Version 1

Aug 05, 2022

Lipidomic analysis of tissue culture cells, tissues, and purified organelles V.1

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dx.doi.org/10.17504/protocols.io.byn2pvge

Harper JW

ABSTRACT

Lipids are a class of molecules that have roles in energy storage, plasma membrane integrity, and signaling events. To gain more understanding of the functions and roles that lipids play in biology, researchers employ discovery analytical approaches, such as mass spectrometry (MS)-based lipidomics. The main objective of this protocol is to provide directive on how to extract lipids from plasma, cells, tissue, and purified organelles for analysis by liquid chromatography (LC)-MS. This analysis will typically yield quantitative data for more than 200 lipids, depending on the sample type analyzed, across a range of lipid classes: phospholipids, cardiolipins, sphingolipids, di- and triacylglyerols, and cholesteryl-esters.

ATTACHMENTS

MSMS exclusion list.pdf

DOI

dx.doi.org/10.17504/protocols.io.byn2pvge

PROTOCOL CITATION

Annie Jen, Lia Serrano, Katherine A. Overmyer, Joshua J. Coon 2022. Lipidomic analysis of tissue culture cells, tissues, and purified organelles . **protocols.io** https://dx.doi.org/10.17504/protocols.io.byn2pvge

Citation: Annie Jen, Lia Serrano, Katherine A. Overmyer, Joshua J. Coon Lipidomic analysis of tissue culture cells, tissues, and purified organelles https://dx.doi.org/10.17504/protocols.io.byn2pvge

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FUNDERS ACKNOWLEDGEMENT

NIH
Grant ID: P41GM108538

KEYWORDS

ASAPCRN

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CREATED

Sep 29, 2021

LAST MODIFIED

Aug 05, 2022

PROTOCOL INTEGER ID

53690

GUIDELINES

During the extraction process, both samples and extraction solvents should be maintained on ice throughout, with as little exposure to ambient temperature as possible.

MATERIALS TEXT

Materials and Equipment

Equivalent materials or equipment may be used unless otherwise noted.

1. Adjustable micropipettors
2. Ultrasonic bath or homogenizer
3. 1.5 mL microfuge polypropylene tubes
4. Glass vials with cap
5. Microcentrifuge capable of reaching 4°C and 14,000 x g
6. Vortexer
7. Centrifuge capable of vacuum drying
8. Ice
9. Ice bucket (e.g. made of ethylene-vinyl acetate or styrofoam)
10. Mortar and pestle
11. Liquid nitrogen source
12. Amber autosampler vials with fused 350 µL vial insert, Thermo Fisher Scientific, part C4000-LV2W
13. 9 mm autosampler vial screw thread caps, Fisher Scientific, part 03-376-481

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14. Heat block capable of reaching and maintaining 60°C
15. Fume hood
16. 5 mm steel beads, Qiagen, part 69989 (optional, for tissue or cells)
17. Laboratory mill (optional, for tissue or cells)
18. Acquity CSH C18 Column, 100 mm x 2.1 mm, 1.7 µm, 130Å; Waters, part number 186005297
19. Acquity CSH C18 Pre-column
20. Thermo Vanquish ultra-high-pressure LC, or similar system with chilled autosampler, heated column compartment, and pump pressure limits above 900 bar.
21. Thermo Scientific Q Exactive HF Orbitrap, or similar high-resolution mass spectrometry system
with heated electrospray ionization and MS/MS data acquisition capabilities in both positive and negative mode.

Solutions

1. Lipid Resuspension Solvent (65:30:5 Acetonitrile:Isopropanol:Water)
In a glass vial, combine 6.5 mL Acetonitrile, 3 mL Isopropanol, and 0.5 mL Water.
Mix well.

2. Mobile Phase A (60% Acetonitrile, 10 mM Ammonium Acetate, 0.025% Acetic Acid)
In a 50 mL conical tube, dissolve 3.083 g (± 0.038 g) NH4Ac in 50 mL H2O by vortexing.
In a 4L amber bottle, combine 1150 mL of H2O and 2800 mL acetonitrile.
Stir solution on a stir plate with a stir rod.
Add NH4Ac buffer slowly while stirring.
Add 1 mL acetic acid.
Allow to stir overnight.

3. Mobile Phase B (90% Isopropanol, 10% Acetonitrile, 10 mM Ammonium Acetate, 0.025% Acetic Acid)
In a 10 mL conical tube, dissolve 3.083 g (± 0.038 g) NH4Ac in 10 mL H2O by vortexing.
In a 4L amber bottle, combine 3600 mL 2-propanol with 400 mL acetonitrile.
NOTE: This is an endothermic reaction.
Begin mixing mixture on a heated stir plate at 50°C for 30 minutes or until room temperature.
Slowly add acetate buffer drop-wise with burette or in 0.5 mL increments every 2-3 minutes.
Add 1mL acetic acid to buffered solution.
Turn off heat and allow to mix for at least 1 hour before use.

Reagents

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Compound	CAS Number	Minimum Grade/Purity	Suggested Source/Catalog
Water	7732-18-5	Milli-Q	In-house
Methanol	67-56-1	LC-MS	Fisher Chemical/A456
Acetonitrile	75-05-8	LC-MS	Fisher Chemical/A955
tert-Butyl methyl ether (MTBE)	1634-04-4	ACS	Sigma Aldrich/443808
Isopropanol	67-63-0	LC-MS	Fisher Chemical/A461
Ammonium acetate	631-61-8	LC-MS	Sigma Aldrich/73594
Acetic acid	64-19-7	ACS	Sigma Aldrich/695092
Toluene	108-88-3	HPLC	Sigma Aldrich/34866

SAFETY WARNINGS

Direct skin/eye contact or inhalation of liquid nitrogen should be avoided, at risk of frostbite, cold burns, or permanent damage to the body. Please wear appropriate PPE while handling.

Pipette tips and other equipment that has contact with non-denatured biological sample material should be either disposed of in biohazard bins or cleaned appropriately if not disposable. It is recommended to clean the affected bench area and equipment with 10% bleach solution after working with biological materials.

Sample Preparation Procedures

1 Plasma Preparation

1.1 Remove plasma samples from frozen conditions, immediately place on ice, and allow to thaw.

Transfer 10 µL into a 1.5 mL microfuge tube using a pipette. Ensure to maintain samples on ice for the remainder of the extraction.

2 Cell Pellet Preparation

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2.1 A minimum of $10x10^6$ cells should be present in the cell pellet for extraction for sufficient peak intensity on the MS. For smaller sample amounts, extraction solvent volumes can be scaled appropriately to maintain sufficient signal.

2.2 No additional workup required if the frozen cell pellet has already been washed and the supernatant removed. Extraction is best performed directly with frozen cell pellet.

2.3 If the cell pellet requires washing (i.e. residual media present):
1. Chill a vial of wash solution. For lipidomics, an ammoniated buffer (such as 0.3% ammonium acetate, 0.3% ammonium formate, 0.9% NaCl, 1 M PBS, and 100 mM HEPES [1]) may be more appropriate for washing to be amenable to electrospray ionization on MS.
2. Add 1 mL wash to the pellet and resuspend by pipetting up and down.
3. Centrifuge at 14,000 x g for 2 minutes at 4°C, then decant supernatant.
4. Repeat steps 2-3 as necessary; it is recommended to wash a total of 2-3 times.
5. On ice, allow cell pellet to dry for 5 minutes.
6. Cell pellet can be used immediately for extraction (maintain cell pellet on ice throughout).

Otherwise, the washed cell pellet can be snap frozen by plunging into liquid nitrogen until after the nitrogen stops boiling and stored at -80°C for later use.

[1] Ulmer, C., Yost, R., Chen, J., Mathews, C., Garrett, T.: Liquid Chromatography-Mass Spectrometry Metabolic and Lipidomic Sample Preparation Workflow for Suspension Cultured Mammalian Cells using Jurkat T lymphocyte Cells. J Proteomics Bioinform. 8(6): 126-132. doi: 10.4172/jpb.1000360

3 Tissue Preparation

Note: Throughout tissue preparation procedure, tissue samples must remain frozen; as such, each step must be performed in a timely manner to prevent thawing prior to extraction.

3.1 Pulverize tissue samples in a ceramic mortar and pestle cooled with liquid nitrogen, ideally into a fine powder.

3.2 Weigh approximately 15 mg of the frozen pulverized tissue into an appropriate tube (e.g. 1.5 mL polypropylene microcentrifuge tube). Record weight (“frozen wet weight”) for later data normalization purposes.

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4 **Purified organelle (mitochondria, nuclei, lysosomes, endosomes, etc.) preparation.**
Purification of organelles typically is achieved through differential centrifugation or immunopurification approaches. For both approaches, it is best that the sample is washed well with a low-salt or an ammoniated buffer, for example 100 mM ammonium acetate, that is compatible with electrospray ionization. It should be noted that high sucrose conditions commonly used in differential centrifugation can affect the extraction procedure. Immunopurification using magnetic beads (or similar approach) is suitable for direct extraction of lipids; there is no need to elute the organelles from the beads.

4.1 Lysosomes are purified from 293 cells expressing endogenously tagged TMEM192-HA as described in protocol: dx.doi.org/10.17504/protocols.io.byi9puh6

4.2 Endosomes are purified from 293 cells stably expressing FLAG-EEA1 as described in protocol: dx.doi.org/10.17504/protocols.io.byi9puh6

4.3 Similar to cultured cells, purified organelles can be directly extracted if sample has been sufficiently washed to remove buffers used during organelle purification.

4.4 Extraction volumes should be adjusted to account for the lower material yield of organelle purification. For organelle yields of ~25 µg of protein, expect to use a final extraction volume of ~300 µL. It is recommended to preform a dilution series and adjust volumes as needed to achieve highest number of lipids quantified within linear quantification range.

Extraction Procedures

5 General guidlines: For liquid samples of significant volume, the liquid sample volume may be used to substitute part or all of the water portion of the extraction solvent mixture, providing that the overall extraction solvent component proportions are preserved. The aqueous component should be added last to induce phase separation.

The overall total extraction volumes are guidelines and can also be adjusted based on sample type and amount, providing that the overall extraction solvent component proportions are preserved.

5.1 **Extraction Method 1: MTBE extraction**
For liquid samples of significant volume, the liquid sample volume may be used to substitute part or all of the water portion of the extraction solvent mixture, providing that the overall extraction solvent component proportions are preserved. The aqueous component should be added last to induce phase separation.

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The overall total extraction volumes are guidelines and can also be adjusted based on sample type and amount, providing that the overall extraction solvent component proportions are preserved.It is recommended with new sample types that several dilutions are evaluated for linear response and number of lipid identifications.

5.2 Matyash [2] method solvent proportions: 10/3/2.5 v/v/v, MTBE/methanol/water

Note: Tubes used should be sufficiently translucent to visually evaluate separation between two liquid layers in the sample solution.

[2] Matyash, V., Liebisch, G., Kurzchalia, T., Shevchenko, A., Schwudke, D.: Lipid Extraction by Methyl-*tert*-butyl Ether for High-Throughput Lipidomics. J Lipid Res. 49(5): 1137-1146. doi: 10.1194/jlr.D700041-JLR200

5.3 Chill vials of MTBE, methanol, and water on ice, and maintain cold throughout analysis.

5.4 Add 225 µL chilled methanol to the sample. Vortex for 10 seconds.

5.5 Add 750 µL chilled MTBE to the sample. Vortex for 10 seconds.

5.6 To induce phase separation, add 187.5 µL chilled water to the sample.

5.7 If lysis is required (for cells, tissues, or organelles): there are several options that can be pursued and are detailed below.
1. Steel beads: Add a single 5mm stainless steel bead to each sample tube with screw cap. Using a Retsch MM400 mixer mill in a cold room environment (4°C), bead beat the samples at a frequency of 25 hz for 5 minutes.
2. Probe sonicator: in a cold room environment (4°C), probe sonicate the samples for 10 seconds, avoiding generation of excessive heat during sonication process.
3. Ultrasonic bath: with the flowing water as cool as possible, sonicate the cells with the following settings: 5 minutes process time, 20 s pulse-on, 10 s pulse-off, amplitude 30.

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If lysis is not required: vortex the samples for 10 seconds, then allow the samples to incubate on ice for at least 10 minutes.

5.8 Allow samples to incubate on ice for at least 10 minutes.

5.9 Centrifuge the samples at 14,000 x g for 2 minutes at 4°C.

5.10 Visually evaluate the sample. The resulting solution should contain two distinct layers; the upper organic layer (enriched with lipids) and the lower aqueous layer (enriched with metabolites). A pellet may also be present at the bottom of the tube; avoid contact or agitation during layer removal.

5.11 **Lipid fraction:** Transfer 100 µL of the upper organic layer into an autosampler vial with fused glass insert, and dry down in a vacuum concentrator at ambient temperature. The dried lipids can be frozen and resuspended at a later date.

Dried Fractions Preparation for Mass Spectromtery Evaluation

6 Lipidomics Sample Resuspension for LC-MS Analysis

Volumes may be adjusted to suit the amount of dried lipids in sample.

6.1 Add 50 µL Lipid Resuspension Solvent (9:1 MeOH:toluene, prepared as described above.) to each vial containing dried lipid extract. Cap vial.

6.2 Vortex for at least 10 seconds to resuspend contents.

6.3 Briefly centrifuge vials to spin down any liquid on the caps.

6.4 Maintain the prepared sample solutions at 4°C prior to analysis via LC-MS/MS.

9. Lipidomics LC-ESI-MS/MS Analysis

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7 Instrument Preparation

7.1 On the LC system, purge mobile phase lines with their respective mobile phase solutions to remove potential air bubbles.

7.2 Connect the Waters Acquity CSH C18 column and corresponding pre-column to the LC system and ensure pressure readings are appropriate for the flow rates and no leaks are present in the system.

7.3 Ensure that MS evaluations have passed and mass calibrations have been performed on the system. Calibrate system components as necessary.

Sample Analysis

8 Instrument Parameters

8.1 Inject 10 µL of sample onto column using autosampler. Samples will be separated at a constant flow rate of 0.4 mL/min at 50°C, with the multi-step gradient program shown below:

LC Gradient		
Time (min)	**%B**	**Ramp**
0	2	5
2	2	5
5	30	5
6	50	5
20	85	5
21	99	5
28	99	5
28.25	2	5
30	2	5

8.2 MS acquisition will be performed with a Thermo Scientific heated electrospray ionization (HESI II) probe in Ion Max API source housing operating in both positive and negative mode. Source parameters should be set as follows: capillary temperature at 350 °C, sheath gas flow rate at 25 units, aux gas flow rate at 15 units, sweep gas flow rate at 5 units, spray voltage at |3.5 kV|, and S-

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lens RF at 90.0 units. The MS should be operated in a polarity switching mode acquiring positive and negative full MS1 scans followed by data-dependent MS2 spectra (Top2) within the same injection, using the exclusion list in Appendix 1. Data acquisition for full MS1 scans in both modes should be collected at 30,000 resolution, 1×10^6 automatic gain control (AGC) target, 100 ms ion accumulation time (max IT), and 200 to 2000 m/z scan range. Data dependent (dd-MS2) scans in both modes should be collected at 30,000 resolution, 1×10^5 AGC target, 50 ms max IT, 1.0 m/z isolation window, and stepped normalized collision energy (NCE) at 20, 30, 40, with a 10.0 s dynamic exclusion.

Spectral Searching

9 The resulting LC–MS/MS data is processed using Compound Discoverer 2.1 or higher (Thermo Scientific) and LipiDex **[3]**, an in-house-developed software suite. The LipiDex software and tutorials can be found at https://github.com/coongroup/LipiDex.

[3] Hutchins, P., Russell, J., Coon, J.: LipiDex: An Integrated Software Package for High-Confidence Lipid Identification. Cell Systems. 6(5): 621-625. doi: 10.1016/j.cels.2018.03.011

10 File Conversion: Convert raw data files to centroid and .mgf format via Proteowizard msConvertGUI, a file converter software that accepts a majority of vendor-specific file formats.

10.1 Spectral matches with a dot product score greater than 500 and a reverse dot product score greater than 700 are recommended for further analysis; however, values can be adjusted to suit needs of analysis.

10.2 Open the Spectrum Searcher module in LipiDex and add the .mgf files generated per Step 10.

10.3 It is recommended to use the “Lipidex_HCD_acetate” library. This library containing in silico generated fragment ions for the major classes of lipids found in mammalian samples.

10.4 Update the MS/MS parameters as necessary, with the parameters listed as follows:

MS1 Search Tolerance: the absolute mass tolerance used to search for valid lipids for the precursor of each MS/MS spectrum.

MS2 Search Tolerance: the absolute mass tolerance used for spectral similarity scoring.

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Max Search Results Returned: the maximum possible number of search results returned for each spectrum.

MS2 Low Mass Cuttoff: the minimum m/z value in each ms/ms spectra which will be used for spectral similarity scoring. This value is useful for removing low mass acetate or formate fragments which are ubiquitous and can skew spectral similarity scoring.

10.5 Select “Search Spectra”; MS/MS search results will be written to the same directory as the input .mgf files.

Compound Discoverer 3.1 Peak Finding

11 For LipiDex to accurately associate MS/MS identifications with the actual feature retention times, it is necessary to generate both a retention time aligned and unaligned feature table.

11.1 Open Compound Discoverer and select “New Study and Analysis” from the main menu, and select “Next”.

11.2 Fill in the appropriate study fields and select the directory where the new study will be created, then select “Next”.

11.3 Select “Add Files”, then add all .raw files to the study.

Note: Because of the file identifiers used by Compound Discoverer, any subsequent changes to this file list will require a new study to be created. If any changes are made to the file list, LipiDex will not be able to associate File ID numbers with the correct .raw file.

11.4 Select “Next”, and identify any blank matrix files as blanks.

11.5 Select “Next”, then “Finish”.

Note: Because of the file identifiers used by Compound Discoverer, do not apply any sample groups to the study.

11.6 **Aligned Workflow creation**: in the Workflow tab, add and connect the correct workflow nodes as follows, then add the following parameters to each node:

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"Input Files" → "Select Spectra" → "Align Retention Times" → "Detect Unknown Compounds" → "Group Unknown Compounds" → "Fill Gaps" → "Mark Background Compounds"

11.7 1. Suggested node parameters are detailed below.

Node: **Select Spectra**	
Parameter	**Value**
Precursor Selection	Use MS (n-1) Precursor
Lower RT Limit	0.2
Upper RT Limit	21
First Scan	0
Last Scan	0
Ignore Specific Scans	(Leave blank)
Lowest Charge State	0
Highest Charge State	0
Min. Precursor Mass	100 Da
Max. Precursor Mass	5000 Da
Total Intensity Threshold	0
Minimum Peak Count	1
Mass Analyzer	Any
MS Order	Any
Activation Type	Any
Min. Collision Energy	0
Max. Collision Energy	1000
Scan Type	Is Full
Polarity Mode	(Not specified)
S/N Threshold (FT-only)	1.5
Unrecognized Charge Placement	1
Unrecognized Mass Analyzer Replacement	FTMS
Unrecognized MS Order Replacements	MS1

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Unrecognized Activation Type Replacements	HCD
Unrecognized Polarity Replacements	+
Unrecognized MS Resolution Replacements	60000
Unrecognized MSn Resolution Replacements	15000

Node: Align Retention Times	
Parameter	**Value**
Alignment Model	Adaptive Curve
Alignment Fallback	Use Linear Model
Maximum Shift [min]	0.6
Shift Reference File	True
Mass Tolerance	7.5
Remove Outlier	True

Node: Detect Unknown Compounds	
Parameter	**Value**
Mass Tolerance (ppm)	10
Intensity Tolerance (%)	100
S/N Threshold	3
Min. Peak Intensity	500000
Ions	[M+H]+1; [M+TFA-H]-1
Min. Element Counts	C H
Max. Element Counts	C100 H190 N10 Na2 O15 P2 S2
Filter Peaks	True
Max. Peak Width [min]	0.5
Remove Singlets	True
Min # Scans per Peak	6
Min # Isotopes	4

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Node: Group Unknown Compounds	
Parameter	**Value**
Mass Tolerance	10
RT Tolerance [min]	0.5
Rule #1	Unspecified
Rule #2	Unspecified
Preferred MS Order	MS1
Preferred Iom	[M+H]+1; [M+TFA-H]-1

Node: Fill Gaps	
Parameter	**Value**
Mass Tolerance	10
S/N Threshold	3
Use Real Peak Detection	True

Node: Mark Background Compounds	
Parameter	**Value**
Max. Sample/Blank	3
Max. Blank/Sample	0
Hide Background	True

After completion, select “Run” and “Ignore” the message about file grouping to start the data analysis process.

11.8 **Unaligned Workflow creation**: in the same study, create a new workflow. Add and connect the correct workflow nodes as follows, then add the following parameters to each node:

“Input Files” → “Select Spectra” → “Detect Unknown Compounds”

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To ensure correct merging of the unaligned and the aligned datasets, use the same parameters as those used in the aligned workflow.

After completion, select “Run” to start the data analysis process.

9.4. Compound Discoverer (2.1 or 3.1) Feature Table Export

12 From the job queue, select the aligned experiment and select "Open Results" to view the aligned data.

12.1 Right click on the data matrix. Select "Export", then "Export to Excel".

12.2 To ensure all the necessary aligned peak data is imported into LipiDex, select the following options:

Level 1: Compounds
Level 2: Compounds per File
Level 3: Features

12.3 Select “Export” to generate the aligned .xlsx data table.

12.4 With the unaligned experiment, repeat steps 12 and 12.1 to open the unaligned workflow export menu.

12.5 To ensure all the necessary unaligned peak data is imported into LipiDex, select the following options:

Level 1: Features
Levels 2 and 3 remain blank.

12.6 Select “Export” to generate the unaligned .xlsx data table.

12.7 Save each exported .xlsx data table as a separate comma separated value (.csv) sheet for use in Peak Finder.
Note: Large data sets might be too large to export at one time due to Excel’s row limits. In this case, use checked boxes to export subsets of the data, “use

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checked boxes" in export to Excel. These smaller files can be converted to .csv and then merged using a text editor.

9.5. Lipid Chromatographic Peak Identification

13 Open the Peak Finder module in LipiDex. For the peak table type, select "Compound Discoverer".

13.1 In the "Upload peak tables" section, upload the aligned and unaligned .csv peak feature tables generated from Compound Discoverer by selecting the "Add" buttons.

13.2 In the "Upload MS/MS result files" section, select the "Load Files" button to bring up the "Results Uploader" window. Select the appropriate data acquisition type, then add the .csv files generated by Spectrum Searcher.

Note: If "Separate Polarity Analysis" is selected, edit the "File ID" field in the table. The positive and negative polarity experiments to be merged should share the same File ID number.

13.3 Update the MS/MS filtering parameters as necessary (defalt settings are recommended), with the parameters listed as follows:

Min. Lipid Spectral Purity: the minimum spectral purity needed to annotate a lipid at the molecular composition level (PC 16:1_18:1) rather than at the sum composition level (PC 34:2).

Min. MS2 Search Dot Product: the minimum spectral similarity score needed to use an MS/MS identification.

Min. MS2 Search Rev. Dot Product: the minimum reverse spectral similarity score needed to use an MS/MS identification.

FWHM Window Multiplier: the maximum allowed retention difference between the apex of the chromatographic peak and the MS/MS spectra in terms of the FWHM of the chromatographic peak.

Max. Mass Difference: the maximum relative mass difference (ppm) allowed to associate a lipid identification with a chromatographic peak.

13.4 Update the Result Filtering Parameters section as needed, with the parameters listed as follows:

Max. RT M.A.D. Factor: the maximum allowed retention time difference between a lipid identification and the all other identified lipids of the same

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class in terms of multiples of the median absolute retention time deviation of the lipid class. A value of 3.5 is used as default.

Feature Found in n Files: the minimum number of times the specific feature was identified to be included in the final peak table. A value of 1 is used. For large data sets, values higher than 1 are recommended.

13.5 Select “Identify Chromatographic Peaks” to begin filtering peaks. Upon completion, all result files are written to the same directory as the peak tables, and are labelled as follows:

Final_Results.csv: contains the filtered peak table.
Unfiltered_Results.csv: includes all peaks.
Sample_Information.csv: contains sample-specific metrics.

Note: If individual fatty acid substituents could not be resolved, then identifications were made with the sum of the fatty acid substituents.

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