Chapter 15

Exometabolomic Mapping of *Caenorhabditis elegans:* A Tool to Noninvasively Investigate Aging

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Abstract

Metabolomic analyses can provide valuable information about the internal metabolism of an organism; however, these studies can become quickly complicated by the large number of metabolites that are often detected. Overcoming this limitation requires high-resolution analytical separation techniques, coupled with high-power deconvolution software. Additionally, much care must be taken in metabolomic sample preparation to quench active enzymes and avoid artifactual changes in the metabolome. Here we present a relatively simple and straightforward technique, exometabolome mapping, which bypasses each of these concerns, is noninvasive, and provides a concise summary of the key metabolic processes operative in an organism. We illustrate our method using the nematode *C. elegans*, an organism which has been widely exploited in aging studies; however, with only minimal modification, our technique is extendible to other sample types, and indeed we have successfully used it both to perform yeast footprinting and to study the excreted metabolic end products of human kidney cancer cell lines.

Key words Metabolomics, Metabonomics, Aging, Exometabolome mapping, *Caenorhabditis elegans*

1 Introduction

Metabolomics is an "omics" science which seeks to examine the metabolome—the ensemble of metabolic reactants, intermediates, and products of a cell, tissue, or organism. In a hierarchical sense, metabolomics falls at the very bottom of genomics (DNA), transcriptomics (RNA), and proteomics (proteins). Understanding which metabolic pathways are operative in a system, and how these pathways are interwoven into networks, is changing how we view disease and aging.

Caenorhabditis elegans is a well-studied model organism which has been exploited over the past several decades to make advances in multiple fields, including aging, genetics, and neurobiology [1–4]. When grown in liquid culture, *C. elegans* excretes metabolites directly into its surrounding medium. This ensemble of excreted metabolites—the exometabolome—is distinct from

defecatory waste products and can be easily collected and mined. Since *C. elegans* defecates continually every 45 s [5], the intestine is cleared prior to the end of our washing procedure, ensuring that only excreted metabolites are collected. Because the exometabolome of *C. elegans* consists of only a few hundred types of metabolites (compared with potentially thousands of internal ones), and because the exometabolome is essentially free of other interfering compounds, including metabolic enzymes, to a first approximation the key operational elements of intermediary metabolism can be reconstructed.

The abundance of aging studies which have been accomplished using *C. elegans* stems from its short lifespan (15 days at 25 °C, [6]), the ease with which genetic manipulations can be carried out, and its high reproductive rate. Moreover, the great abundance of genetic mutants, RNAi clones, and environmental interventions that significantly alter worm lifespan [7–11] have made *C. elegans* one of the premier model organisms used to investigate aging processes. Ongoing studies in our laboratory have revealed that several long-lived genetic variants of *C. elegans* share a unique type of metabolism. These findings raise the possibility that metabolic configuration, per se, may contribute to lifespan extension in this organism. In this regard, we have previously reported that the exometabolome of long-lived mitochondrial (Mit) mutants of *C. elegans* can be distinguished from that of wild-type animals using HPLC [12]. Here we present detailed methods for the collection, derivatization, and analysis of the exometabolome from *C. elegans* using gas chromatography–mass spectrometry (GC-MS). Raw GC-MS dataset files for wild-type worms and long-lived *eat-2(ad465)* mutants are provided as training tools. The *eat-2(ad465)* mutant strain has impaired pharyngeal pumping, resulting in slowed eating, and is used to study calorie restriction, an established intervention to increase lifespan, in *C. elegans* [13].

2 Materials

All solutions should be prepared using the highest-purity water available. We recommend using double-distilled water. Unless otherwise noted, all solutions can be stored at room temperature. All solvents should be LC/MS grade or better.

2.1 Bacterial Food Stock

1. *E. coli* bacteria (OP50): Purchased from the Caenorhabditis Genetics Center, Minneapolis, MN (http://www.cbs.umn.edu/CGC/).

2. Luria Bertani (LB) broth: Dissolve 10 g bacto-tryptone, 5 g yeast extract, and 5 g sodium chloride in 800 mL water, and then add water to bring to a total volume of 1 L. Aliquot into

2 × 1 L glass autoclavable bottles (500 mL/bottle) and autoclave for 30 min using liquid cycle settings (*see* **Note 1**).

3. Once LB has cooled to room temperature, transfer 500 mL to a 2-L Erlenmeyer flask.

4. Inoculate LB with a single colony of *E. coli* (OP50), and incubate overnight at 37 °C on a platform shaker set at 200 rpm (*see* **Note 2**).

5. Following incubation, use a spectrometer to measure the absorbance of the bacterial stock (1:10 dilution in LB) at 595 nm. If the optical density (OD_{595}) of the diluted stock is less than 0.3, the culture should be concentrated by centrifugation (4,250 × g, 10 min, 4 °C) and resuspended in a lesser volume of LB.

6. Dilute OP50 to a final OD_{595} of 2.5 (undiluted), aliquot into 50-mL conical tubes, and store aliquots at 4 °C.

2.2 BNGM Agar Plates

For our experiments we culture animals on BNGM agar plates. These differ from traditionally used nematode growth medium (NGM) plates in that they contain a greater amount of bacteriological peptone, which allows for growth of a thicker lawn of bacteria.

1. Bacteriological peptone (Sigma-Aldrich).

2. Sodium chloride.

3. Agar.

4. Polystyrene petri dishes, 100 mm × 15 mm (Fisher).

5. Calcium chloride, 1 M: Dissolve 147.02 g calcium chloride monohydrate in 800 mL water, and then add water to bring to a total volume of 1 L. Aliquot into 500-mL glass autoclavable bottles (200 mL/bottle), and autoclave for 30 min using liquid cycle settings (*see* **Note 1**).

6. Cholesterol, 5 mg/mL: In a 500-mL beaker, dissolve 1.0 g cholesterol in 200 mL 95 % ethanol with gentle heating. Filter sterilize the solution using a polystyrene filter system with a 0.22-μm polyethersulfone membrane (Corning).

7. Magnesium sulfate, 1 M: Dissolve 120.4 g anhydrous magnesium sulfate in 800 mL water, and then add water to bring to a total volume of 1 L. Aliquot into 500-mL glass autoclavable bottles (200 mL/bottle), and autoclave for 30 min using liquid cycle settings (*see* **Note 1**).

8. Potassium phosphate, 1 M: Dissolve 98.0 g anhydrous potassium phosphate (monobasic) and 48.0 g anhydrous potassium phosphate (dibasic) in 800 mL water. Check the pH with pH paper or a pH meter, it should be near 6. Add water to bring to a total volume of 1 L, and aliquot into 500-mL glass autoclavable bottles (200 mL/bottle). Autoclave for 30 min using liquid cycle settings (*see* **Note 1**).

9. In a 4-L polypropylene beaker (Nalgene), mix 20 g peptone, 6 g sodium chloride, 40 g agar, and 2 L water. Using a magnetic stir bar, stir to partly dissolve. Autoclave contents, with stir bar, for 1.75 h using liquid cycle settings.

10. Following autoclave cycle, allow heated solution to partially cool by stirring at room temperature for 30 min. Then add 2 mL 1 M calcium chloride, 2 mL 5 mg/mL cholesterol, 2 mL 1 M magnesium sulfate, and 50 mL 1 M potassium phosphate.

11. Using an automatic liquid dispenser (Wheaton Omnispense), or manually, dispense 10 mL of solution into each 10-cm dish, until all solution is used (*see* **Note 3**). Cover dishes and allow to dry at room temperature for 2 days.

12. Dilute OP50 (OD$_{595}$ 2.5) 1:750 using LB; you will need 0.5 mL of this solution per plate.

13. Using an automatic pipet with a sterile tip, spot 500 μL of OP50 dilution per plate, six at a time. Once plates have been spotted, spread the bacteria evenly across the plates using a sterile 60-mm cell spreader (Fisher). Before starting, and after each set of six plates, sterilize the spreader by submerging in 95 % ethanol. Then remove the spreader and pass it through a flame to burn off the alcohol.

14. Allow the bacteria to dry on the plates for 1 day. Plates that are to be used within 1 week can be stored at room temperature. Plates that will not be immediately used can be stored at 4 °C for 1–2 months.

2.3 S-Basal Buffer

1. In a 1-L beaker, mix 4.4 g anhydrous potassium phosphate (dibasic), 3.4 g anhydrous potassium phosphate (monobasic), and 5.85 g sodium chloride.

2. Add water to the beaker to fill to approximately 800 mL and stir until all salts are dissolved. The pH should be between 6 and 7.

3. Add water to bring to a final volume of 1 L.

4. Aliquot into 500-mL autoclavable glass bottles (300–400 mL per bottle).

5. Loosely cap bottles and autoclave for 30 min (liquid cycle). Let cool to room temperature prior to removing from the autoclave. Store cooled S-Basal at room temperature. At least one bottle should be stored at 4 °C, for use in sucrose flotation.

2.4 Sucrose Flotation and Exometabolome Collection

1. S-Basal (25 and 4 °C).

2. Polypropylene conical tubes, 15 and 50 mL (*see* **Note 4**).

3. Pasteur pipets.

4. Sucrose, 50 % w/v: In a 50-mL gradated polypropylene tube, weigh 25 g sucrose. Fill with water to the 40-mL line, and then vortex or sonicate until sucrose is completely dissolved. Fill to the 50-mL line with water, and then sterilize the solution by passing it through a 25-mm Nalgene syringe filter with a 0.2-μm cellulose acetate membrane (Thermo). Store the sterilized solution at 4 °C.

5. Gelatin, 0.5 % w/v: Dissolve 50 mg gelatin in 10 mL water.

6. Glass petri dishes (custom-made from Kimax) (3-cm inner diameter), sterilized.

7. Polymer filter membranes, 0.2-μm nylon-66 membrane (Life Science Products, Cat. No. 6502-413X; *see* **Note 5**).

2.5 Protein Quantification Components

1. Sodium dodecyl sulfate (SDS), 1.25 % w/v: Dissolve 2.5 g SDS in 200 mL water.

2. Pierce BCA protein assay kit (Thermo; *see* **Note 6**).

2.6 Standards

1. 3,4-Dimethoxybenzoic acid (Sigma-Aldrich). In a 15-mL conical polypropylene tube (Becton Dickinson), mix 18.2 mg 3,4-dimethoxybenzoic acid with 9 mL water and adjust the pH to 10 with 1 M NaOH, and then bring the final volume to 10 mL with water (10 mM final concentration). Protect the solution from light by wrapping the tube with aluminum foil and store at room temperature.

2. Sodium phenylpyruvate monohydrate (Alfa Aesar): Dissolve 10.2 mg sodium phenylpyruvate monohydrate in 10 mL water (5 mM). Partition into 1 mL aliquots and store in 2-mL conical polypropylene tubes (Eppendorf, Cat. No. 022363352; *see* **Note 5**). Further partition 1 mL of solution into 100 μL aliquots and store all aliquots at −80 °C. Each time a 100 μL aliquot is used, it should be disposed of afterwards. One milliliter aliquots can be thawed once and partitioned into 100 μL aliquots as needed.

3. L-Norvaline (Alfa Aesar): Dissolve 5.9 mg L-norvaline in 10 mL water (5 mM). Partition into 1 mL aliquots and store in 2-mL conical polypropylene tubes (Eppendorf, Cat. No. 022363352; *see* **Note 5**). Further partition 1 mL of solution into 100 μL aliquots and store all aliquots at −80 °C. Each time a 100 μL aliquot is used, it should be disposed of afterwards. One milliliter aliquots can be thawed once and partitioned into 100 μL aliquots as needed.

4. 3-Methylvaleric acid (Sigma-Aldrich): In a 15-mL conical polypropylene tube (Becton Dickinson), mix 12.5 μL 3-methylvaleric acid with 10 mL water (10 mM). Store at 4 °C.

5. Amino Acid Standard H (Thermo): 2.5 mM each (in 0.1 N HCl) ammonia, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine

HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-valine; 1.25 mM L-cystine.

6. Group A standards: 100 μL each (10 mM stocks, prepared in water at 25 °C unless otherwise noted) 3-methyl-2-oxopentanoic acid, L-malate, fumaric acid (made neutral with NaOH), lactate, DL-2-hydroxybutyrate, hypoxanthine (hot water), 400 μL S-Basal. Separate into 100 μL aliquots and store at −80 °C.

7. Group B standards: 100 μL each (10 mM stocks, prepared in water at 25 °C unless otherwise noted) 4-methyl-2-oxovalerate, succinate, pyruvate, xanthine (hot water), ethanolamine, 3-hydroxy-3-methylbutyrate, 400 μL S-Basal. Separate into 100 μL aliquots and store at −80 °C.

8. Group C standards: 100 μL each (10 mM stocks, prepared in water at 25 °C unless otherwise noted) 3-methyl-2-oxobutanoic acid, citrate, α-ketoglutarate, 2-oxobutyrate, citramalate, urea, 400 μL S-Basal. Separate into 100 μL aliquots and store at −80 °C.

9. Volatiles standards: In 50 mL S-Basal, dissolve each of the following (1 mM each)—4.8 mg sodium propionate, 4.2 μL methacrylic acid, 4.6 μL butyric acid, 4.5 μL isobutyric acid, 6.3 mg sodium 3-hydroxybutyrate, 5.4 mg lithium acetoacetate, 5.0 mg tiglic acid, 5.0 mg 3-methylcrotonic acid, 6.3 μL hexanoic acid, 5.5 μL valeric acid, 5.5 μL isovaleric acid, 4.4 mg acetoin, 5.5 μL 2-methylbutyric acid. Store at 4 °C.

2.7 Derivatization Reagents

1. Acetonitrile.

2. Diethyl ether (*see* **Note 7**).

3. Hydrochloric acid (1 M).

4. GC-MS autosampler vials (specific for MS instrument).

5. Conical glass inserts (Supelco, Cat. No. 24703).

6. Nitrogen gas (or other inert gas) cylinder with regulator.

7. Rubber tubing (should fit gas regulator).

8. Pasteur pipets.

9. Methoxylamine hydrochloride (Sigma-Aldrich). This should be prepared fresh prior to derivatizing each batch of samples by dissolving 20 mg in 1 mL of anhydrous pyridine (*see* **Note 8**). This volume will be sufficient to derivatize up to 25 samples. Store unused methoxylamine hydrochloride (dry powder) in a desiccator. Unused methoxylamine hydrochloride/pyridine solution should be properly disposed.

10. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide + 1% *tert*-butyldimethylchlorosilane (MTBSTFA + TBDMS, Thermo Scientific). This should be purchased as 1 mL aliquots in glass ampules. Discard opened ampules after use. Store unopened ampules at 4 °C.

2.8 Software	The following software will be used to analyze GC-MS spectral data. All programs listed here are free to download and use and are available at the URLs listed below. In the following section, we have chosen to present a highly supervised method for analysis, to aid the novice researcher with limited experience in manipulating the multidimensional data produced by GC-MS; however, it should be noted that alternate software programs are available which will largely automate this process (*MetaboliteDetector* [14], *Metabo Analyst* [15]).

1. *Automated Mass Spectral Deconvolution and Identification System* (*AMDIS*)—http://chemdata.nist.gov/mass-spc/amdis/downloads/

2. *MSSearch*—http://chemdata.nist.gov/mass-spc/ms-search/downloads/

3. *MET-IDEA*—http://bioinfo.noble.org/download/

4. *LIB2NIST*—http://chemdata.nist.gov/mass-spc/ms-search/Library_conversion_tool.html

5. *Python* (optional)—http://www.python.org/

3 Methods

3.1 Exometabolome Collection	1. Synchronous populations of animals should be grown on 10-cm BNGM agar plates spotted with OP50 bacteria (*see* **Note 9**). Animals should be plated at a density of 10,000/10-cm plate (11,000 worms can be used for mutant strains of smaller adult size), to allow them to reach gravid adulthood without starving. We use 120,000 animals (approximately 0.3 g) for each analysis (60,000 minimum; *see* **Note 10**). The number of animals needed will depend on the sensitivity of the mass spectrometer used for the analysis. Plates should be stored in a 20 °C incubator until animals reach gravid adulthood.

2. Using S-Basal (10 mL per plate), wash animals off of BNGM agar plates. Wash plates five at a time, and combine the washes in a 50-mL conical polypropylene tube. Allow animals to settle by gravity, and then remove and discard the supernatant. Combine animals into a single 50-mL tube, and then wash five more times with 50 mL S-Basal. It will take approximately 5–6 min for the animals to settle with each wash. This method is preferable to centrifugation, since it selectively removes eggs and L1 larvae from the animal pool.

3. *This should be done concurrently with* **step 2**: Gelatin passivate two 15-mL conical polypropylene tubes (one should be pre-weighed) by adding 0.5 mL gelatin to each tube, bringing the total volume to 10 mL with distilled water, and capping and

inverting to coat the inside of the tube. This solution should also be used to passivate the inside of two glass Pasteur pipets. This procedure helps to prevent animals from sticking to the inside of glass and plasticware. Thoroughly wash tubes and pipets by rinsing five times with double distilled water.

4. *This should be done concurrently with* **step 2***:* To the pre-weighed tube, aliquot 10 mL cold (4 °C) S-Basal. To the other tube, aliquot 7 mL 50 % sucrose. Keep both tubes on ice until ready to use.

5. Following the final wash, reduce the total volume in the tube to 3 mL. This is best done by aspirating the supernatant down to the 5-mL gradation line and then removing 2 mL with an automatic pipet.

6. Pour the cold sucrose into the 50-mL tube, and then transfer back into the gelatin-passivated tube. Immediately centrifuge at $2,400 \times g$ for 3 min at 4 °C. *The centrifuge should be prechilled prior to this step* (*see* **Note 11**). Use the cold S-Basal as a balance.

7. Using a gelatin-passivated Pasteur pipet, transfer the floating animals to the second passivated tube, containing cold S-Basal (*see* **Note 11**).

8. Pellet animals at 1,200 rpm (2 min, $280 \times g$) at 4 °C. *The centrifuge should be prechilled prior to this step* (*see* **Note 11**).

9. Remove and discard supernatant. Wash the pellet two more times by filling the tube with room temperature S-Basal and centrifuging.

10. Carefully remove the supernatant down to the worm pellet, and weigh the tube to determine the mass of the loosely packed animals.

11. Add S-Basal to bring the total volume to 1.4 mL (loosely packed worms have a density near 1 g/mL) and transfer to a 3-cm glass dish using the second gelatin-passivated Pasteur pipet. Place the dish (uncovered) in a loosely sealed container with moist paper towels to minimize sample loss through evaporation. Then place the entire assembly on an orbital shaker set at 100 rpm and agitate at room temperature for 18 h. This step is necessary to prevent the animals from going anoxic during the collection period. If available, the shaker can be placed in an incubator set at 20 °C for the 18-h period.

12. At the end of the incubation period, transfer the animals and supernatant to a 1.5-mL polypropylene microcentrifuge tube using a gelatin-passivated Pasteur pipet (*see* **step 3**). Centrifuge for 3 min at $2,000 \times g$, and filter the supernatant through a 0.2 μm nylon syringe filter. Aliquot into 100 μL portions into 1.5-mL polypropylene tubes (Eppendorf, Cat. No. 022363352; *see* **Note 5**), and store the worm pellet and the aliquotted supernatant at −80 °C.

3.2 Protein Extraction and Quantification

To control for variations in the number of animals per experiment, as well as for the fact that different genetic mutants will have different adult sizes, the intensity of metabolite GC peaks will be normalized to the total protein content of the animals contributing to the sample.

1. Thaw frozen worm pellets at room temperature. Add S-Basal to adjust the total volume of each tube to 1.0 mL and transfer to 15-mL conical polypropylene tubes.

2. Add 1.25 % SDS to bring the total volume in each tube to 5 mL. This will result in a final SDS concentration of 1.0 % w/v.

3. Puncture the lids of the polypropylene tubes with a hot needle. Place tubes in boiling water for 5 min. Allow tubes to cool briefly (30–60 s) and then mix by briefly vortexing (2 s). Return to boiling water for an additional 5 min, and then remove and allow to briefly cool before proceeding.

4. Place samples in a centrifuge, and centrifuge for 5 min at $2,400 \times g$, and then remove the supernatant, *carefully avoiding the loose cuticle pellet* (*see* **Note 12**).

5. Quantify the total protein in each sample using a commercially available protein assay kit. The manufacturer's instructions on the assay kit should be followed. If samples are not to be used immediately for protein quantification, they can be stored at $-80\ ^{\circ}$C (*see* **Note 13**).

3.3 Derivatization Procedure for Standard (Nonvolatile) Metabolites

Internal standards should be added to samples and samples dried overnight, *the night prior to derivatization and analysis by GC-MS.*

1. Thaw previously frozen aliquots (100 µL) of exometabolome samples to be analyzed on ice. In 1.5-mL polypropylene tubes (Eppendorf, Cat. No. 022363352; *see* **Note 5**), prepare each of the following. Blank, 100 µL S-Basal; full standards, 100 µL S-Basal, 10 µL group A standards, 10 µL group B standards, 10 µL group C standards, and 5 µL amino acid standards.

2. To each sample to be derivatized (including blank and standards samples), add 575 µL acetonitrile (*see* **Note 14**) and 10 µL each of 3,4-dimethoxybenzoate, phenylpyruvate, and norvaline.

3. Dry samples overnight in a vacuum centrifuge (no heat).

4. *The following morning*, add 40 µL methoxylamine HCl to each dried sample and top tubes with dry nitrogen gas. This is done by inserting a glass Pasteur pipet into a piece of rubber tubing connected to the gas tank regulator and using the tip of the pipet to blow nitrogen or inert gas into samples tubes for 5–10 s. Vortex samples briefly (2–5 s) and incubate for 90 min at 30 °C. Samples should be mixed by vortexing at least once during this period.

5. Add 60 μL MTBSTFA + TBDMS to each sample and top with dry nitrogen. Incubate for 1 h at 80 °C (*see* **Note 15**).

6. Pellet any insoluble material (mostly salt) by centrifugation, and transfer 50 μL to a glass insert inside a glass autosampler vial. Top with dry nitrogen and seal.

3.4 Derivatization Procedure for Volatile Metabolites

This procedure is for derivatization of the compartment of the exometabolome which contains highly volatile metabolites (e.g., short-chain fatty acids) that would be lost during the drying step of the standard derivatization procedure. All steps in this procedure should be performed *the same day as samples are to be analyzed by GC-MS.*

1. Thaw previously frozen aliquots (100 μL) of exometabolome samples on ice. In 1.5-mL polypropylene tubes (Eppendorf, Cat. No. 022363352; *see* **Note 5**), prepare each of the following. Blank, 100 μL S-Basal; full standards, 100 μL S-Basal and 10 μL volatiles standards.

2. To each sample, add 20 μL 1 M HCl; the pH should be between 1 and 2.

3. Extract metabolites by adding 300 μL diethyl ether to each sample and vortexing for 30 min at room temperature.

4. Centrifuge samples at 800 × g for 5 min to separate phases. Remove and save the upper organic layer.

5. Using a gentle stream of nitrogen, concentrate the organic phase to approximately 50 μL.

6. Add 40 μL methoxylamine HCl to each sample and top tubes with dry nitrogen gas. Vortex samples and incubate for 90 min at 30 °C. Samples should be mixed by vortexing at least once during this period.

7. Add 30 μL MTBSTFA + TBDMS to each sample and top with dry nitrogen. Incubate for 30 min at 80 °C (*see* **Note 15**).

8. Transfer 50 μL of each sample to glass inserts inside glass autosampler vials. Top with dry nitrogen and seal. Keep samples cool until analysis.

3.5 GC-MS and Analysis of Mass Spectral Data

We highly recommend that novice GC-MS users send their samples to a mass spectrometry facility for analysis. Many institutions will have an on-site facility equipped for GC-MS; however, if samples must be shipped off-site for analysis, we have found that overnight shipping on dry ice results in insubstantial loss of sample integrity.

We analyze our samples using a Thermo Fisher TRACE DSQ single quadrupole mass spectrometer. The following parameters will allow a competent technician to reproduce the analysis. GC: column, ZB-5MS (Phenomenex; Torrance CA), 30 m × 0.25 mm, 0.25-μm film thickness; carrier gas, helium; linear velocity, 1 mL/min (constant flow); injection, split, 10-mL/min split flow; injector

temperature, 220 °C; column temperature program (standard metabolites), initial temperature of 70 °C held for 1 min followed by an increase to 310 °C at 5 °C/min; column temperature program (volatile metabolites), initial temperature of 50 °C held for 1 min, followed by an increase to 80 °C at 10 °C/min. Hold for 3 min at 80 °C and then increase to 275 °C at a rate of 30 °C/min. MS conditions: ionization, electron impact (70 eV); detection, positive ion; full scan analyses, *m/z* 50—*m/z* 700 at 2 scans/s.

Analysis of GC-MS data consists of (1) peak deconvolution, (2) peak integration, (3) preprocessing the dataset (e.g., removing artifactual peaks and low-abundance features), (4) metabolite identification, and (5) data visualization and clustering. Here we will present a basic flow-through for processing raw GC-MS data. Due to the limited space available, we will only cover the basic functions needed to process the data; however, many of the software programs that are employed have functionalities beyond what is described here. We have provided a comprehensive software tutorial as a supplementary online file to address some of these functions. This tutorial is presented in a format that would be understandable by a new graduate student entering a lab and who has no prior experience in MS. Before proceeding, make sure all of the software programs listed in Subheading 2.8 are installed on your computer. Detailed installation instructions for these programs can be found in the supplementary tutorial file. The following section assumes a basic familiarity with GC-MS terminology; those researchers with no prior experience with GC-MS are advised to skip this section and go straight to the online tutorial.

1. The format of the raw GC-MS data files will depend on the instrument manufacturer. For example, data acquired on Thermo instruments will be in the format ".RAW." These will need to be converted into a universal format before continuing. For our purposes, we will require data to be in the ".CDF" format. The proprietary software that comes with your instrument should be able to convert its MS data into this format. If you do not have access to this software, you can request that the MS facility provide you with data in ".CDF" format.

2. The software program *AMDIS* will be used to identify peaks and to deconvolute peaks that partially co-elute. Using this program, open one of your data files, or, alternatively, use one of the example files provided with the tutorial.

3. Select the "Settings" tab under the "Analyze" menu—note that this will be inaccessible unless a file is open. A complete discussion of the settings which can be optimized is given in the supplementary software tutorial. Make sure that the correct instrument type is selected under the "Instrument" tab. When you are finished, click save and then press the button that says "Run."

Table 1
Example of a partial ion retention time list

287.1	27.85	100066	False	False
250.1	27.92	100144	True	True
299.15	27.95	100067	False	False
198.16	28.69	100070	False	False
345.19	28.83	100072	False	False
303.14	29.13	100074	False	False
171.1	29.28	100075	False	False
272.11	30.85	100077	False	False
357.09	30.93	100174	False	False
332.42	31	100170	False	False
218.05	31.1	100078	False	False
292.12	31.1	100079	False	False
239.02	31.28	100080	True	True
288.17	31.44	100081	False	False
271.17	31.91	100165	False	False

4. *AMDIS* will attempt to identify unique peaks in the GC trace. Once one of these peaks is selected, the center panel will display the *total ion count* peak, as well as several representative masses which best describe the shape of that peak. Write down the retention time of each peak, and the mass which best describes it. Try to avoid using masses 73, 75, and 147—these are due to rearrangement of the derivatizing group and are common to many peaks.

5. In order to integrate the peaks in your GC trace, you will need to create an ion retention time (IRT) list. Using standard spreadsheet software, such as *Microsoft Excel*, create a new spreadsheet. The first two columns should contain the masses (column 1) and retention times (column 2) of each of the peaks you identified using *AMDIS*. The third column will contain a unique ID which you assign to each peak. The last two columns will indicate whether a particular metabolite is to be used as a calibration standard for mass (column 4) or retention time (column 5). Identify your three internal standards and enter values of "TRUE" in both of these columns. For all other metabolites enter "FALSE" in both columns (*see* **Note 16**). An example of what this file should look like is given in Table 1. Save the file as a tab-delimited text file with the extension ".ION" and exit *Excel*. Close *AMDIS*; you do not need to

save the ".FIN" and ".ELU" files that this program will automatically create.

6. Following identification and deconvolution in *AMDIS*, you will open your data in *MET-IDEA*. All of your data (including runs for your blank and standards) should be in the same folder as the IRT list which you just created.

7. Once opening *MET-IDEA*, you can adjust the settings by selecting "Parameter Setup" under the "Tools" menu (see the supplementary tutorial file for a description of these settings). After adjusting the settings, select "Start" under the "MET-IDEA" menu.

8. Browse to find your IRT list (you will need to change the file extension type to make this file visible). Select the boxes for "Show intermediate results," "Calibrate Retention Time," and "Calibrate Mass Data," and then press "OK."

9. After prompting you to save your calibration data, the program will integrate the peaks in your IRT list for each of the spectra in the folder, and read out an integrated peak area in arbitrary units. Check the integration limits on each of these peaks and adjust them accordingly (*see* **Note 17**). The amount of time this takes will depend on how well you optimized your parameters. A description of common integration errors is found in the software tutorial.

10. After you have checked your peaks, transpose your data, then select "OK," and save your results. *Met-IDEA* will save these as an ".OUT" file, which can be opened as a delimited text file in *Excel*. This will contain a list of peaks with their integrated values for each dataset.

11. Before visualizing your data, you will need to do some preprocessing on the ".OUT" file. First, you should identify an appropriate noise cutoff for your data. A good way to do this is to integrate several areas of data which do not contain any well-defined peaks. Select integration limits appropriate to the average peak width on your instrument (typically around 0.1 min). For our instrument, we have determined that an integrated intensity of less than 10,000 is likely noise. Any peak which has an integrated intensity less than your experimentally determined threshold in all samples can be considered noise, and these rows removed from the data file. Also, any peaks which are due to artifacts (including phosphate) should be removed at this point.

12. Normalize your data by dividing every peak in a sample by the intensity of 3,4-dimethoxybenzoate and by the total amount of protein (experimentally determined, in mg) for that sample and then multiplying by 100,000,000 to return the data to its original scale. This can be done either in *Excel* or by running

a script using the programming language *Python,* as described in the online tutorial file. After doing this, remove the lines corresponding to your exogenously added internal standards. You may also remove the data columns for your blank and standards samples.

13. Reformat the first column to remove the retention time and mass for each component. Where possible, replace the ID numbers with appropriate names. Identities of compounds can be determined through library matching with *MSSearch* or *AMDIS* if an appropriate mass spectral library is available. Most available MS libraries are in ".MSP" format; if using *AMDIS* for library matching, you will need to use the program *LIB2NIST* to convert your library to the correct format for this software. The MS library used in our laboratory is included in the online supplemental files. Identities can also be confirmed by running standards. More information on MS libraries and the types of fragmentation patterns produced by *t*-BDMS derivatized compounds can be found in the supplementary tutorial file.

14. We use the online program *GenePattern* for visualization and clustering of data. This software is free to use after registration. To use this program, your data must be reformatted to ".GCT" format. This can be done manually in *Excel*; details on this file format can be found at: http://www.broadinstitute.org/cancer/software/genepattern/tutorial/gp_fileformats.html. Alternatively, one can use a *Python* script to reformat the data, as detailed in the online tutorial. Go to http://www.broad-institute.org/cancer/software/genepattern/ and select "GP @ Broad" to run an analysis on the server at the Broad Institute.

15. Select "Clustering," then "Hierarchical clustering" in the pop-up window that appears. Under "Step 2" select "Open Hier-archicalClustering."

16. Select the location of your ".GCT" file, and then set "column distance measure" and "row distance measure" both to "Pearson correlation." For "clustering method" select "Pairwise complete-linkage." Do not change any other settings. Click "Run."

17. When the analysis is complete, three files will be generated. Access the drop-down box to one of these files by clicking on the arrow that appears next to it. Select HierarchicalCluster-ingViewer. When the new dialogue comes up, select "Run." A clustered heat map of your data will be generated. It is possible to toggle the display between a global array (across all cells) or a relative array (across rows). Export your data as an image file (Fig. 1).
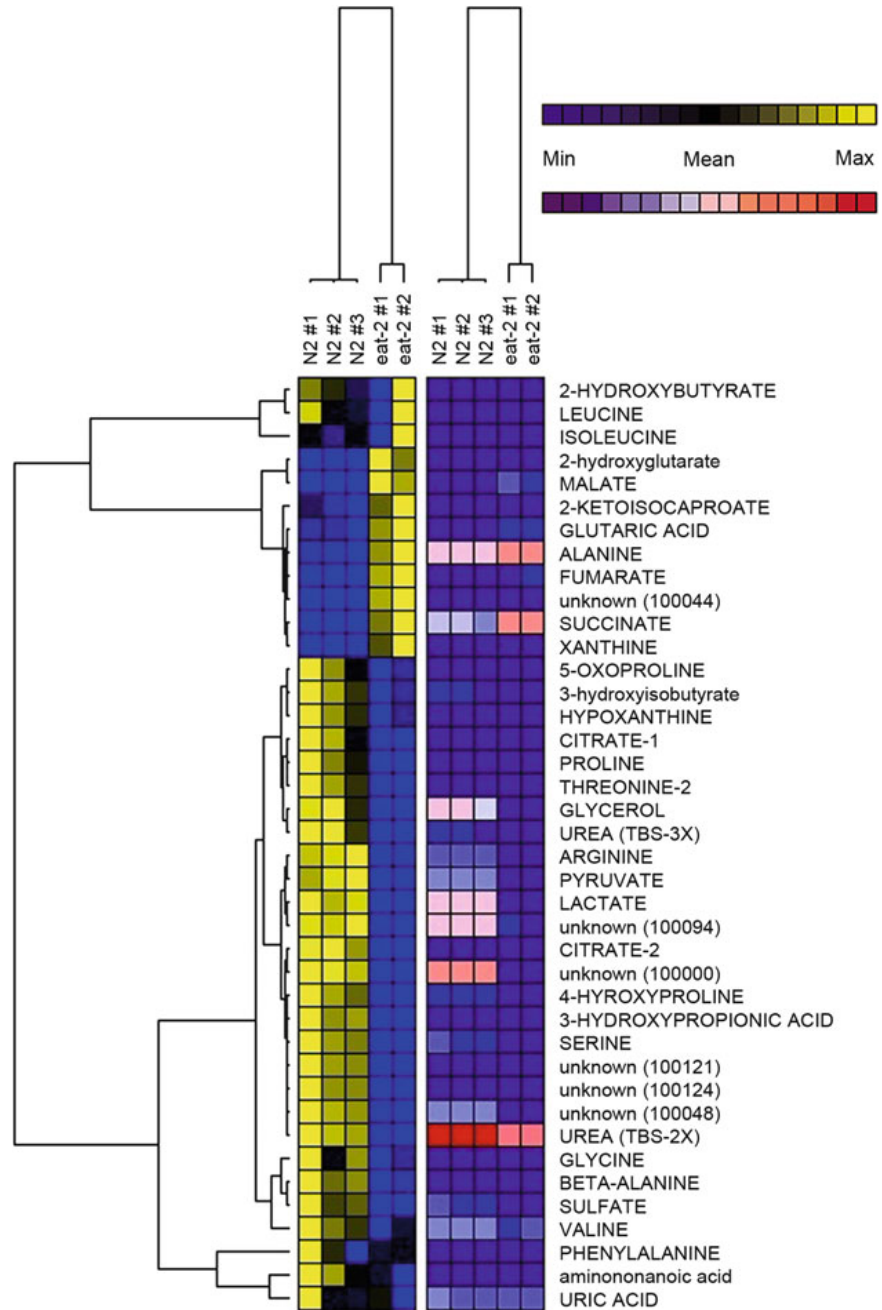
Fig. 1 Hierarchical clustering was used to segregate exometabolome samples from wild-type (N2) animals and *eat-2(ad465)* mutants. Integrated peak areas for each metabolite were normalized to an internal standard and to total protein. Low-abundance metabolites were removed prior to clustering. Heat maps are colored according to (1) individual metabolite variation across the sample set (*blue–yellow*) and (2) global variation among metabolites over the entire exometabolome dataset (*blue–red*)

4   Notes

1. When autoclaving liquids, caps should be loosely fitted to the glassware. Tightening the caps all the way can result in a buildup of pressure inside the bottle and in extreme instances bottle breakage. Solutions can become superheated in the autoclave and should be allowed to cool to room temperature prior to handling, to avoid sudden boiling.

2. To control for exogenous bacterial contamination, 5 mL of LB should be removed from the flask prior to inoculation and transferred to a 15-mL conical tube using sterile techniques. This tube should be taped to the side of the flask and the two incubated together overnight. If the control tube appears cloudy the next day, it indicates a contamination in the LB, and the entire batch should be discarded.

3. All components of the liquid dispenser which will come in contact with the agar solution (rubber tubing, nozzle) must be sterilized by autoclave prior to use. After dispensing all of the solution, the rubber hose should be immediately cleaned by pumping hot water through it. If this is not done, agar will solidify inside the tubing, clogging it and making it difficult to clean.

4. We have previously used 15-mL polystyrene tubes and found these to be susceptible to cracking during centrifugation. Therefore, it is recommended to use polypropylene only, since these are better able to stand high centrifugation speeds.

5. When using plasticware, it is important to be sure that no plasticizers leech out into solution. We have tested this particular brand and part number for leeching and found that it does not interfere with our analyses.

6. If an alternate protein assay is employed, it is critical that the investigator be sure that the reagents are compatible with concentrations of sodium dodecyl sulfate up to 1.25 % w/v.

7. Diethyl ether can potentially decompose over time, forming explosive peroxides. We recommend storing this reagent at 4 °C for up to a year. Expired reagent, or any bottle with white deposits around the cap, should be considered dangerous and disposed of properly.

8. Anhydrous pyridine is hygroscopic and care should be taken to prevent introduction of moisture. Water will interfere with the derivatization reactions.

9. In our experience, large populations of synchronous animals can be obtained by placing 10,000 animals (wild type, arrested L1 larval stage), on a 10 cm/OP50 plate and allowing them to starve out the plate. This will allow the animals enough food to

reach gravid adulthood and to lay eggs before starving. Once the eggs hatch, the larvae should not have enough food available to progress past the first larval stage and will arrest as L1s. Do not allow L1 larvae to remain starved beyond 24 h, otherwise they exit arrest at different rates when finally provided with food.

10. For triplicate experiments, we typically prepare 36 plates of worms and collect and pool adult worms prior to washing (Subheading 3.1, **step 2**). Following washing, we split the 360,000 animals into three separate populations for the 18-h metabolite collection step. For fully independent experiments, we recommend pouring three separate batches of 12×10-cm plates and using animals from three independent starting populations of L1 larvae.

11. The purpose of this step is to remove any remaining bacteria clinging to the cuticle of the animals. Exposure to high concentrations of sucrose places the animals under osmotic shock; therefore, these steps should be done as quickly as possible, and at 4 °C. In our hands, the sucrose float plus all subsequent washes should take no more than 15 min. While this is standard procedure in a *C. elegans* laboratory, it should be noted that we have not observed any significant differences in the exometabolome of wild-type animals treated with a sucrose flotation versus untreated animals [16].

12. It is important to aspirate over the cuticle carefully, since the cuticle does not dissolve in SDS, any remaining cuticle may adversely affect the protein analysis (unpublished observation). The GC-MS data generated later will be normalized to crude protein mass, so it is imperative to get an accurate reading.

13. After protein extraction, protein samples can be stored at −80 °C until analysis; however, we have experienced problems reproducing protein estimates when using frozen samples containing SDS. To circumvent this, we advise to reboil any frozen samples prior to analysis.

14. Acetonitrile precipitates enzymes and creates an azeotrope with water to aid in drying.

15. We have noticed that the Eppendorf polypropylene tubes have a tendency to pop open during incubation at 80 °C, possibly leading to contamination. To prevent this, we suggest resting a heating block or heat-resistant tube rack over the tubes to weigh down the lids. Alternatively, boiling locks may be used.

16. The exogenously added internal standards are easily identified, since they should be three of the largest peaks in your GC traces; they will also appear in the blank and standards samples. To aid you in identifying these peaks, the representative masses for each peak are 186.2 (norvaline), 250.1 (phenylpyruvate),

and 239.0 (3,4-dimethylbenzoate). Note that there will also be a large phosphate peak in all samples from the S-Basal buffer; do not confuse this as an internal standard. Phosphate has a representative mass of 383.2. It will be advantageous at this point to start a database which contains a list of ion masses and retention times, along with ID numbers and putative IDs.

17. Some peaks in your GC spectra will be artifacts arising from the derivatization procedure, as opposed to actual metabolites. These peaks can be identified by their presence in a blank sample, and should be noted accordingly as artifacts in your database for future reference. Rather than integrating these peaks, we opt to enter an integrated value of "0" to indicate that that peak was found but not integrated. To distinguish these peaks from actual metabolites, we enter a value of "1" for a metabolite with zero integrated intensity (i.e., the metabolite is not present in a given sample). Some peaks that appear to be artifacts may later turn out to be actual metabolites (e.g., if there is GC column bleed causing high-intensity metabolites to appear in the blank sample), so it is important to keep note of these peaks in case you wish to go back and integrate them later. We recommend, for the sake of time, removing peaks determined to be artifacts from your IRT list so that they are not integrated, but keeping track of them in a separate file. The set of full standards is for comparison and identification purposes, and peaks in this dataset do not need to be integrated properly, since they will not be part of the final analysis.

Acknowledgements

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