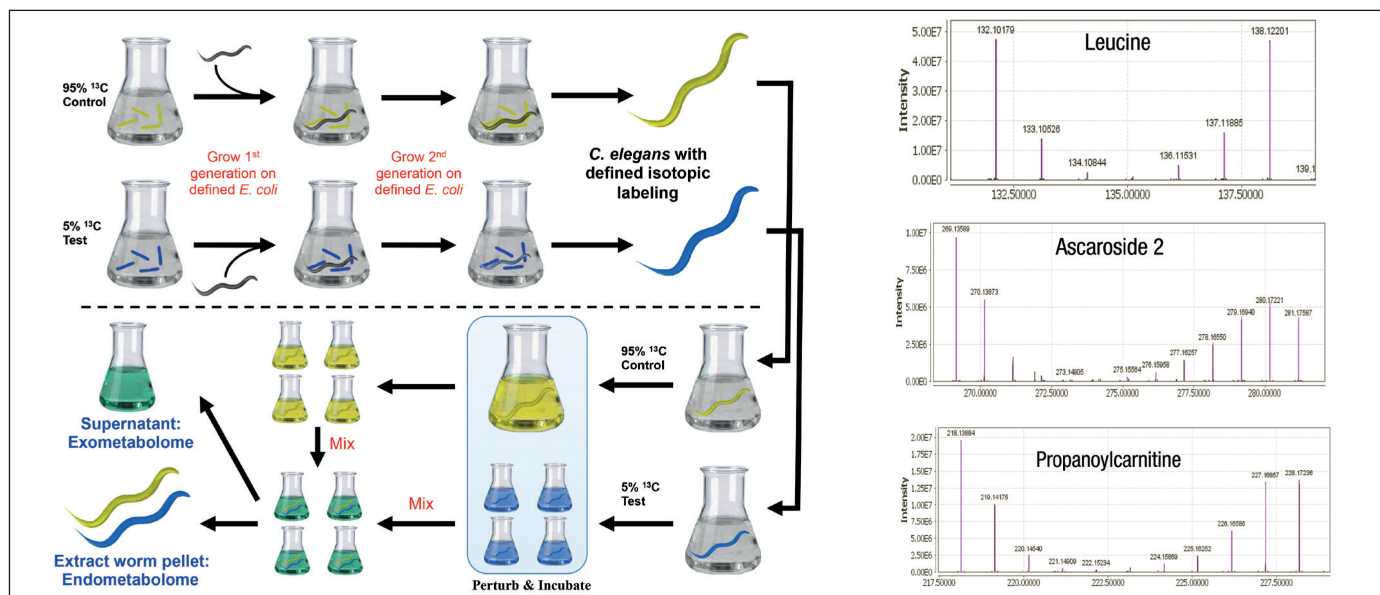

Figure 1. IROA experimental design. The IROA protocol uses ^{13}C-isotopic media to create IROA signatures in all metabolites by allowing the bacterial diet of the worms to grow in either a 5% or a 95% ^{13}C enriched media. Once labeled, the worms and their metabolites are also fully labeled. The 5% labeled worm metabolites contribute the lower mass (left-hand) portion of 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.

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Experimental

Sample Preparation

E. coli grown in minimal media supplemented with 95% or 5% randomly ^{13}C-labeled glucose (IROA media) was fed to wild-type *C. elegans* hermaphrodites. After worms reached young adult stage, the 5%-labeled worm population was split into four replicates and 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% acetonitrile/50% water.

Liquid Chromatography

Samples were separated by injection of 2 µL via reverse-phase LC (RPLC) and hydrophilic interaction liquid chromatography (HILIC) using the Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system. RPLC was performed on a Thermo Scientific™ Hypersil™ GOLD aQ 2.1 mm × 150 mm, 1.9 µm column with a gradient of 0–95% B (A: 0.1% formic acid; B: acetonitrile + 0.1% formic acid) in 12 min. HILIC was performed on a proprietary 2.1 × 150 mm column using a gradient of 10–70% A over 15 min, where A was 10 mM ammonium acetate adjusted to basic pH (B: acetonitrile).

Mass Spectrometry

All data were acquired on the Thermo Scientific™ Q Exactive™ mass spectrometer using external mass calibration. Sample analyses were conducted in both positive and negative electrospray ionization (ESI) modes as separate LC-MS/MS runs. Full-scan MS were collected at a mass resolution of 70,000 FWHM at *m/z* 200. Data-dependent MS/MS scans were acquired at 17,500 FWHM. Relevant source and scan parameters are shown in Tables 1 and 2.

Table 1. Source parameters for Heated Electrospray Ionization probe (HESI-II)

Parameter	Setting	
	HILIC	RPLC
Sheath gas:	45	60
Aux gas:	20	20
Sweep gas	2	2
Vaporizer temperature (°C):	350	450
Capillary temperature (°C):	275	275
S-Lens (%):	40	40
Spray voltage (V)	+3000 (-2250)	+3000 (-2250)

Table 2. Scan parameters for Q Exactive mass spectrometer

Parameter	Setting	
Full-scan MS	HILIC	RPLC
Mass range:	*m/z* 70–1,000	*m/z* 70–1,000
Resolution (FWHM at m/z 200):	70,000	70,000
AGC target:	1 x 10^6	1 x 10^6
Max inject time (ms):	50	50
Data-dependent MS^2	HILIC	RPLC
Resolution (FWHM at m/z 200):	17,500	17,500
AGC target:	1 x 10^5	1 x 10^5
Max inject time (ms):	150	150
Top N MS/MS:	4	2
Isolation width (Da):	1.5	1.5
Collision energy (NCE):	35	35
Stepped NCE (%):	40	40
Underfill (%):	25	50
Dynamic Exclusion duration (s):	6	4
Apex trigger (s):	1–6	0.2–2

Data Analysis

The data were analyzed using IROA ClusterFinder™ software. The software detects and characterizes IROA peaks, which are visible in the mass spectra due to the enriched levels of ^{13}C. As the IROA peaks are all mathematically calculable, the IROA ClusterFinder software algorithms achieved a data reduction of complex raw data to concise, high-value information by characterizing all peaks according to source (artifact, experimental (^{12}C), control (^{13}C), or standard); removing all artifacts; aligning and pairing all remaining peaks across all scans, identifying and determining the relative $^{12}C/^{13}C$ ratios of analytes in each sample. The number of carbons for each biochemical compound was calculated from its mass spectra by the distance between the two monoisotopic peaks, ^{12}C and ^{13}C. IROA components were searched against the KEGG™ database to determine compound identities based on the elemental compositions as established by HR/AM.

Results and Discussion

Removal of Artifacts, Noise, and Sample-to-Sample Analytical Variance

The IROA protocol utilized an enrichment of 95% and 5% ^{13}C for the control and experimental populations. This created readily recognizable isotopic patterns (Figure 1) that were used to 1) discriminate control from experimental samples and thereby allow composite pooling to remove sample-to-sample analytical variance and the effects of ion suppression, and 2) distinguish noise and artifactual peaks from IROA-labeled biological peaks. The later ensured a significant reduction of the number of peaks in the dataset as only biological peaks were interrogated.

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 3 shows a summary of the results.

Table 3. IROA components observed in ^{13}C-labeled hermaphroditic *C. elegans* via HILIC and RPLC

Number of IROA Components Observed	HILIC	RPLC
3 of 4 samples	4021	5090
Named	668	674
2-fold change	1303	1409
2-fold change, named	81	100
Unique to LC method	20	39

The number of IROA peaks identified in three of four samples through either HILIC or RPLC represented a significant reduction from the millions of mass spectral peaks in an average chromatographic run. 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 (for example, fatty acids and ascarosides). 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*.[3] 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 given the dual constraints of the accurate mass and carbon number derived from the distance between the monoisotopic ^{12}C and ^{13}C peaks. This indicates that many of the statistically relevant compounds in these experiments have yet to be positively identified. Given that many of these unknown compounds may be secondary metabolites and potentially novel, this is not surprising. Interpretation of the HR/AM spectra and MS/MS data are on-going.

Figure 2 displays the extracted ion chromatograms (XICs) for carnitine and 2-aminoadipic 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 ultra-high resolution of the Q Exactive mass spectrometer easily mass resolves these two isobaric ions and all their ^{13}C isotopes from the IROA labeling at 70,000 FWHM (Figure 3). Second, these components are chromatographically separated by HILIC. The identities of the major HILIC peaks were confirmed by HR/AM MS/MS with library matching (data not shown). Third, close inspection of the HILIC data show at least two other isomeric species of 2-aminoadipic acid. Data-dependent HR/AM MS/MS of *m/z* 162.0760 at 4.92 min and 5.32 min are presented in Figures 4 and 5, respectively. Neither the Thermo Scientific Metabolomics MS/MS library nor the online 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.

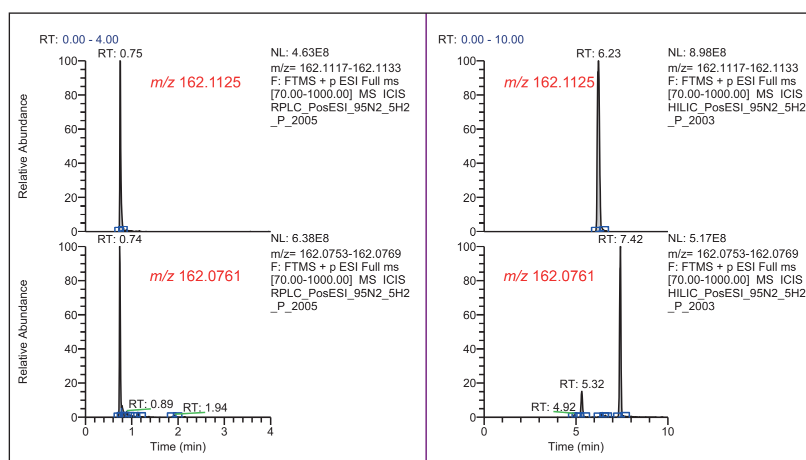

Figure 2. XICs for carnitine and 2-aminoadipic acid by RPLC (left) and HILIC (right). Chromatograms are ±5 ppm.

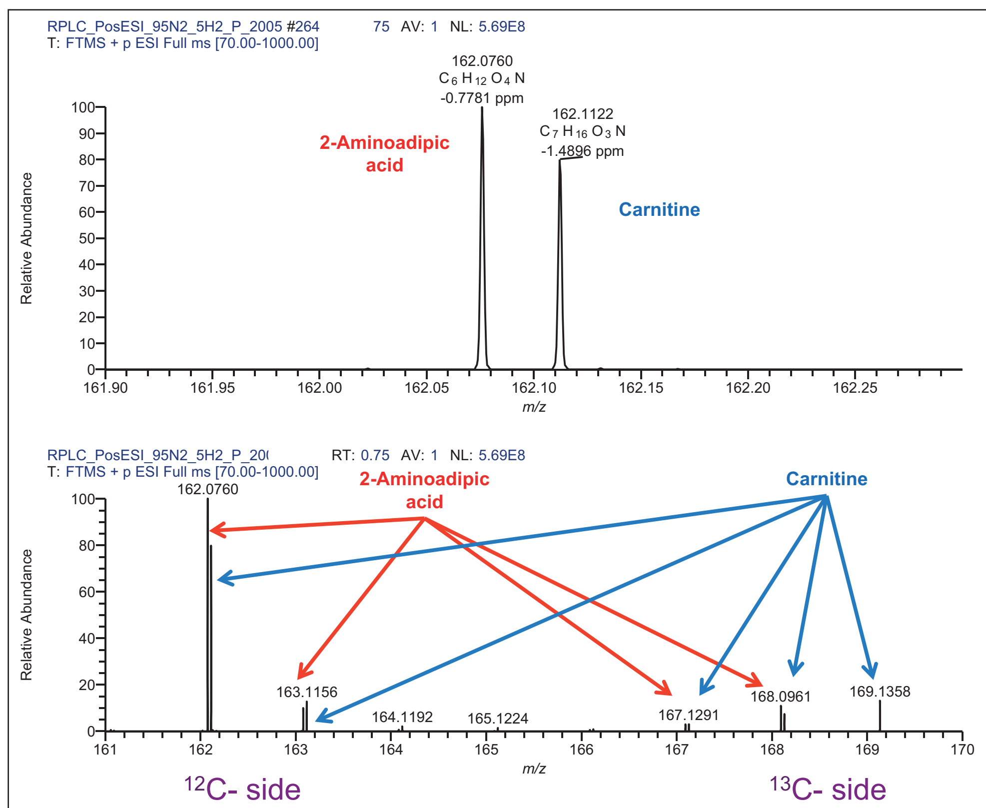

Figure 3. HR/AM of carnitine and 2-aminoadipic acid at 70,000 FWHM by RPLC. The top spectrum is a close-up of monoisotopic masses; the bottom spectrum shows the full IROA envelope.

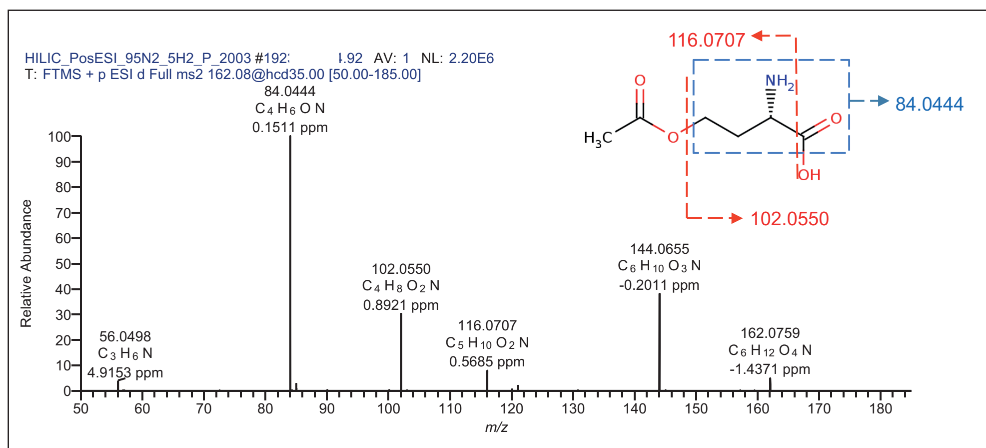

Figure 4. HR/AM MS/MS of 2-aminoadipic acid isomer at 4.92 min by HILIC. Spectrum is consistent with O-acetylhomoserine.

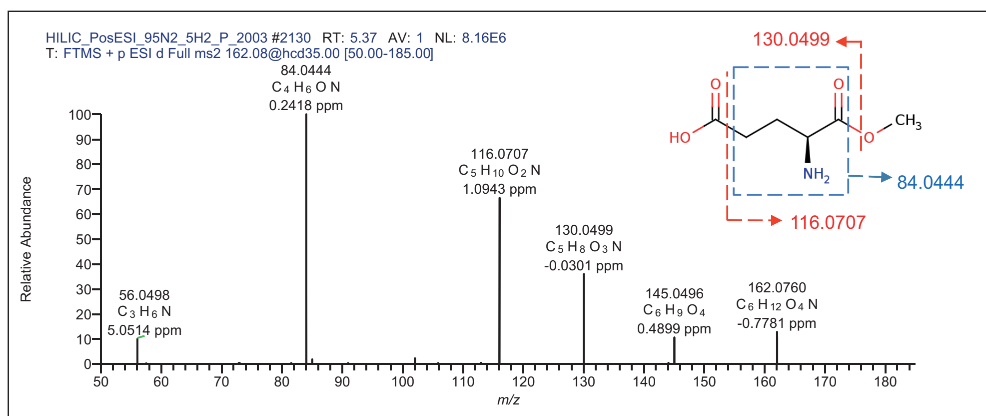

Figure 5. HR/AM MS/MS of 2-aminoadipic acid isomer at 5.32 min by HILIC. Spectrum is consistent with glutamate methyl ester.

Ascarosides

In recent years, ascarosides have been identified as signaling pheromones in *C. elegans*.[3] These pheromones are responsible for various social interactions, including sexual attraction and repulsion in hermaphroditic nematodes. Figure 6 shows the RPLC chromatograms for several ascarosides that are up-regulated in the pellet of the 5% ^{13}C-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 HR/AM MS/MS (Figure 7). The icas#9 ascaroside has been shown to promote attraction and aggregation in hermaphroditic *C. elegans*.[4] 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.

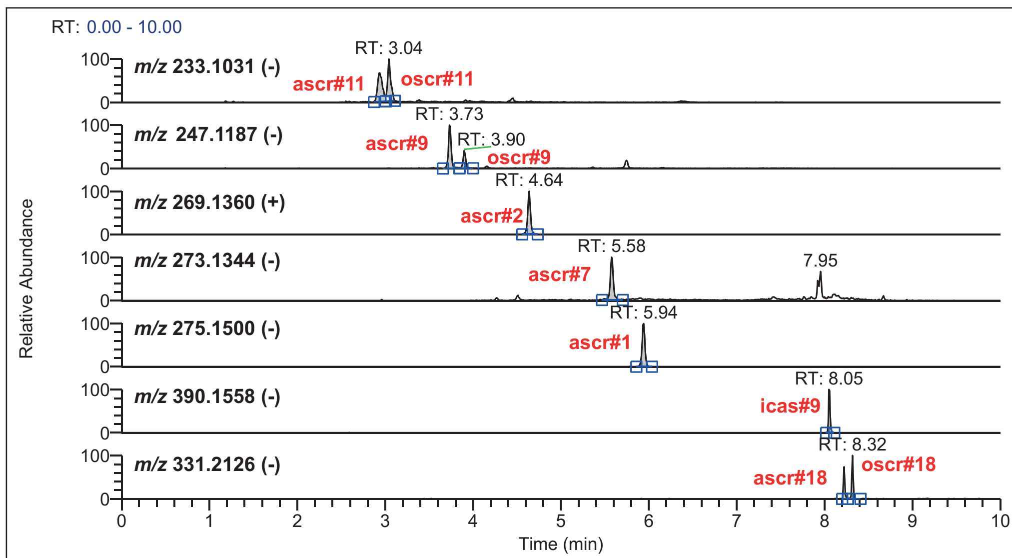

Figure 6. XICs of ascarosides, RPLC. Data from *C. elegans* pellet. Chromatograms are ±5 ppm.

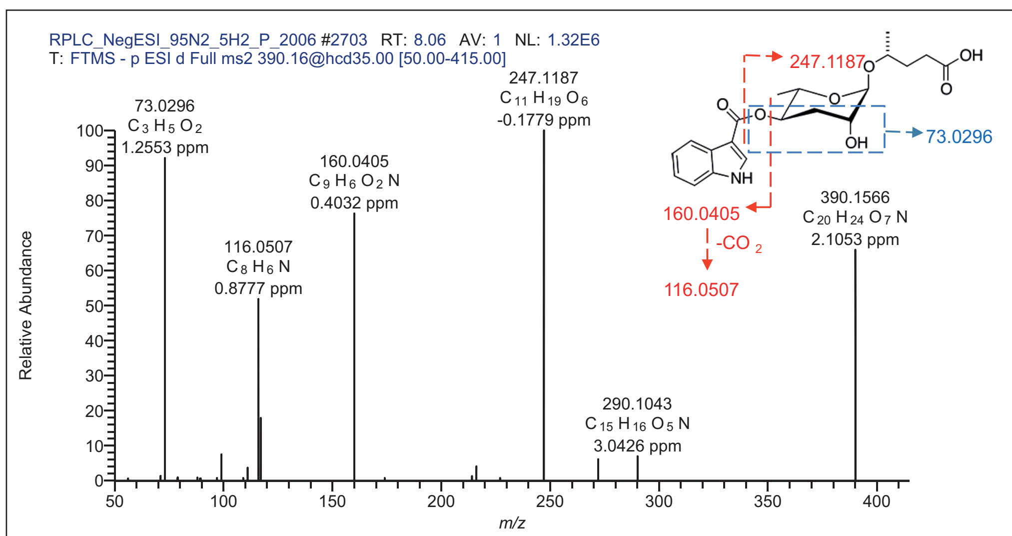

Figure 7. HR/AM MS/MS, icas#9. *m/z* 73.0296 is a diagnostic negative ion fragment for all ascarosides.

Conclusion

The IROA protocol enabled accurate metabolite measurement in the study through the following:

- Pooling control and experimental samples prior to sample extraction and LC-MS/MS analysis and reducing sample-to-sample variance and the impact of ion suppression
- Discriminating biosynthesized molecules and artifacts and noise
- Determining the number of carbons in each biological molecule, constraining the possible number of formulae
- Providing relative quantitation by determining the $^{12}C/^{13}C$ ratios of analytes in each sample

IROA labeling and the ClusterFinder software together with HR/AM on the Q Exactive mass spectrometer 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 to provide a complete metabolomics picture for hydrophilic and lipophilic compounds. The HR/AM MS/MS data provided 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.

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