Preparation of Human Tissues Embedded in Optimal Cutting Temperature Compound for Mass Spectrometry Analysis
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1   **TITLE:**
2   Preparation of Human Tissues Embedded in Optimal Cutting Temperature Compound for Mass
3   Spectrometry Analysis
4
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21 **KEYWORDS:**
22 lipidomics, sphingolipid, biorepository, OCT, lung cancer, lung adenocarcinoma, non-small cell
23 lung cancer, tumor, optimal cutting temperature compound, LC-ESI-MS/MS, mass spectrometry
24

25 **SUMMARY:**
26 Sphingolipids are bioactive metabolites with well-established roles in human disease.
27 Characterizing alterations in tissues with mass-spectrometry can reveal roles in disease etiology
28 or identify therapeutic targets. However, the OCT-compound used for cryopreservation in
29 biorepositories interferes with mass-spectrometry. We outline methods to analyze sphingolipids
30 in human tissues embedded in OCT with LC-ESI-MS/MS.
31

32 **ABSTRACT:**
33 Sphingolipids are cellular components that have well-established roles in human metabolism and
34 disease. Mass spectrometry can be used to determine whether sphingolipids are altered in a
35 disease and investigate whether sphingolipids can be targeted clinically. However, properly
36 powered prospective studies that acquire tissues directly from the surgical suite can be time
37 consuming, and technically, logistically, and administratively challenging. In contrast,
38 retrospective studies can take advantage of cryopreserved human specimens already available,
39 usually in large numbers, at tissue biorepositories. Other advantages of procuring tissues from
40 biorepositories include access to information associated with the tissue specimens including
41 histology, pathology, and in some instances clinicopathological variables, all of which can be used
42 to examine correlations with lipidomics data. However, technical limitations related to the
43 incompatibility of optimal cutting temperature compound (OCT) used in the cryopreservation
44 and mass spectrometry is a technical barrier for the analysis of lipids. However, we have

45 previously shown that OCT can be easily removed from human biorepository specimens through
46 cycles of washes and centrifugation without altering their sphingolipid content. We have also
47 previously established that sphingolipids in human tissues cryopreserved in OCT are stable for up
48 to 16 years. In this report, we outline the steps and workflow to analyze sphingolipids in human
49 tissue specimens that are embedded in OCT, including washing tissues, weighing tissues for data
50 normalization, the extraction of lipids, preparation of samples for analysis by liquid
51 chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS), mass
52 spectrometry data integration, data normalization, and data analysis.
53
54 **INTRODUCTION:**
55 Sphingolipids are bioactive metabolites known for their roles in human metabolism and
56 disease[1,2]. They regulate complex cellular processes such as cell migration, cell survival and death,
57 cell movement, vesicular trafficking, cellular invasion and metastasis, angiogenesis, and the
58 production of cytokines[1–9]. Defects in the regulation of sphingolipid metabolism contribute to the
59 initiation and progression of cancers, determine how aggressive cancers are, and how cancers
60 respond to and develop resistance to therapy[3,10]. Therefore, because of these broad impacts on
61 the etiology of disease, analytical methods that can precisely establish disease-specific
62 sphingolipid alterations are important tools. Mass spectrometry (MS) is the most accurate and
63 reliable method to analyze sphingolipid alterations.
64
65 Human specimens that can be used for the analysis of sphingolipid alterations can be obtained
66 prospectively from the surgical suite or retrospectively from tissue biorepositories. Fresh tissues
67 from surgery are advantageous because they can be directly analyzed by MS or other analytical
68 methods. However, acquiring tissues prospectively has administrative, technical, and logistical
69 hurdles, and collecting sufficient specimens to reach statistical power can be challenging.
70 Obtaining tissues from biorepositories is advantageous because they can be acquired
71 retrospectively, in large numbers, and biorepositories confirm histology and pathology, use
72 standard operating procedures to cryogenically preserve and store tissues, and can provide
73 clinicopathological data that can be used for correlation analyses. However, to preserve
74 molecular and structural features, biorepositories may cryopreserve tissues by embedding them
75 in optimal cutting temperature (OCT) compound, which we have shown interferes with data
76 normalization assays and the quantification of sphingolipids by liquid chromatography
77 electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS)[11]. It has also been shown
78 that polyvinyl alcohol and polyethylene glycol, the primary components in OCT compound, result
79 in ion suppression in other MS analysis platforms[12–15]. Therefore, OCT compound must be
80 removed from tissues prior to sphingolipidomic analysis by MS.
81
82 In a previous report, we have validated a protocol for the removal of OCT compound from human
83 specimens for LC–ESI–MS/MS analysis[11] and the methodology used for weighing tissues for data
84 normalization[11]. Here, we detail the steps of the sphingolipidomic OCT compound-removal
85 protocol (sOCTrP) and show representative data from human lung adenocarcinoma tumors and
86 normal adjacent uninvolved tissues.
87
88 **PROTOCOL:**

89 De-identified human lung tissues were obtained from the Virginia Commonwealth University
90 (VCU) Tissue and Data Acquisition and Analysis Core under an internal review board (IRB)
91 approved protocol (#HM2471). The use of mice for research and harvesting of mice tissues was
92 approved by the VCU institutional animal care and use committee (IACUC).
93
94 **1. Preparation of materials**
95
96 NOTE: These steps should be performed a day prior to the tissue washing.
97
98 1.1. Pre-label and weigh 1.5 mL polypropylene centrifuge tubes with concise but clear identifier
99 codes for each sample that will be processed the next day. Use a chemically resistant permanent
100 marker (see **Table of Materials**) that will not fade under the repeated tube handling.
101
102 1.2. Open an electronic spreadsheet and label three consecutive columns with the following
103 column headers: tube identifier (ID), tube alone, tube w/tissue, and total tissue. Enter the tube
104 identifiers that will be processed into the column labeled tube ID.
105
106 1.3. Pre-calibrate and zero an analytical scale. Place a clean 10 mL Erlenmeyer flask in the center
107 of the balance plate (the flask will act as a tube holder). Close the weighing chamber door and
108 tare the scale with the flask.
109
110 1.4. Weigh 1.5 mL centrifuge tubes. Carefully place the tube in the flask and close the weighing
111 chamber door. Allow the weight to stabilize and record the weight in the column labeled tube
112 alone.
113
114 NOTE: Avoid moving the 10 mL Erlenmeyer flask when weighing tubes. If the flask is moved, re-
115 center the flask and re-tare the balance. Place the closed tubes in a rack and store until needed.
116
117 1.5. Pre-label 13 x 100 mm screw-top glass tubes and caps with the identifier codes and fill them
118 with 2 mL of LC-MS grade methanol. Place them in a tube rack, cap the tubes, and store them in
119 a refrigerator (4 °C) until use.
120
121 NOTE: Use a bottle top solvent dispenser (see **Table of Materials**). If one is not available, use
122 glass pipettes to avoid the use of plastics when dispensing organic solvents.
123
124 1.6. Pre-label 13 x 100 mm glass test-tubes. Cover and store the test tubes at room temperature.
125
126 1.7. Pre-label autoinjector vials with identifier codes. Cover and store the autoinjector vials at
127 room temperature until use.
128
129 1.8. Prepare tissue-drying wicks.
130
131 1.8.1. Begin by cleaning surgical scissors by immersing them in LC-MS grade methanol for 5 min,
132 and then wipe them with a clean laboratory grade tissue.

133

134 1.8.2. Using powder free gloves, twirl the end of a laboratory grade tissue (see **Table of Materials**)
135 until a finely tapered 30–40 mm wick is formed.

136

137 1.8.3. Using methanol-cleaned scissors, cut the wick on the thick end. Place wicks in a clean
138 carboard box. Make double the number of wicks than will be needed to process all the samples.

139

140 1.9. Prepare shortened 1 mL pipette tips. Using methanol-cleaned surgical scissors, cut 4–5 mm
141 off the tip of a 1 mL pipette tip, and place the tip back in the tip holder. Prepare twice as many
142 tips as samples to be processed.

143

144 NOTE: These will be used in the retrieval of washed tissues from tubes. Do not touch the end of
145 the tip.

146

147 1.10. Pre-chill molecular biology grade phosphate buffered saline (PBS) to 4 °C (0.5 L is sufficient).
148 This will be used to retrieve specimens. For each sample that will be processed, pre-chill (4 °C) 60
149 mL of ultra-pure deionized water.

150

151 1.11. Prepare mass spectrometry internal standards. Dissolve the following lipids in
152 ethanol:methanol:water (7:2:1; v/v/v) at a concentration of 25 nmol/mL: d17:1-sphingosine,
153 (2S,3R,4E)-2-aminoheptadec-4-ene-1,3-diol; d17:1-sphingosine-1-phosphate, heptadecasphing-
154 4-enine-1-phosphate; C12-Ceramide (d18:1/C12:0), N-(dodecanoyl)-sphing-4-enine; C12-
155 sphingomyelin (d18:1/C12:0), N-(dodecanoyl)-sphing-4-enine-1-phosphocholine; C12-
156 glucosylceramide (d18:1/C12:0), N-(dodecanoyl)-1-β-glucosyl-sphing-4-eine; C12-
157 lactosylceramide (d18:1/C12:0), N-(dodecanoyl)-1-ß-lactosyl-sphing-4-ene.

158

159 **2. Tissue washing**

160

161 NOTE: Completing all steps in section 1 one day prior allows a skilled researcher to process up to
162 40 samples per day, and novices ~10–15 samples per day. Begin early in the morning and do not
163 stop until all steps in sections 2.1–3.8 are completed.

164

165 2.1. On the day that the tissues will be processed, prepare the biosafety cabinet work area by
166 equipping it with a vortexer, a fully charged serological pipettor, and an ice tray. For all steps in
167 this section, wear appropriate PPE, including a lab coat, a double layer of gloves, and protective
168 eyewear.

169

170 CAUTION: Human tissues and fluids should be considered and treated as biohazardous materials.

171

172 2.2. Pre-chill (4 °C) a swinging bucket centrifuge, any adapters needed for holding 15 mL conical
173 centrifuge tubes, and centrifuge biocontainment lids. Pre-chill (4 °C) a fixed angle centrifuge
174 capable of holding 1.5 mL centrifuge tubes.

175

176 2.3. If tissues are not aliquoted into 15 mL polypropylene centrifuge tubes, use a methanol

177 cleaned spatula (clean for each new tissue sample) to transfer them to pre-labeled 15 mL
178 polypropylene tubes. Label the 15 mL caps also.

179

180 2.3.1. Place a tube rack capable of holding 15 mL centrifuge tubes in an ice tray and fill the tray
181 with ice. Thaw tissues that will be processed that day by placing them in the rack on ice.

182

183 NOTE: A minimum of 3–4 mg of tissue should be requested from the biorepository for processing
184 as it has been previously shown that tissue washing and weighing protocols are accurate and
185 reliable at these weights[11].

186

187 2.4. Move the ice tray with the samples into the biosafety cabinet.

188

189 2.5. Perform three sOCTrP cycles[11] as discussed below (i.e., perform steps 2.5.1–2.5.5 three
190 times).

191

192 2.5.1. Add 10 mL of ice-cold ultra-pure deionized water to each tube and cap them tightly. Allow
193 the tubes to incubate for 10 min.

194

195 2.5.2. Vigorously vortex each tube for 10–20 s or until all the tissue deposited on the walls of the
196 tube, or a tissue pellet, is completely resuspended.

197

198 NOTE: In sOCTrP cycles 2–3, it is critical that the pellet be resuspended so that any OCT in the
199 pellet is diluted into the wash solution.

200

201 2.5.3. Transfer the tubes to the pre-chilled (4 °C) swinging bucket centrifuge. Centrifuge samples
202 at 4,000–5,000 x *g* for 10 min at 4 °C.

203

204 NOTE: Avoid the generation of and exposure to biohazardous aerosols generated during
205 centrifugation by sealing the centrifuge carriers with a biocontainment lid (see **Table of**
206 **Materials**).

207

208 2.5.4. Retrieve samples from the centrifuge and transfer to the ice tray. Place the tray in a
209 biosafety cabinet and uncap the tubes. Save the caps.

210

211 2.5.5. Carefully aspirate the wash solution. Avoid aspirating the tissue material in the pellet by
212 leaving ~0.5 mL of the supernatant in the tube. Cap the tubes and avoid cross-contamination by
213 matching labeled caps with tubes.

214

215 NOTE: The wash solution (the supernatant) in step 2.5.5 is a biohazardous material; handle and
216 dispose of it following biosafety guidelines appropriate for specimens of human origin.

217

218 **3. Tissue weighing (for data normalization)**

219

220 3.1. After the last sOCTrP wash cycle, carefully aspirate most of the wash solution but avoid

221 disturbing the pellet. Leave ~0.5 mL of the wash solution in the tube.

223 3.2. Using the shortened pipette tips (prepared in step 1.9), take-up 500 µL of ice-cold PBS and
224 add it to the tube.

226 3.2.1. Using the same tip, gently resuspend the pellet and retrieve all tissue. Avoid turbulent
227 pipetting to decrease tissue loss by its adherence to the tube and pipette tip walls.

229 3.3. Add the resuspended tissue to its corresponding pre-labeled and pre-weighed 1.5 mL
230 centrifuge tube and place the tube on ice. Repeat for all tissues in the data set.

232 3.4. Place the 1.5 mL tubes into a pre-chilled centrifuge and pellet the tissues at 7,000 x *g* for 5–
233 7 min and 4 °C.

235 3.4.1. Retrieve tubes and carefully aspirate as much of the PBS as possible without disturbing the
236 pellet. Place the tube back on ice.

238 3.5. Centrifuge tubes for an additional 3 min at 7,000 x g to ensure tissues are well pelleted.
239 Transfer the tubes to ice.

241 NOTE: The additional centrifugation step is important to prevent tissue loss when removing all
242 wash solution and wicking. If processing more than five samples, repeat the 3 min, 7,000 x *g*
243 centrifugation step after every fifth sample is processed to ensure tissues remain pelleted.

245 3.6. Using the wicks prepared in step 1.8, gently remove any remaining wash solution. Use a clean
246 wick for each sample. It is acceptable to gently dab the tissue with the wick, which will help
247 remove most of the wash solution but avoid losing any tissue by adherence to the wick. Close the
248 tube and place it on ice.

250 3.7. Weigh each tube in the recently calibrated, tared, and zeroed analytical balance.

252 3.7.1. Place each tube in the glass Erlenmeyer flask and close the chamber door. When the weight
253 has stabilized, record it in the electronic spreadsheet column labeled tube w/tissue.

255 3.7.2. After weighing, place the tube back on ice.

257 3.8. Add 300 µL of ice-cold PBS. Using a shortened pipette tip (step 1.9), carefully retrieve all
258 tissue and deposit in a pre-labeled screw-top glass tube (prepared in step 1.5) containing 2 mL of
259 ice-cold LC-MS grade methanol (add the tissue to the methanol, not to the side of the tube).

261 3.9. Calculate the amount of tissue that was washed by subtracting the tube alone from the tube
262 w/tissue value. Record this number in the total tissue column. The total tissue value will be used
263 to normalize mass spectrometry data in step 6.12.

265   3.10. Store the samples at -80 °C until ready to perform steps 4.1–4.15.

266

267   NOTE: This is a good stopping point, and the samples can be stored safely for a couple of weeks
268   at -80 °C. For other tissue normalization methods, such as protein concentration or lipid
269   phosphate content, refer to Rohrbach et al.[11].

270

271   **4. Lipid extraction**

272

273   NOTE: It is important to begin these steps in the morning, particularly when many samples will
274   be processed. Before starting, pre-heat a water bath or incubator to 48 °C.

275

276   4.1. Retrieve the samples prepared in step 3.8 and the MS lipid standards (prepared in step 1.11)
277   from the freezer. Allow them to equilibrate to room temperature for 20 min.

278

279   4.2. Vortex and immerse the internal standard solution in an ultrasonic water bath (see **Table of**
280   **Materials**) for 2–3 min or until the MS lipid standards are fully dissolved prior to dispensing into
281   samples. This will avoid errors in data normalization.

282

283   4.3. Using a repeating pipette, add 250 pmol (10 µL from the 25 nmol/mL solution prepared in
284   step 1.11) of internal mass spectrometry standards to each tube. Re-cap the tubes lightly.

285

286   4.4. To facilitate homogenization, adjust the methanol volume to 2 mL. Only use LC-MS/MS grade
287   methanol.

288

289   4.5. Working one tube at a time, use a homogenizer to triturate the tissue until no clumps are
290   visible (typically 5–20 s). Move the homogenizer tip in a circular motion and gently press against
291   the tube wall to assist trituration. Re-cap the tube.

292

293   NOTE: Ensuring all tissues are finely triturated will maximize lipid extraction. Do not add
294   chloroform to samples prior to homogenization as the disposable homogenizer tips are made of
295   plastic that will dissolve in solutions containing chloroform.

296

297   4.6. Immerse the tube at a 45° angle in an ultrasonic water bath for 5–10 s.

298

299   4.6.1. If no tissue clumps form upon immersing in the ultrasonic bath, cap it, and move on to the
300   next tube.

301

302   4.6.2. If tissue clumps form when the tube is immersed in the ultrasonic bath, re-homogenize,
303   and target the clumps.

304

305   4.6.3. Repeat this process (homogenization/immersion in an ultrasonic bath) iteratively until all
306   large tissue clumps are broken-up.

307

308   4.7. In a fume-hood, add LC-MS grade chloroform and methanol to each tube (use a bottle top

309   dispenser) to achieve a ratio of 2:1:0.1 methanol:chloroform:water. Using clean caps, seal the
310   tubes tightly and leave on the benchtop until the end of the day.

312   4.7.1. For tissues weighing 50 mg or less, use a total extraction volume of 4 mL, and adjust using
313   this ratio (50 mg:4 mL extraction solution) for larger specimens.

315   4.8. Before leaving that evening, place the tightly capped tubes in a 48 °C water bath or incubator
316   and incubate them overnight.

318   NOTE: It is critical that the tubes be tightly capped so that the solvent ratios do not change due
319   to evaporation.

321   4.9. The next morning, retrieve the samples from the 48 °C water bath and pellet any solvent-
322   insoluble debris by centrifugation at 4,000–5,000 x g at 4 °C for 20 min.

324   4.10. Working in a fume-hood, carefully decant the supernatant into the pre-labeled 13 x 100
325   mm borosilicate glass test-tubes. Tilt the 13 x 100 mm tube at a 30–45° angle to facilitate
326   decanting.

328   NOTE: If processing more than 12 samples, do not allow the centrifuged tubes to sit for extended
329   periods of time as the pellets will soften and insoluble debris will be transferred when performing
330   step 4.10. To avoid this, only take 12 tubes out of the centrifuge at a time and continue
331   centrifuging the other tubes until you are ready to decant them.

333   4.11. Transfer the tubes to a vacuum concentrator capable of holding 13 x 100 mm tubes.
334   Evaporate all solvent with an initial 1 h heating step (40 °C) and run under the maximum vacuum.

336   4.12. Remove tubes from the vacuum concentrator and add 500 µL of LC-MS grade methanol
337   (use a bottle top dispenser) to each tube.

339   4.13. Resuspend the dried lipid extracts by vortexing for 5–10 s, followed by immersing tubes at
340   a 45° angle in an ultrasonic water bath for 2 min while rotating the tube. Re-vortex for 5 s and
341   immerse in ultrasonic water bath for an additional minute.

343   NOTE: This is a critical step to ensure the maximal recovery of extracted lipids. Do not move
344   ahead until all the dried material on the wall of the tube has been resuspended.

346   4.14. Place the 13 x 100 mm tubes in a pre-chilled (4 °C) centrifuge and remove any insoluble
347   debris by centrifugation at 4,000–5,000 x g at 4 °C for 20–30 min.

349   4.15. Carefully decant the clarified supernatant into a pre-labeled autoinjector vial. Tilting the
350   autoinjector vial at a 30–45° angle will facilitate decanting. Ensure that the entire contents of the
351   13 x 100 mm tube are decanted into the autoloader vial.

352

353

355

356   **5. LC-ESI-MS/MS analysis**

357

358   NOTE: The following procedure uses a binary pump system coupled to an autoinjector, degasser,
359   and LC-MS/MS system operating in a triple-quadrupole mode. The column temperature was
360   maintained using a column oven. See **Table of Materials** for details on the equipment used.

361

362   5.1. Prepare mobile phase A1 ($CH_3OH:H_2O:HCOOH$, 58:41:1, v:v:v, with 5 mM ammonium
363   formate) and mobile phase B1 ($CH_3OH:HCOOH$, 99:1, v:v, with 5 mM ammonium formate). Use
364   only LC-MS grade solvents, water, and reagents.

365

366   5.2. For each set of samples, prepare four Blank autoloader vials containing 500 µL of LC-MS
367   grade methanol.

368

369   5.3. For each set of samples prepare two Internal Standard (label as IS) autoloader vials by diluting
370   10 µL (250 pmol total each standard) of the internal standard solution prepared in step 1.11 in
371   500 µL of LC-MS grade methanol.

372

373   5.4. Set the column oven temperature to 60 °C, and the ion source temperature to 500 °C.

374

375   5.5. For the MS/MS analysis, utilize the triple-quadrupole mode of the spectrometer. Set the first
376   quadrupole (Q1) to pass precursor ions, while setting the third quadrupole (Q3) to pass
377   molecularly distinctive product ions (or a scan across multiple m/z in Q1 or Q3) using N2 to
378   collisionally induce dissociations in quadrupole 2 (Q2), which is offset from Q1 by 30-120 eV.

379

380   5.6. Set the multiple reaction monitoring pairs (MRM) detection window at 120 s with a target
381   scan time of 1.0 s. Refer to **Table 1** for a listing of MRM pairs, ionization energies, and collision
382   energies.

383

384   5.7. Use the following parameters to resolve sphingolipids in the LC step: using a flow rate of 0.7
385   mL/min, equilibrate a 2.1 (i.d) x 50 mm C18 reverse phase column for 0.5 min with a solvent
386   mixture of 95% mobile phase A1 and 5% mobile phase B1.

387

388   5.7.1. After sample injection, maintain the mobile phase A1/B1 ratio at 95/5 for 2.25 min,
389   followed by a linear gradient to 100% B1 over 1.5 min, which is then held at 100% B1 for 5.5 min,
390   followed by a 0.5 min gradient return to 95/5 A1/B1.

391

392   5.7.2. After the run, re-equilibrate the column with a mixture of 95/5 A1/B1 for 0.5 min.

393

394   5.8. Use the following settings for the sample autoloader: rinsing volume, 500 µL; needle stroke,
395   52 mm (this should be adjusted depending on vial size and volume in each vial); rinsing speed, 35
396   µL/s; sampling speed, 5.0 µL/s; rinse dip time, 2 s; rinse mode, before and after aspiration; rinse

397   time,2 s.

398

399   5.9. Place samples prepared in step 4.15 onto an autoloader tray in the following order: Blank;
400   IS; Blank; Samples prepared in step 4.15; Blank, IS, Blank.

401

402   5.10. Analyze 5–10 µL of each sample by LC-ESI-MS/MS. Analyze the same volume for all samples
403   in the data set.

404

405   **6. Data processing, integration, and normalization**

406

407   NOTE: Although the following steps outline a procedure for specific software (see **Table of**
408   **Materials**), similar procedures on equivalent LC-MS instruments and software can be used to
409   integrate MRM pairs for the analyzed lipid classes and corresponding internal standards.

410

411   6.1. Open the quantitation software. In the **Quantitate** tab double click on the **Quantitation**
412   **Wizard**. In the pop-up window, in the left-most workspace, navigate to the correct data set and
413   left click on it. The available samples will be shown in the middle workspace.

414

415   6.2. Highlight the samples to be analyzed and then click on the right-handed arrow to add
416   samples to the **Selected Samples** workspace. Click on **Next** to navigate to the settings and query
417   screen where the default settings are typically used.

418

419   6.3. Hit **Next** to navigate to the integration method selection screen. Select an appropriate
420   integration method containing MRM transition pairs for lipids to be analyzed. Refer to the **Table**
421   **1** for MRM transition pairs. Hit **Finish**.

422

423   NOTE: Retention times will vary depending on instrumentation and individual settings and must
424   therefore be verified for each individual setup.

425

426   6.4. In the navigation bar, click on the **Peak Review** pane so that MRM pairs can be inspected. To
427   facilitate review, right-click on the **Peak Review** pane, change the **Automatic Zooming** to **100%**
428   **of largest peak**, and a **Zoom Window** of **1.00–2.00 minutes**.

429

430   6.5. Right-click on the **Quantitation Display** window and select **Analyte** and the desired
431   sphingolipid to be examined. Verify that there are no peaks in the Blank samples and that the
432   correct peak has been integrated in the IS samples.

433

434   6.6. Begin integrating unknown samples. Working one sphingolipid class at a time, inspect the
435   retention time for the MRM pair of the internal standard (i.e., C12:0 ceramide) and ensure that
436   the software has integrated the correct peak.

437

438   6.7. Continue onto the analytes in the same lipid class (i.e., C14:0 ceramide, C16:0-ceramide,
439   etc.), beginning with the shortest chain-length lipid in the class. Verify for each chain-length lipid
440   that the software routine has integrated the correct MRM pair peak.

441

442 6.8. When all the analytes have been integrated, create an electronic spreadsheet for each
443 sphingolipid species analyzed (e.g., ceramides, sphingomyelins, etc.). Label column headers to
444 match the order of the analytes and chain-lengths in the LC-MS/MS quantitation software. Also
445 include a column header for sample ID and sample normalization weights determined in step 3.7.

446

447 NOTE: As previously described[11], other common normalization measures include total lipid
448 phosphate content or protein amount.

449

450 6.9. Export or copy the peak areas for all analytes and internal standards to the electronic
451 spreadsheet.

452

453 6.10. Normalize data by dividing the integrated peak area of each chain-length species for a given
454 sphingolipid class by the peak area of its corresponding internal standard. For instance, C14:0
455 ceramide, C16:0 ceramide, C18:1 ceramide, etc., should each be divided by the C12:0 ceramide
456 internal standard peak area.

457

458 6.11. Convert peak area to moles of lipid recovered by multiplying the normalized peak area
459 (calculated in step 6.10) with the amount of internal standard, in picomoles, that was added to
460 the sample in step 4.3. In this protocol, this amount is 250 pmol.

461

462 6.12. To obtain the relative amount of lipid in each sample, divide the picomoles of each lipid
463 chain-length by the tissue weight determined in step 3.7. This will give units of picomoles of lipid
464 per mg of tissue.

465

466 **REPRESENTATIVE RESULTS:**
467 In this protocol, we describe in detail a method to remove OCT from cryo-preserved human
468 tissues and weigh the tissues for analysis by LC-ESI-MS/MS. The materials required for this
469 procedure are listed in **Table of Materials**. Shown in **Figure 1** are results of a typical experiment
470 where 10 human lung adenocarcinoma tumors and 10 normal adjacent tissues were washed to
471 remove OCT and analyzed by LC-ESI-MS/MS. Importantly, as we have previously shown[11], there
472 are various chain-length species of ceramides (**Figure 1A**) and monohexosyl-ceramides (**Figure**
473 **1B**) that are significantly elevated in lung adenocarcinoma tumors as compared to normal
474 adjacent uninvolved tissues.

475

476 In control experiments to assess the effect of OCT on the lipid extraction steps outlined in section
477 4, 200 mg of mice livers (C57BL6; males; 14–16 weeks old) were resuspended in 2 mL of
478 phosphate buffered saline and homogenized until finely triturated. The resulting fine liver
479 suspension was then extensively sonicated with a probe sonicator (three rounds of 1 min
480 sonication on ice, 1 min rest on ice, 60% power; microtip). From this solution, six 300 µL liver-
481 homogenate replicates were prepared. To three replicates, 200 mg of OCT was added (average
482 amount found in biorepository specimens[11]), and 200 µL of water was added to the other three.
483 The samples were then processed in parallel following the steps outlined in section 4.
484 Importantly, following centrifugation of the single-phase Bligh-Dryer solution, samples

485 containing OCT were cloudy whereas the control samples containing water were clear (**Figure**
486 **2A**). The cloudiness in the single-phase lipid extraction solution persisted even after increased
487 centrifugation and extensive sonication (not shown). After the solvent was evaporated, samples
488 containing OCT had large pellets (**Figure 2B**), which could not be reconstituted in methanol even
489 under vigorous vortexing and extensive sonication. Samples containing OCT also showed a large
490 loss of signal for internal standards of all species analyzed (**Figure 3A–G**). We have also previously
491 shown that samples containing OCT showed loss of signal for many of the sphingolipids
492 analyzed[11].

494 Typically, it is expected that the sphingolipid standards will elute sequentially as shown in **Figure**
495 **4** in the following order: d17:1 sphingosine, d17:1 sphingosine-1-phosphate, C12:0 lactosyl-
496 ceramide, C12:0 monohexosyl-ceramide, C12:0 sphingomyelin, and C12:0 ceramide. For each of
497 these sphingolipid species, using the gradient and instrumentation settings described in section
498 5 and previously[11], there will be approximately a +0.15 min shift in the retention time for every
499 2-carbon increase in chain-length. For example, as shown in **Figure 5**, C14:0-ceramide (**Figure 5B**)
500 will elute approximately +0.15 min later than C12:0-ceramide (**Figure 5A**). A double bond in the
501 fatty acid often results in a -0.15 shift in retention time, so C16:0-ceramide (**Figure 5C**) will have
502 a retention time similar to C18:1-ceramide (**Figure 5D**). However, longer chain-length lipids (i.e.,
503 C24:0, C26:0) may have larger retention time shifts (**Figure 5I,K**, respectively).

505 **FIGURE AND TABLE LEGENDS:**

507 **Figure 1: Sphingolipid profiles of lung adenocarcinomas and adjacent uninvolved lung tissues.**
508 Tissues were cryogenically stored in OCT, thawed, processed with three sOCTrP cycles, weighed,
509 and analyzed by LC-ESI-MS/MS. Levels are in pmol per mg of tissue. The indicated acyl chain-
510 length species of ceramide (**A**), monohexosylceramide (**B**), sphingomyelin (**C**), lactosylceramide
511 (**D**), and sphingosine (**E**; Sph), sphingosine-1-phopshate (**F**; S1P), dihydro-Sph (**E**), and dihydro-
512 S1P (**F**) were quantified. n = 10 tumors and n = 10 adjacent uninvolved tissues. Box plots show
513 medians (black line) and whiskers of min to max. ns, not significant; ***, p≤0.005; ****, p ≤
514 0.0005.

516 **Figure 2: Representative images of extracted samples with and without OCT.** Mouse livers were
517 homogenized and sonicated, split into six identical samples: 200 mg of OCT was added to three
518 samples and water to the other three. (**A**) After methanol and chloroform were added to achieve
519 a ratio of 2:1:0.1 methanol:chloroform:water (v:v:v), overnight incubation at 48 °C, followed by
520 centrifugation, the supernatants of samples that contained OCT were cloudy and could not be
521 clarified by sonication, vortexing, or centrifugation. (**B**) Following solvent evaporation, a large
522 pellet was recovered from samples containing OCT that could not be fully reconstituted in 0.5 mL
523 of methanol after extensive sonication and vortexing.

525 **Figure 3: LC–ESI–MS/MS quantification of sphingolipid standards in the presence or absence of**
526 **OCT.** Mouse livers were homogenized, sonicated, and split into six identical samples. To three
527 out of the six samples, 200 mg of OCT was added, while to the other three, water was added.
528 After addition of internal standards, methanol, and chloroform, samples were processed

529 identically and extracted lipids were analyzed by LC-ESI-MS/MS. Shown are multiple reaction
530 monitoring (MRM) pairs of the indicated lipids (**A–F**), and corresponding integrated peak areas
531 (**G**). Abbreviations: Sph, sphingosine; S1P, sphingosine-1-phosphate. Black arrows point to the
532 correct MRM pair for the indicated sphingolipid.

534 **Figure 4: Relative retention times for LC–ESI–MS/MS multiple reaction monitoring pairs of**
535 **sphingolipid standards.** Mouse livers were homogenized and sonicated; internal standards,
536 methanol, and chloroform added; and lipids extracted and analyzed by LC-ESI-MS/MS. Shown are
537 retention times of multiple reaction monitoring pairs of the indicated lipids (**A–F**). Note the
538 relative order in which lipids elute during the liquid chromatography gradient. Abbreviations:
539 Sph, sphingosine; S1P, sphingosine-1-phosphate; SM, sphingomyelin. The X-axis is MRM pair
540 retention time in minutes. The Y-axis is signal intensity for the MRM pairs.

542 **Figure 5: Acyl chain-length dependent shift in retention time of LC–ESI–MS/MS MRM pairs.**
543 Mouse livers were homogenized and sonicated; internal standards, methanol, and chloroform
544 added; and lipids extracted and analyzed by LC-ESI-MS/MS. Shown are retention times and MRM
545 pairs of the indicated lipids. Note the shift in retention time with increasing acyl-chain length for
546 various lipids (**A–K**), which typically corresponds to a +0.15 min retention time per 2-carbon
547 increase. A double bond will result in a -0.15 min retention time shift (**D,H,J**). However, for longer
548 chain lipids, the relative increase in retention time may be longer (**G–K**). The X-axis is MRM pair
549 retention time in minutes. The Y-axis is signal intensity for the MRM pairs.

551 **Table 1: MRM pairs, ionization parameters, and collision energies for LC–ESI–MS/MS analysis.**
552 DP, de-clustering potential; CE, collision energy.

554 **DISCUSSION:**
555 OCT is a common long-term cryo-preservation agent used in biorepositories. However, OCT can
556 result in ion suppression when tissues are analyzed by various mass spectrometry platforms[12-15],
557 or result in loss of signal when samples are analyzed by LC-ESI-MS/MS[11]. OCT in cryopreserved
558 tissues can also interfere with tissue normalization methods such as weighing and protein
559 quantification assays such as Bradford and BCA[11]. Here, we present a detailed protocol to remove
560 OCT from human tissues and subsequently be analyzed by mass spectrometry. The protocol may
561 be modified to use other tissue sources that are not lung, or from mice tissues as we have
562 previously described[11]. In addition, other mass spectrometry data normalization strategies can
563 be used, including those we have previously described[11], as well as other validated methods.

565 An important limitation of this protocol is that it is not adequate for the analysis of tissues that
566 have been previously fixed with formaldehyde or another fixing agent. It is only adequate for
567 tissues that have not been fixed and have been cryopreserved in OCT. In addition, this method is
568 not adequate for processing tissues that weigh less than 1–2 mg as there is always some tissue
569 loss during the sOCTrP steps and transfers between tubes. It will be difficult to weigh such small
570 tissues using a standard analytical scale, and any left-over PBS not removed during the wicking
571 stage will contribute greatly to the experimental error. Below are other important considerations
572 when washing tissues to remove OCT with this method.

573
574 To ensure the smooth execution of this protocol, particularly if many samples will be processed,
575 steps outlined in section 1 should be performed ahead of time, including pre-labeling and pre-
576 weighing 1.5 mL tubes, pre-labeling 13 x 100 mm screw cap tubes and caps, 13 x 100 mm culture
577 tubes, and autoinjector vials, preparing all wicks, pre-shortening 1 mL pipette tips, and pre-
578 chilling wash solutions. This will prevent time-consuming procedures having to be performed on
579 the day that tissues will be washed, which can lead to delays. In general, we find that if many of
580 these steps are performed the day prior, an experienced researcher can wash 30–40 specimens
581 in a normal workday. However, because the first suggested stopping point is after all tissues have
582 been washed, weighed, and placed in methanol and stored at -80 °C, not performing 1.5 mL pre-
583 weighing and labeling ahead of time will impact efficiency and extend the length of time required
584 to wash 30–40 tissues.
585
586 Tissue weighing is one of the most important steps, as errors or carelessness will impact data
587 quality as they will carry over into data normalization. It is critical that the analytical balance used
588 for weighing be calibrated, properly zeroed, and tared. Accordingly, when using wicks, removing
589 as much of the wash solution as possible is important, especially for tissues weighing less than 5
590 mg. At these tissue weights, leftover wash solution will make weighing inaccurate by a large
591 percentage and carry over as an error in data normalization. When removing wash solution, we
592 find that tissues can be gently touched with the wick for several seconds to remove as much wash
593 solution as possible without leading to significant losses of tissue by adhesion to the wick.
594 However, each tissue type being washed should be tested for adhesion to wicks, and these steps
595 in the protocol adjusted accordingly.
596
597 To facilitate the tissue washing workflow, it is recommended that tissue shavings be requested
598 from the biorepository in 15 mL polypropylene tubes. This will reduce the handling of tissues
599 prior to washing.
600
601 When washing tissues, if a gel-like material is observed at the bottom of a tube after the third
602 sOCTrP cycle, this is indicative that not all OCT has been removed and more sOCTrP cycles are
603 needed. Keep track of how many cycles are needed, and for consistency, perform the same
604 number of sOCTrP cycles in all specimens in the data set. We have previously evaluated up to 9
605 sOCTrP cycles and found no effect on depletion of sphingolipids in tissues[11].
606
607 When using wicks to remove wash solution, gently dabbing the tissue will ensure that all wash
608 solution is removed. This increases the accuracy of tissue weight estimation. However, great care
609 must be taken with very small tissues as these can stick to the wick and result in the loss of
610 specimen. It is helpful to use several wicks for the same tube to remove as much solution as
611 possible. To prevent cross-sample contamination, always use a new, and clean, wick for each
612 sample. When making wicks, use laboratory grade tissue (see **Table of Materials**) as we
613 previously tested these and found to contain a negligible amount of contaminating
614 sphingolipids[11]. Wicks from other materials can be used if they are tested as described[11].
615
616 When retrieving specimens from 1.5 mL tubes after weighing, it is critical that all tissue be

617   recovered. Failing to do so will result in errors in data normalization. If more PBS is needed to
618   recover all the tissue, add the equivalent amount of PBS used to all other tubes to avoid
619   differences in extraction efficiency that might result from samples containing different amounts
620   of PBS. Adjust chloroform and methanol volumes to account for any increase in water volume to
621   maintain a 2:1:0.1 methanol:chloroform:water ratio.

623   When thawing specimens, it is important that this step be done on ice to avoid the degradation
624   of labile lipids such as sphingosine-1-phosphate. Fully thawing samples until the OCT is in a liquid
625   state will also facilitate its removal as it will more easily be dissolved in the cold washing solution
626   rather than remaining as a pellet during early wash steps.

628   Occasionally, especially when washing lung tissues, there will be fragments that will not pellet
629   and will float on top of the wash solution after centrifugation. With care, immersing the aspirating
630   tip beneath the floating tissues to remove the wash solution can prevent loss during aspiration.

632   Do not add chloroform to samples prior to homogenization as the disposable homogenizer tips
633   are made of plastic and will dissolve in solutions containing chloroform. This can significantly
634   impact the LC-MS/MS quantification of lipids in the samples. Therefore, in general, the use of
635   plastics that are not resistant to solvents should be avoided when dispensing chloroform and
636   methanol. These solvents are also toxic and should be handled in an appropriate fume hood.
637   Wear appropriate PPE when handling solvent such as methanol and chloroform.

639   Human tissues should be processed in an appropriate biosafety cabinet (e.g., Class II). Users
640   should be trained in the handling of biohazardous materials and fluids of human origin and follow
641   safety protocols such as wearing appropriate personal protective equipment and
642   decontaminating any surface or equipment that comes in contact with specimens. Users should
643   thoroughly disinfect (see **Table of Materials** for suggested decontaminant) any surface or
644   equipment (centrifuges, centrifuge carriers and adapters, pipettors, vortexers, fume hood
645   surfaces, balances, glassware, ice trays, computer keyboards, computer mouse, etc.) used during
646   the wash procedure. Lab members should not use equipment or hoods used for tissue washing
647   until they have been thoroughly disinfected. Avoid the use of bleach on steel surfaces and
648   electronics.

650   When processing many samples, use caution to prevent cross contamination using clean caps in
651   re-capping steps during the lipid extraction steps. Caps can also be re-used if they are labeled.
652   However, in practice, we find that markers stand out poorly on the black caps, and adhesive
653   labels fall off tubes during the overnight 48 °C incubation.

655   Sphingolipids are demonstrated important players in the etiology of disease and human cancers.
656   Therefore, there is great interest in precisely defining the sphingolipid metabolism alterations in
657   these diseases as they may reveal therapeutic targets. However, many of the tissues that are
658   easily accessible to researchers are cryopreserved in OCT, which is not compatible with mass
659   spectrometry analysis. Thus, the detailed protocol described here will expand the repertoire of
660   tissues adequate for quantitative sphingolipidomic analysis, and thus help increase our

understanding of the biology of lipid metabolism alterations in human disease and cancer.

DISCLOSURES:
The authors have nothing to disclose.

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706   analysis of fresh frozen and optimal cutting temperature (OCT) embedded primary non-
707   small cell lung carcinoma by LC-MS/MS. *Methods*. **81**, 50–55 (2015).
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709   *Analytical Biochemistry*. **469**, 27–33 (2015).

Figure 1   Click here to access/download;Figure;Figure 1.tiff ±

Figure 2

Click here to access/download;Figure;Figure 2.tif
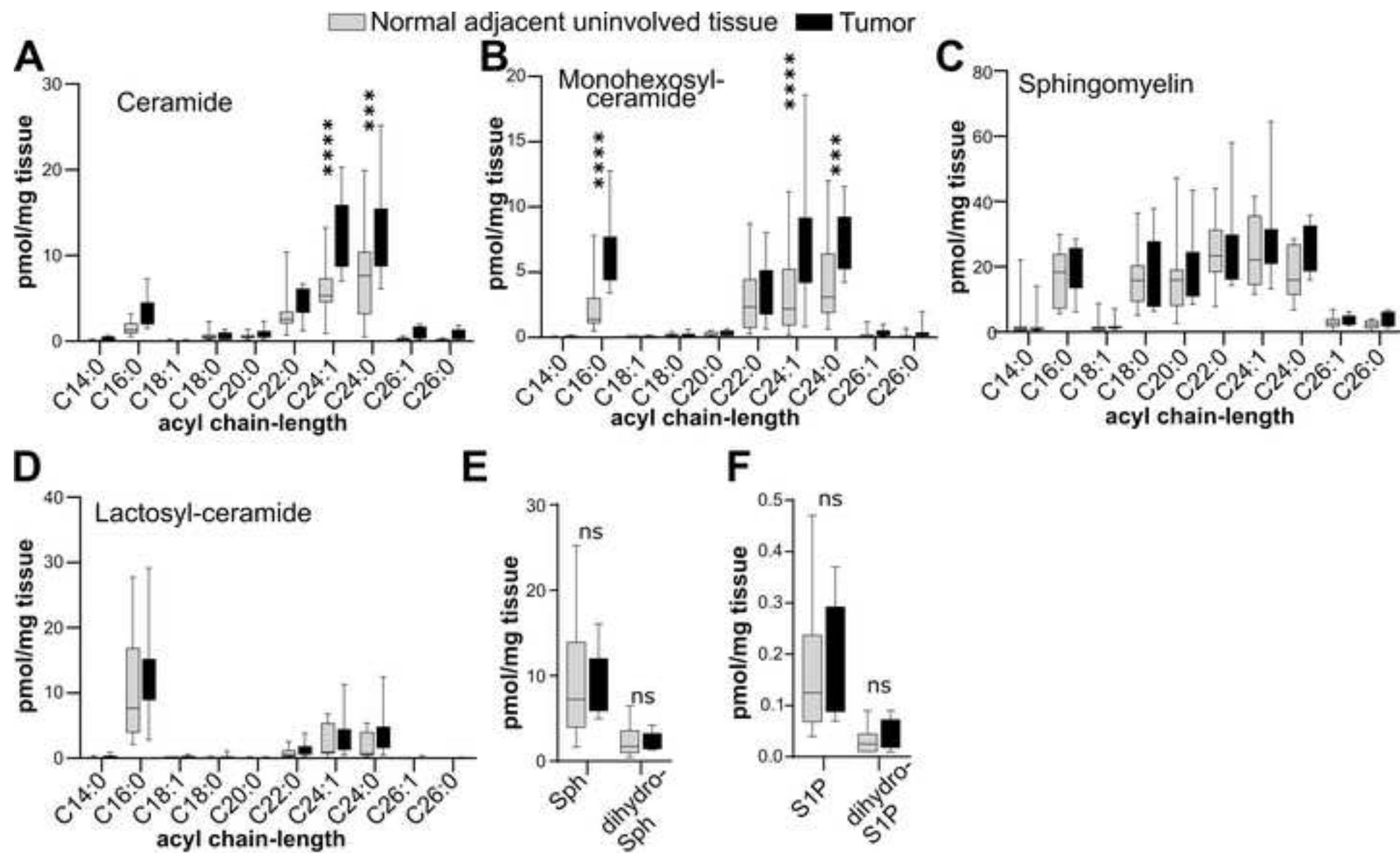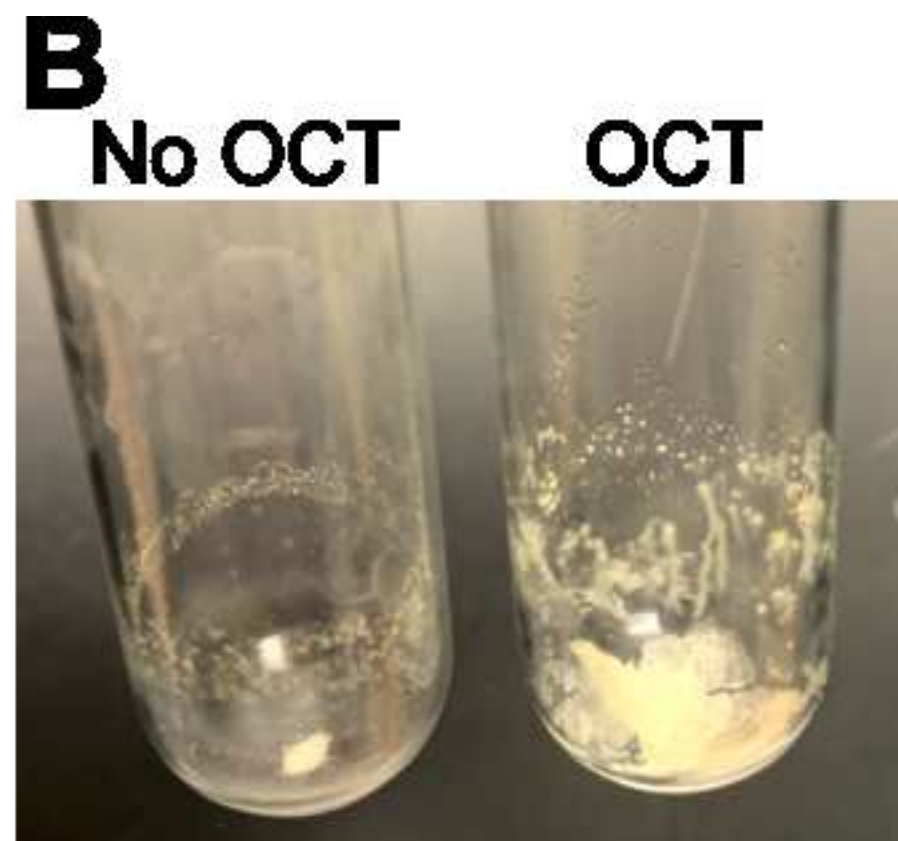
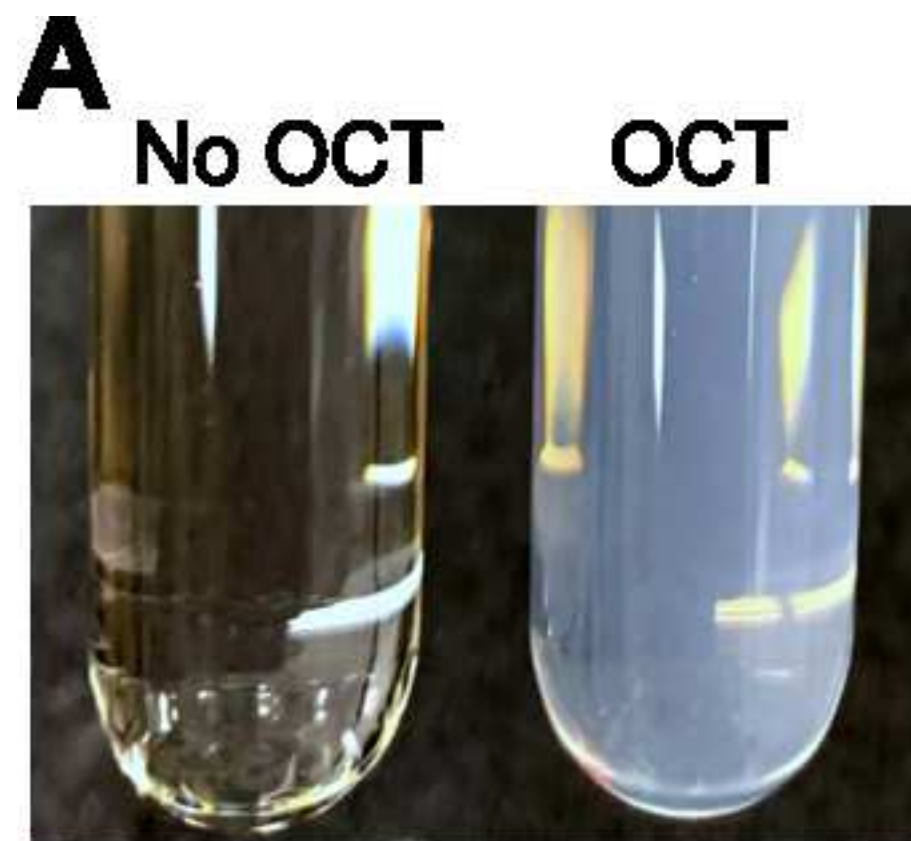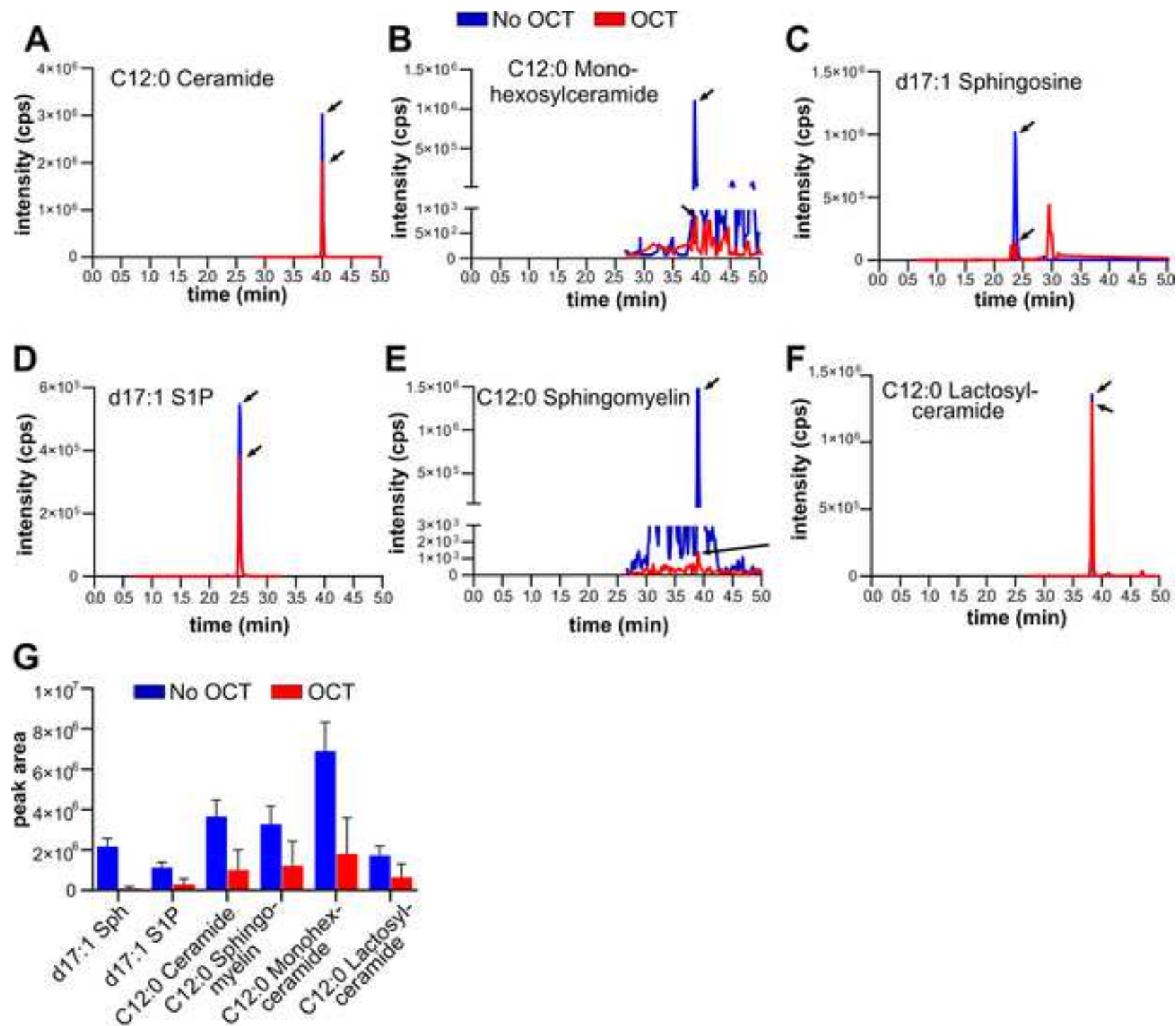Figure 3   Click here to access/download;Figure;Figure 3.tiff ⬇

Figure 4

Click here to access/download;Figure;Figure 4.tiff ⬇

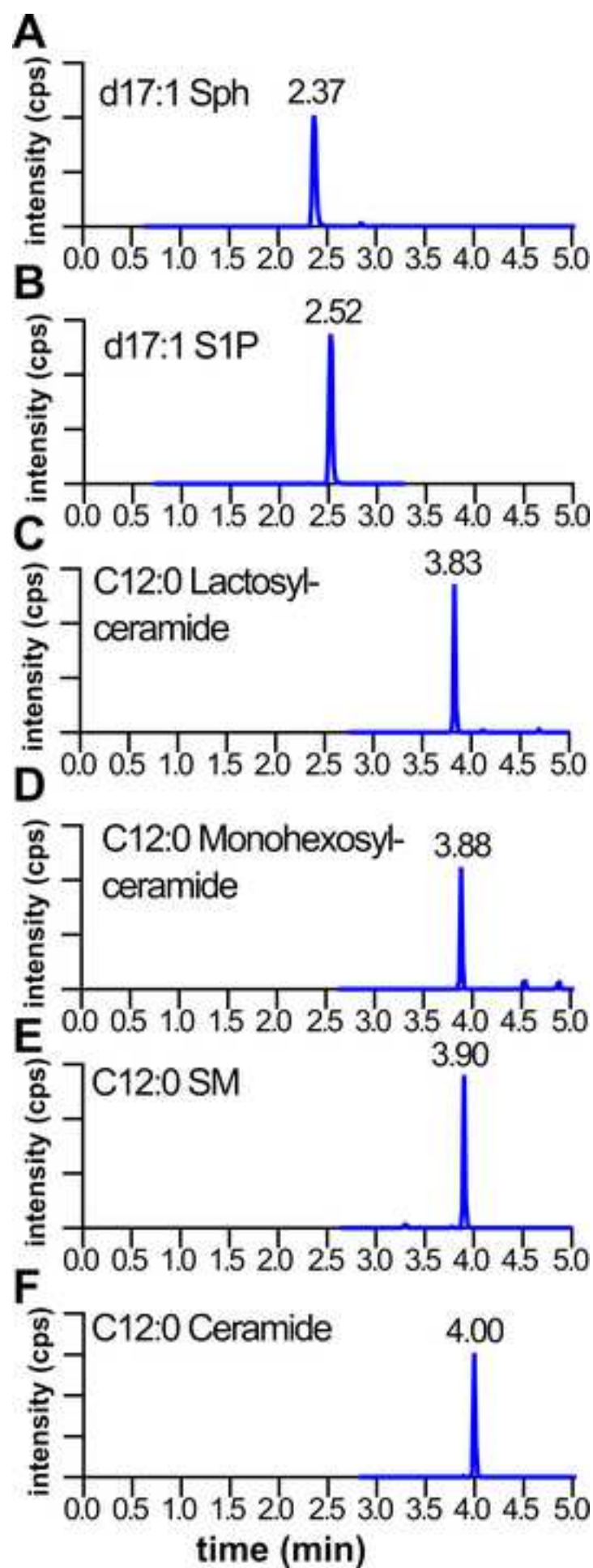**A** d17:1 Sph 2.37

B d17:1 S1P 2.52

C C12:0 Lactosyl-ceramide 3.83

D C12:0 Monohexosyl-ceramide 3.88

E C12:0 SM 3.90

F C12:0 Ceramide 4.00

time (min)

Figure 5

Click here to access/download;Figure;Figure 5.tiff

Table 1
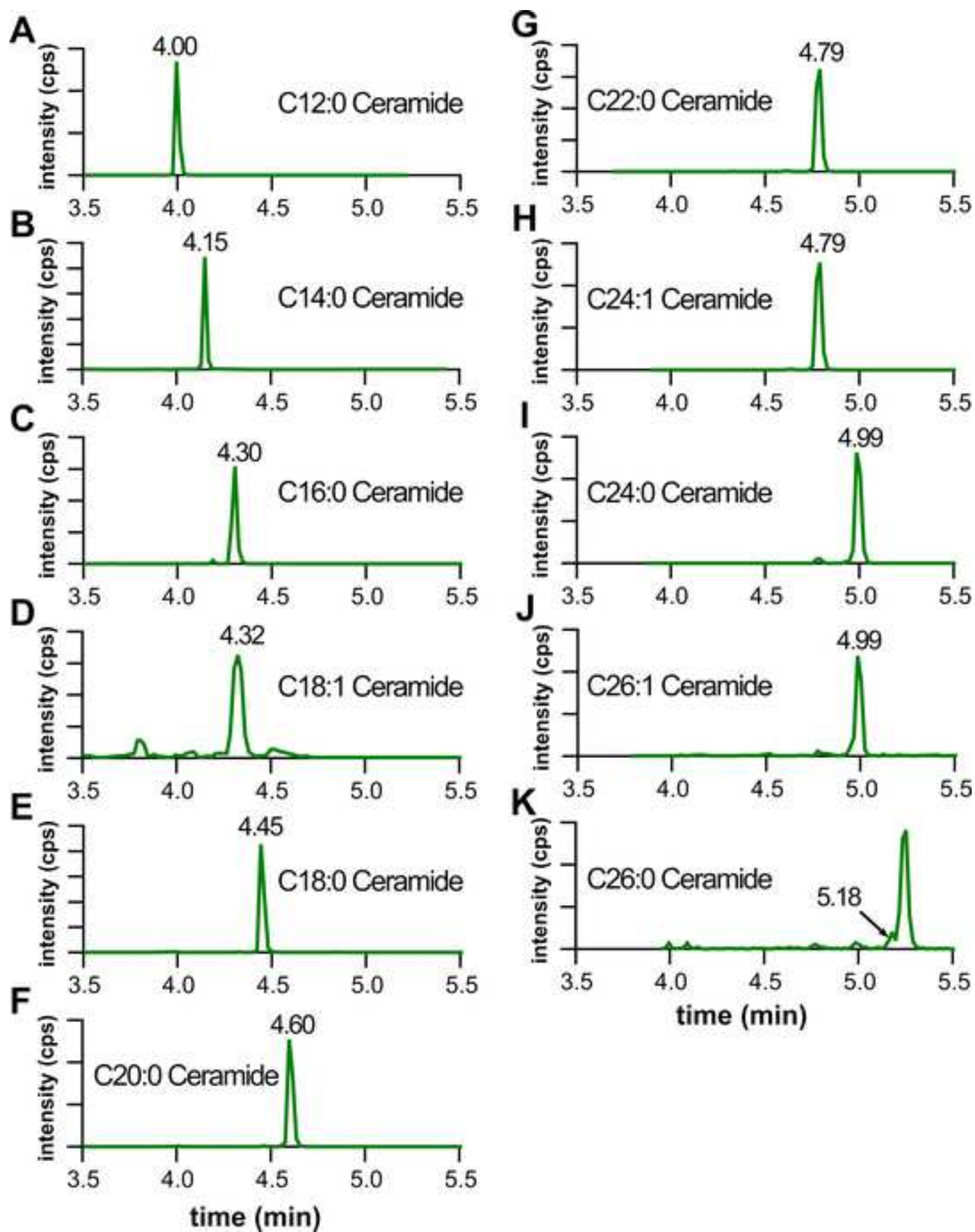
Lipid ID	Q1 Mass (Da)	Q3 Mass (Da)	DP	CE
d17:1 So	286.4	268.3	100	15
d17:0 Sa	288.4	270.4	100	21
d18:1 So	300.5	282.3	100	21
d18:0 Sa	302.5	284.3	100	21
d17:1 So1P	366.4	250.4	90	23
d17:0 Sa1P	368.4	252.4	90	23
d18:1 So1P	380.4	264.4	90	25
d18:0 Sa1P	382.4	266.4	90	25
C12:0 Cer	482.6	264.4	80	41
C14:0 Cer	510.7	264.4	80	43.5
C16:0 Cer	538.7	264.4	80	46
C18:1 Cer	564.7	264.4	80	48.5
C18:0 Cer	566.7	264.4	80	48.5
C20:0 Cer	594.7	264.4	80	51
C22:0 Cer	622.8	264.4	80	53.5
C24:1 Cer	648.9	264.4	80	56
C24:0 Cer	650.9	264.4	80	56
C26:1 Cer	676.9	264.4	80	58.5
C26:0 Cer	678.9	264.4	80	58.5
C12:0 MonHex	644.6	264.4	80	41
C14:0 monHex	672.6	264.4	80	43.5
C16:0 MonHex	700.7	264.4	80	46
C18:1 MonHex	726.7	264.4	80	48.5
C18:0 MonHex	728.7	264.4	80	48.5
C20:0 MonHex	756.7	264.4	80	51
C22:0 MonHex	784.8	264.4	80	53.5
C24:1 MonHex	810.9	264.4	80	56
C24:0 MonHex	812.9	264.4	80	56
C26:1 MonHex	838.9	264.4	80	58
C26:0 MonHex	840.9	264.4	80	58
C12:0 SM	647.7	184.4	80	41
C14:0 SM	675.7	184.4	80	43.5
C16:0 SM	703.8	184.4	80	46
C18:1 SM	729.8	184.4	80	48.5
C18:0 SM	731.8	184.4	80	48.5
C20:0 SM	759.9	184.4	80	51
C22:0 SM	787.9	184.4	80	53.5
C24:1 SM	813.9	184.4	80	56
C24:0 SM	815.9	184.4	80	56
C26:1 SM	841.9	184.4	80	58
C26:0 SM	843.9	184.4	80	58
C12:0 LacCer	806.6	264.4	80	41
C14:0 LacCer	834.6	264.4	80	43.5
C16:0 LacCer	862.7	264.4	80	46
C18:1 LacCer	888.7	264.4	80	48.5
C18:0 LacCer	890.7	264.4	80	48.5

C20:0 LacCer	918.7	264.4	80	51
C22:0 LacCer	946.8	264.4	80	53.5
C24:1 LacCer	972.9	264.4	80	56
C24:0 LacCer	974.9	264.4	80	56
C26:1 LacCer	1000.9	264.4	130	47
C26:0 LacCer	1002.9	264.4	130	47

Name of Material/ Equipment	Company	Catalog Number
1 mL polypropylene pipette tips	NA	NA
1.5 mL polypropylene centrifuge tubes	NA	NA
10 mL Erlenmeyer flask	VWR	89091-116
AB Sciex Analyst 1.6.2	Sciex	NA
Ammonium formate	Fisher Scientific	A11550
Analytical scale	NA	NA
Bottle top dispenser	Sartorius	LH-723071
C12-Ceramide (d18:1/C12:0); N-(dodecanoyl)-sphing-4-enine	Avanti Polar Lipids	LM2212
C12-glucosylceramide (d18:1/12:0); N-(dodecanoyl)-1-β-glucosyl-sphing-4-eine	Avanti Polar Lipids	LM2511
C12-lactosylceramide (d18:1/12:0); N-(dodecanoyl)-1-ß-lactosyl-sphing-4-ene	Avanti Polar Lipids	LM2512
C12-Sphingomyelin (d18:1/C12:0), N-(dodecanoyl)-sphing-4-enine-1-phosphocholine	Avanti Polar Lipids	LM2312
CHLOROFORM OMNISOLV 4L	VWR	EM-CX1054-1
ClickSeal Biocontainment Lids	Thermo Scientific	75007309
Conflikt	Decon Labs	4101
CTO-20A/20AC Column Oven	Shimadzu	NA
d17:1-Sphingosine; (2S,3R,4E)-2-aminoheptadec-4-ene-1,3-diol	Avanti Polar Lipids	LM2000
d17:1-Sphingosine-1-phosphate; heptadecasphing-4-enine-1-phosphate	Avanti Polar Lipids	LM2144
DGU20A5R degasser	Shimadzu	NA
Disposable Culture Tubes 13x100mm	VWR	53283-800
Heated water bath	NA	NA
Homogenizer 150	Fisher Scientific	15-340-167
Homogenizer Plastic Disposable Generator Probe	Fisher Scientific	15-340-177
Kimwipes	Kimtech	34120
Methanol LC-MS Grade 4L	VWR	EM-MX0486-1
Nexera LC-30 AD binary pump system	Shimadzu	NA
Permanent marker	VWR	52877-310
Phenolic Screw Thread Closure, Kimble Chase (caps for disposable culture tubes)	VWR	89001-502
Phosphate bufffered saline	Thermo Scientific	10010023
Repeater pipette	Eppendorf	4987000118
Screw Caps, Blue, Red PTFE/White Silicone	VWR	89239-020
Screw Thread Glass Vials with ID Patch	VWR	46610-724

SIL-30AC autoinjector	Shimadzu	NA
SpeedVac	Thermo Scientific	SPD2030P1220
Supelco 2.1 (i.d.) x 50 mm Ascentis Express C18 column	Sigma Aldrich	53822-U
Triple Quad 5500+ LC-MS/MS System	Sciex	NA
Ultrasonic water bath	Branson	Model 2800
Vortexer	NA	NA
VWR Culture Tubes Disposable Borosilicate Glass	VWR	47729572
Water Hipersolve Chromanorm LC-MS	VWR	BDH83645.400

Comments/Description
Used to retrieve specimens

Used for tube and tissue weighing
Software to analyze and integrate MS data
For LC mobile phases
Scale that is accurate to 0.1 mg
Used for dispensing solvents
Internal standard
Internal standard
Internal standard
Internal standard

To prevent biohazard aeresols during centrifugation
Decontaminant
For LC
Internal standard
Internal standard

13x100 mm screw top tubes
For overnight lipid extraction
triturate tissues
for homogenization
Laboratory grade tissue used to make wicks

For LC-MS

13x100 mm screw top tube caps
To retrieve specimens from tubes after washing
To dispense LC-MS internal standards
Autoinjector vial caps
Autoinjector vials

For drying solvents

For LC-MS

For LC-ESI-MS/MS

for homogenization and resuspension of extracted and dried lipids

For sOCTrP and resuspending dried lipids

13x100 glass culture tubes

Dear Dr. Bajaj,

Thank you for your suggestions and edits. We have gone through and addressed all the comments and edits that you suggested. To this end, we have added "Replies" to your comments to facilitate your review. We have also followed your guidelines to address sections where the "how" of the steps was not clear. Therefore, there are some new steps and notes that had to be added to improve the clarity of the "how". We hope that this is acceptable at this stage. After completing your suggestions and accepting your edits, we believe they have made our manuscript better and the methods much clearer.

We have also removed highlights from all "notes", and ensured that any "note" that needed to be filmed was converted into a step. We also checked that there was only one note per step.

Finally, we have added the sections to the discussion that you have requested.

Thank you again,

Santiago Lima