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Qualitative Characterization of the Rat liver Mitochondrial Lipidome using All Ion Fragmentation on an Exactive Benchtop Orbitrap MS

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Summary

Untargeted lipidomics profiling by liquid chromatography-mass spectrometry (LC-MS) allows researchers to observe the occurrences of lipids in a biological sample without showing intentional bias to any specific class of lipids and allows retrospective re-analysis of data collected. Typically, and in the specific method described, a general extraction method followed by LC separation is used to achieve non-specific class coverage of the lipidome prior to high resolution accurate mass (HRAM) MS detection. Here we describe a workflow including the isolation of mitochondria from liver tissue, followed by mitochondrial lipid extraction and the LC-MS conditions used for data acquisition. We also highlight how, in this method, all ion fragmentation can be used to identify species of lower abundances, often missed by data dependent fragmentation techniques. Here we describe the isolation of mitochondria from liver tissue, followed by mitochondrial lipid extraction and the LC-MS conditions used for data acquisition.

Keywords

mitochondria; lipidomics; LC-MS; cardiolipins; HCD

1. Introduction

Mitochondria are intracellular membrane-enclosed organelles that play crucial role in bioenergetics, biosynthesis of critical cellular constituents, the regulation of cell survival, and the execution of cell death pathways (1,2). Lipids are essential to mitochondrial viability, and they are involved in the regulation of the wide range of mitochondrial functions, such as maintenance of membrane structural composition and fluidity, membrane fusion and fission, electron transport and oxidative phosphorylation, signal transduction, interaction with other cellular constituencies such as proteins and glycoproteins, and energy

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storage (3–5). The role of lipids in mitochondrial function is, for example, exemplified by mitochondrial phospholipid cardiolipin (CL). CL, which is almost exclusively found in the inner mitochondrial membrane, plays multiple key roles in the regulation of mitochondrial metabolism, including regulation of essential enzymatic activities involved in electron transport and oxidative phosphorylation, and assembly of respiratory supercomplexes (5–7). Recent studies suggest a critical role of mitochondrial lipid cardiolipin in apoptotic cell death pathway (8).

Mitochondrial lipids are also both a major target for oxidative damage by reactive oxygen species produced during respiration and a major source of lipid peroxides and peroxidation byproduct (e.g., hydroxyalkenals, γ -isoketoaldehydes) production that serve to amplify oxidative damage under pathophysiological conditions (9–13). Development of analytical methods for accurate quantitative and qualitative analysis of all lipid classes and species that contribute to and reflect mitochondrial function is, therefore, highly important.

Liquid chromatography with high resolution accurate mass (HRAM)-MS is widely used to study the biochemical species (proteins, metabolites, lipids etc) that comprise a biological sample (14–16). These techniques offer the speed, sensitivity and specificity necessary to determine the biochemical composition of a system by providing both qualitative and relative quantitative results. This is imperative for a successful non-targeted lipidomics analysis, where all lipid species are considered of potential biological importance and therefore there can be no deliberate analytical bias is given toward any of the 8 lipid categories established and defined by LIPID MAPS (17).

By using HRAM instrumentation, such as the Exactive benchtop Orbitrap MS, for both full scan and all ion fragmentation (AIF), a broad picture of the lipidome is captured and the electronic data record obtained can easily be used for both primary and retrospective analysis. LC separation followed by HRAM detection in combination with MS/MS fragmentation allows both known and unknown species to be structurally elucidated and monitored. Using the method described herein, containing both LC and MS with AIF MS/MS, the identification of more than 350 unique lipids in serum, mitochondria and premature infant fecal samples have been found and reported (18–21).

2. MATERIALS

2.1. Liver Mitochondria Isolation

1. Isolation buffer IB1: 240 mM Sucrose, 10 mM HEPES, pH 7.4, 1 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) stock with pH 7.2 adjusted by KOH, 5 g/1L bovine serum albumin (BSA, Sigma-Aldrich, fatty acid free). (See Notes 1–5)
2. Petri dish
3. 50 ml plastic disposable beaker.
4. Potter-Elvehjem glass-Teflon homogenizers 40 ml and 15 ml volume
5. 50 ml round-bottom centrifuge tubes.

6. Fixed angle JA-20 centrifuge rotor and appropriate centrifuge, (both, Beckman, USA), both pre-chilled to 4 °C.
7. Secure the pestle in the chuck of the electrical motor.
8. Isolation buffer IB2: 82.152 g/1L sucrose, 2.38 g/1L HEPES, pH 7.4, adjusted by KOH)
9. Bicinchoninic acid kit (Sigma-Aldrich, USA) to determine mitochondrial protein concentration and 1.0 mg/mL BSA as a standard solution (*See Note 6*)
10. Spectrophotometer, Uvikon 943, or equivalent.
11. Washing buffer: 125 mM KCl, 10 mM HEPES, pH 7.4 adjusted by KOH.

2.2. Mitochondrial Lipid Extraction

1. 1.5 mL Fisher brand siliconized low-retention microcentrifuge tubes (Fisher Scientific, Pittsburgh PA)
2. Microcentrifuge floating rack
3. Mechanical ultrasonic cleaner
4. 10 mL Falcon tubes
5. Thermo Scientific Savant SPD111V P1 ()
6. ACS grade dimethyl sulfoxide (DMSO)
7. ACS grade dichloromethane (DCM)
8. LC-MS grade methanol (MeOH)
9. LC-MS grade isopropanol (IPA)
10. LC-MS grade acetonitrile (ACN)
11. All water (H₂O) was deionized to attain a sensitivity of 18 M Ω cm at 25°C
12. Lipid internal standard solution: 1.5 mL, enough for 50 samples; Combine 37.50 uL of a 2mg/mL solution of 1,1',2,2'-tetraoleoyl cardiolipin (CL(18:1)4) (Avanti Polar Lipids), 30 uL each of a 2.5mg/mL solution of, 2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0) and 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (PG(14:0/14:0) (Avanti Polar Lipids), 15 uL of a 5mg/mL solution of 11-O-Hexadecyl-sn-glycero-3-phosphocholine (lysoPC(16:0)) (Enzo Chemicals) and 75 uL of a 1 mg/mL solution of 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (PS(16:0/16:0) (Avanti Lipids) followed by 1297.5 uL of 2:1 DCM:MeOH. All standards are dissolved in 2:1 mixture of DCM:MeOH prior to mixing and stored at -20 °C
13. LC-MS internal standard: Create 100 mL of a 5ug/mL working concentration solution of LC-MS internal standard 1,2-diheptadecanoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (PG 17:0/17:0) by adding 200 uL of a 2.5 mg/mL stock solution to 100 mL of a 63:30:5 ACN:IPA:H₂O mixture.

2.3. LC-MS Analysis

1. LC-MS grade acetonitrile with 0.1% formic acid
2. Ascentis Express C₁₈ 2.1 × 150 mm 2.7µm column (Sigma-Aldrich, St. Louis, MO)
3. Column hot pocket thermostated column heater (Thermo Fisher Scientific, San Jose, CA)
4. Thermo Fisher Scientific PAL autosampler, (Thermo Fisher Scientific, San Jose, CA)
5. Accela quaternary HPLC pump (Thermo Fisher Scientific, San Jose, CA)
6. Exactive benchtop Orbitrap mass spectrometer with HCD (Thermo Fisher Scientific, San Jose, CA) and heated electrospray ionization (HESI) probe.
7. Mobile phase A was prepared by dissolving 630 mg of ammonium formate into a mixture of 600 mL Water and 400 mL ACN with 0.1% formic acid. This bottle was then sonicated for several minutes to remove any gases from mixing solvents.
8. Mobile phase B was prepared by dissolving 630 mg of ammonium formate into 1mL of water before adding to 900 mL of IPA and 100 mL of ACN with 0.1% formic acid. This bottle was then sonicated for several minutes to facilitate dissolution and remove any gases from mixing solvents.
9. SIEVE v 1.3 Differential Analysis Software (Thermo Fisher Scientific, San Jose CA)

3. METHODS

3.1. Liver Mitochondria Isolation

1. All isolation steps have to be done on ice, and all operations have to be carried out at 0–4°C as described previously (22). (See **Note 7**) Alternative mitochondrial isolation approaches from liver and/or other tissue are also expected to provide suitable starting material, although the user should be aware of the characteristics of any given preparation with regards, for example, to purity. The use of other preparations would replace steps 1–12 of this protocol.
2. Take liver tissue (8–10 g) from the animal (See **Note 8**) and place it immediately in Petri dish containing ice-cold 20–30 ml of the isolation buffer #1 (IB1). Wash out blood.
3. Transfer liver tissue in a 50 ml plastic disposable beaker containing 20–30 ml of IB1, and mince the tissue with scissors, or use a press.
4. Pour the suspension in Potter-Elvehjem glass-Teflon homogenizer and add IB1 to a total volume of 40 ml.

5. Homogenize the suspension using 7–8 up-and-down strokes with the pestle rotating at 500–600 rpm. (*See Notes 9,10*) Suspension should look homogeneous.
6. Transfer the obtained homogenate into 50 ml round-bottom centrifuge tubes. Centrifuge the tubes at 1,000 g x 10 min. (*See Note 11*)
7. Collect the supernatant and centrifuge it at 11,000 g x 10 min. (*See Note 12*)
8. Collect the pellet and resuspend it in 15 ml of IB1 in the Potter-Elvehjem glass-Teflon homogenizer using 4–5 up-and-down strokes by hand. Suspension should look homogeneous. Adjust volume to 40 ml, mix, and centrifuge the suspension at 11,000 g x 10 min.
9. Resuspend the pellet in 15 ml of isolation buffer #2 (IB2) in the Potter-Elvehjem glass-Teflon homogenizer with 4–5 up-and-down strokes by hand. Suspension should look homogeneous. Adjust volume to 40 ml, mix, and centrifuge the suspension at 11,000 g x 10 min.
10. Resuspend final pellet in 0.5 ml of IB2 and store on ice. (*See Note 13*)
11. Add 10 μ l of mitochondrial suspension into 990 μ l of IB2 to measure protein concentration by bicinchoninic acid -based method using BSA as a standard (see sigmaaldrich.com for details).
12. Aliquot mitochondrial sample by 1 mg of protein, resuspend it in 125 mM KCl, 10 mM HEPES, pH 7.4 buffer, centrifuge at 11,000 g x 10 min, remove supernatant and freeze as dry pellets at -80°C for further analysis.

3.2. Mitochondrial Lipid Extraction

1. Add 40 μ L DMSO to each aliquot of mitochondria (containing 1 mg of protein). (*See Notes 14*)
2. Place each mitochondria/DMSO sample into the floating microcentrifuge rack and sonicate for 1 hour to disrupt the membranes.
3. Pipette 10 μ L from each sample into a 10mL Falcon tube to create a mitochondrial pool sample. The pool samples will be processed for quality control (QC) and lipid identification studies.
4. Add 5 μ L of lipid internal standard to each 30 μ L mitochondria sample, this includes samples from the study and pool samples created in step 3.
5. Add 190 μ L of MeOH to each sample and vortex for 10 seconds.
6. Next add 380 μ L of DCM and vortex for 20 seconds. (*See Notes 15,16*)
7. Finally, add 120 μ L of water to induce phase separation.
8. Vortex samples for 10 seconds and allow to equilibrate at room temperature for 10 minutes.
9. Centrifuge each sample at 8000 g for 10 minutes at 10°C . (*See Note 17*)

10. Using a 500 μ L pipette, transfer 320 μ L of the lower lipid-rich DCM layer to a clean microcentrifuge tube. Be careful to push the pipette tip along the side of the container when piercing the protein disk as not to disrupt the phase separation or transfer any protein with the lipid rich DCM layer.
11. Evaporate the samples to dryness under vacuum using a speedvac. (See **Note 18**)
12. Reconstitute samples in 300 μ L of LC-MS internal standard (PG (17:0/17:0)) before LC-MS analysis. (See **Note 19**)

3.3. LC-MS Conditions

1. Separate 10 μ L lipid extracts on an Ascentis Express C₁₈ 2.1 $\times$ 150 mm 2.7 μ m column connected to a Thermo Fisher Scientific PAL autosampler, Accela quaternary HPLC pump and an Exactive benchtop Orbitrap mass spectrometer equipped with a heated electrospray ionization (HESI) probe. (See **Notes 20,21**)
2. Run separations for 30 minutes with mobile phase A and B consisting of 60:40 Water:ACN in 10mM ammonium formate and 0.1% formic acid and 90:10 IPA:ACN also with 10mM ammonium formate and 0.1% formic acid, respectively.
3. The gradient starts at 32% B for 1.5 minutes; from 1.5 to 4 min increases to 45% B, from 4 to 5 min increases to 52% B, from 5 to 8 min to 58% B, from 8 to 11 min to 66% B, from 11 to 14 min to 70% B, from 14 to 18 min to 75% B, from 18 to 21 min to 97% B, during 21 to 25 min 97% B is maintained; from 25–30 min solvent B is decreased to 32% and then maintained. The flow rate is 260 μ L/min.
4. Maintain the column oven temperature at 45 °C and set the temperature of the autosampler to 4 °C. The same LC conditions and buffers are used for all MS experiments.
5. Set the spray voltage to 3.5 kV, whereas the heated capillary and the HESI probe are held at 250 °C and 350 °C, respectively.
6. Set the sheath gas flow to 25 units and the auxiliary gas to 15 units.
7. Hold these conditions constant for both positive and negative ionization mode acquisitions.
8. The instrument is tuned by direct infusion of PG (17:0/17:0) in both positive and negative mode and external mass calibration is performed using the standard calibration mixture and protocol from ThermoFisher approximately every five days.

3.4. Full scan profiling experiments

1. Operate the MS in high resolution mode, corresponding to a resolution of 60k and a 2 Hz scan speed and hold the scan range between m/z 120–2000.

2. Mitochondrial lipid extracts are profiled by injecting each sample once, in randomized order, with pool samples, blanks and IS mixture injections spread throughout the analysis.

3.5. Lipid identification studies

1. Run HCD experiments on the pool, lipid IS mixture and blank samples only.
2. Perform these experiments by alternating between full scan acquisitions and HCD scans, both run at 2 Hz. Three different HCD energies, 30, 60 and 100 eV, are used in separate experiments in both positive and negative mode.

3.6. Data Analysis

All LC-MS profiling samples are analyzed using the MS label free differential analysis software package SIEVE v 1.3 (Thermo Fisher Scientific and Vast Scientific, Cambridge, MA). (See **Notes 22,23**)

The frame m/z values were used to do batch searches on the Metlin database (23), the human metabolome database (HMDB) (24) and the LIPID MAPS database (25) and those matches were confirmed using the intact molecule's exact mass observed during the analysis, RT regions based on the lipid IS mixture elution times run throughout the sequence and HCD fragmentation patterns that chromatographically align with the intact exact mass extracted ion chromatograms for the parent compound. (See **Note 24**)

4. Notes

1. Use de-ionized water (Milli-Q, "Millipore") for mitochondrial buffer preparation.
2. Air-tight glass containers or Thermo Scientific Nalgene disposable bottles are strongly recommended for storage of mitochondrial buffers.
3. Sterilize all buffers by filtering through 0.22 μ m membrane (we recommend to use Thermo Scientific Nalgene disposable filter units that come with bottles). The buffers are stable for at least 3 months if kept sterile.
4. Do not freeze isolation and incubation buffers.
5. The buffer composition for mitochondria isolation and incubation may vary. For example, examination of the literature will show changes in relative concentrations of sucrose and/or mannitol, in the choice of buffering agent and counter ion, the inclusion/exclusion of divalent cation chelators, osmolarity, and ionic strength. The buffers described have been used by our lab for upstream respiration and/or calcium overload experiments and subsequent downstream lipidomics studies.
6. Alternative protein concentration approaches can be used, but linear range and potential interference must be examined

7. Quality and purity of isolated mitochondria is very important, because contamination of mitochondrial preparation with cytosolic structures can interfere/mask lipid profiling results.
8. Animals have to be sacrificed, and liver tissue has to be removed and placed in ice-cold buffer as quickly as possible.
9. During tissue homogenization steps, do not draw a vacuum and avoid bubble creation.
10. Avoid very fast pestle rotation during homogenization.
11. Do not use detergents to clean centrifuge tubes
12. Carefully remove fat from centrifuge tube walls with a delicate wiper after second centrifugation.
13. Make final mitochondria suspension rather concentrated than diluted (we recommend a concentration of 60–80 mg/ml of mitochondria).
14. Minimize contamination in mass spectrometry:

Do not store organic solvents used for the sample preparation in plastic tubes. PEG contaminants and plasticizers such phthalates can leach out of plastic into the sample and make their way to the LC-MS system. Always use glass bottles.

If you have to use detergent to wash the glassware, be sure to rinse them with large volume of deionized water before use. Detergents contain PEG materials which can contaminate reversed phase columns and cause ion suppression in mass spectrometry.
15. Dichloromethane is a harmful organic solvent with some evidence of carcinogenic effects. It must be handled wearing safety gloves and in a fume hood at all times. Be sure to read dichloromethane MSDS before use.
16. Dichloromethane has a high vapor pressure at room temperature that makes it difficult to be accurately measured and transferred using pipettes. To minimize loss of DCM through evaporation during pipetting, pipette up and down several times in DCM to ensure the inside of pipette tip is saturated with CDM vapors.
17. Centrifugation of the sample at 4°C helps keep the DCM layer cool. Transfer the DCM layer while it's still cool. Cold DCM is easier to transfer using pipette tips and less prone to loss via evaporation.
18. Before drying the lipid extracts in a vacuum centrifuge, cool the extracts down in a –20 °C freezer. This will help minimize exposure to DCM vapors when opening and placing sample tubes in the vacuum centrifuge, in case the instrument is not placed in a fume hood.
19. Reconstitute the samples with the appropriate solvent right before LC-MS analysis. Avoid keeping the reconstituted samples for prolonged time before LC-

MS analysis to minimize potential lipid degradation. Store the extra samples in a -80°C freezer.

- 20.** MS Preparation for Lipidomics Profiling: Mass calibration: Mass calibration should be done to the instrument vendor's specifications. On the Exactive in our laboratory, masses calibrations are done weekly. Calibrations are done separately in positive and negative ion modes.

MS Tuning: Tuning of the MS is done to optimize ion transmission through the ion optics to the analyzer. Tuning is therefore performed at the operating LC FLOW rate and the compound chosen as the tuning compound should have similar ESI characteristics as your molecules of interest and should be at the same concentration as a majority of molecules being analyzed. As an example, for our LC-MS analysis, we tee-in (using a splitter) $2.5\text{ }\mu\text{g/ml}$ of PG 17:0/17:0 flowing at $10\text{ }\mu\text{/minute}$ and 50:50 of mobile phase A: Mobile Phase B at a flow of $250\text{ }\mu\text{l/minute}$ (total flow into MS is $260\text{ }\mu\text{l/ml}$). The final concentration of PG 17:0/17:0 into the MS is $\sim 100\text{ ng/ml}$.

LC Solvents: Always use LC-MS grade solvents to avoid contamination of mass spectrometer with impurities. To assist with solubilization of ammonium formate in mobile phase B, first dissolve it in $\sim 1\text{ ml}$ of deionized water.

- 21.** Analysis of lipid samples by reversed-phase LC-MS: Lipids, (especially triglycerides) are very hydrophobic and thus they tend to stick to the injector port which leads to carry-over from sample-to-sample. To avoid carry-over, it is important to flush the injector port thoroughly. We usually flush the injector port with 50:50 acetonitrile: isopropanol and 50:50 isopropanol: dichloromethane (10X with each). The isopropanol in dichloromethane wets the needle and prevents drying (which can ruin the needle plunger). It is also important to ensure that the solvent used for flushing area is miscible.
- 22.** The framing parameters in these experiments are set at 0.01 Daltons for the m/z window and 1.00 minute for the RT window; 1000 is used at the intensity threshold. Peaks under this intensity will normally be background or too low to quantify robustly. These parameters can be adjusted based on your own data. A pool from the middle of the sample sequence should be used as a qualitative reference and for relative quantitation, and frames built off the reference are then applied to all samples in the experiment. If a different reference is used, the intensity values may change slightly, however, the overall lipid ratios should be the same.
- 23.** Chromatographic alignment and framing using Sieve: For Sieve analysis on the LC-MS lipidomics data, the small molecule, chromatographic alignment and framing and the non-differential single class analysis options are used. Positive and negative ion data are analyzed separately and only the full scan raw data is used for alignment and framing. The framing parameters used are determined by the user. The m/z window chosen depends on the mass accuracy of the instrument used. The retention time window chosen should be large enough to

enable correct chromatographic alignment to account for retention time drifts over time, but not too large so as to minimize false positives. We therefore typically use 1 minute for the retention time window and an intensity threshold of 1000 (to ensure that as many features as possible are identified but without picking out the noise).

24. Lipid Identification: To assist with lipid identification of the lipids obtained from the framing data, HCD analysis (done separately in the positive and negative modes) is performed on a pooled sample. HCD fragmentation is done at three energies 30 eV (Low energy), 60 eV (Medium energy) and 100 eV (high energy).

Lipid identification is done by matching the retention time of extracted ion chromatogram of a particular exact mass and its fragments obtained using the all ion fragmentation data. This alignment helps in distinguishing lipids with the same exact mass but different fatty acyl chains.

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FINANCIAL DISCLOSURES

SSB currently works for ThermoFisher

IGS, RMG and FT have no financial disclosures

BSK is the inventor on general metabolomics-related IP that has been licensed to Metabolon via Weill Medical College of Cornell University and for which he receives royalty payments via Weill Medical College of Cornell University. He also consults for and has a small equity interest in the company. Metabolon offers biochemical profiling services and is developing molecular diagnostic assays detecting and monitoring disease. Metabolon has no rights or proprietary access to the research results presented and/or new IP generated under these grants/studies. BSK's interests were reviewed by the Brigham and Women's Hospital and Partners Healthcare in accordance with their institutional policy. Accordingly, upon review, the institution determined that BSK's financial interest in Metabolon does not create a significant financial conflict of interest (FCOI) with this research. The addition of this statement where appropriate was explicitly requested and approved by BWH.

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