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Lipidomics Profiling by High Resolution LC-MS and HCD Fragmentation: Focus on Characterization of Mitochondrial Cardiolipins and Monolysocardiolipins

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Abstract

A liquid chromatography-mass spectrometry (LC-MS) method was used for separation of lipid classes as well as both qualitative and semi-quantitative detection of individual lipids in biological samples. Data were acquired using high resolution full scan MS and high energy collisional dissociation (HCD) all ion fragmentation. The method was evaluated for efficient separation and detection in both positive and negative ionization mode using standards spanning 6 lipid classes. Platform linearity and robustness, related to the mitochondrial lipid cardiolipin (CL), were assessed using extracted ion chromatograms with mass tolerance windows of 5ppm or less from full scan exact mass measurements. The platform CL limit of detection was determined to be 5 pmol (0.9 μM) on the column, with mass accuracy <1.5 ppm, retention time coefficients of variation (CV) < 0.5%, and area CV < 13%. This mass accuracy was critical to the identification of unknown CL species in mitochondria samples, through the elimination of false positives. In addition to detection and relative quantitation of CL species in mitochondria, CL structures were characterized through the use of alternating HCD scans at different energies to produce diagnostic fragmentations on all ions in the analysis. The developed lipid profiling method was applied to mitochondrial samples from an animal study related to the linkages between diet, mitochondrial function and disease. The analysis identified 28 unique CL species and 2 monolysocardiolipin species that are often associated with mitochondrial stress and dysfunction.

Keywords

lipidomics; mitochondria; cardiolipin; LC-MS; mass spectrometry; metabolomics profiling; lipids

INTRODUCTION

Lipidomics—the analysis of the overall lipid composition of a system—has been limited in its development by both the unclear definition of lipid species and inadequate analytical technology.[1] The LIPID Metabolites And Pathways Strategy (LIPID MAPS) consortium (www.lipidmaps.org) has systematically defined and classified lipids into eight categories, with multiple subclasses of each covering both eukaryotic and prokaryotic sources.[2] This standardization has assisted the progress in developing analytical methods for lipidomic studies. Technological advances in mass spectrometry (MS) such as the combinations of

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high mass resolution, high mass accuracy, faster scan times, and quantitative reproducibility in relatively affordable and easy to use instrumentation, have facilitated the technique to form the basis of the majority of these methods due to its quantitative and qualitative advantages.

MS-based lipidomics profiling ideally yields comprehensive, quantitative and reproducible analytical data in an efficient and robust manner from any given biological sample, without deliberate bias or optimization for a specific lipid class or species. By its very nature, the type of untargeted profiling addressed herein is inherently different from a targeted approach, which focuses analysis on one or more pre-determined lipids or lipids classes. This type of approach optimizes the platform, sample preparation, separation and detection, for these analytes only. There is a continuum of targeted approaches that can focus on one or at most a few lipids, to those that address a single subclass of lipids, eg., cardiolipins, to those that capture a class of lipids, eg, glycerophospholipids. This is in comparison to broader profiling approaches, such as our own, that seek to capture multiple classes of lipids with minimal bias. Profiling approaches are affected by two distinct sources of potential analytical complications. The first being the qualitative and quantitative complexity of the sample to be analyzed, including the inherent diversity of the lipid composition in terms of both structural class and concentration.[2] The second consists of the requirements for the analytical platform, including lipid extraction, separation (if used), qualitative identification, and quantitation. Both of these sources of potential complexity are addressed by the method described.

The combination of liquid chromatography (LC) coupled with high resolution (HR) MS facilitates the separation of multiple lipid classes while also providing quantitative and qualitative information. HR instruments have the ability to resolve ions with the same nominal mass by achieving mass resolutions of 60,000 or greater while also making accurate mass measurements within 5 parts per million (ppm). HR LC-MS techniques using electrospray ionization (ESI) and reversed phase chromatography (RPC) have successfully been used to profile plasma lipids in both human populations (eg., for an obesity study[3]) as well as in laboratory animal studies (eg., a p53 mutant mouse model[4]). In these examples, lipids were profiled and identified using exact mass measurements on the parent molecule made from HR-MS instruments, with fragmentation data provided in either additional experiments or from a lower resolution scan on the MS. Coupling LC-MS profiling methods with fragmentation data also acquired on an accurate mass HR MS enhances our ability to probe the lipidome, improving lipid identification and relative quantitation at times when chromatographic resolution is lacking.[5, 6]

HR-MS detection, via a benchtop orbitrap mass spectrometer, adds a further level of spectral resolution. This resolution is obtained through high energy collisional dissociation (HCD) experiments, during which full MS scans are alternated with HCD scans to yield comprehensive fragmentation of all lipid ions in the analysis.[5-7] In comparison, data dependent fragmentation methods, such as those referenced above, isolate the high abundant ions in the chromatogram for MS/MS analysis and fragmentation information on the other species is lost, Through the use of several different HCD energies to produce key fragmentations that are specific to both the class of the lipid and the lipid itself. these fragmentations can be used for characterization of multiple lipid species in a single run on all ions in the chromatogram, not just the most abundant. Additionally, the sensitivity, dynamic range, and potential to retroactively re-analyze HR-LC-MS experiments are all important advantages of discovery-focused profiling, making such approaches applicable to numerous sample types and organisms where a broad-based survey of the lipidome is required.

The current report demonstrates this approach in the context of profiling lipids from the cellular membrane-enclosed organelle, mitochondria. In mitochondria, lipids are central to normal function at all levels, ranging from the biophysical effects of mitochondrial membrane fluidity on respiratory chain activity[8], to the normal quaternary structure of the organelle[9], to the activity of specific electron transport complexes and enzymes.[8, 10] These organelles are both major sources and major targets of attack from reactive species, and damage from these agents can lead to mitochondrial dysfunction.[10]The analytical method used to assess the composition of the mitochondrial lipidome must be qualitatively and quantitatively accurate across the inherent diversity of lipids in terms of structural class, fatty acyl (FA) side chain differences and overall lipid concentration.[2]

Cardiolipins (CLs) comprise a subclass of glycerophospholipids normally found exclusively in the mitochondria inner membrane that play central roles in regulating normal mitochondrial function. These roles include maintaining proper mitochondrial quaternary structure and regulating certain enzymatic activities involved in electron transport and oxidative phosphorylation (complex I (NADH ubiquinone oxidoreductase), Complex III (ubiquinone cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase) and complex V (ATP synthase)).[11-13] Changes to overall CL concentration and/or to FA chain composition have been implicated in multiple situations associated with suboptimal mitochondrial function, including Barth's disease[14], gliomas,[15] hypothyroidism,[16] heart failure, and aging[17] These observations make both qualitative and quantitative detection of CLs a critical aspect of any mitochondrial lipidomic analysis. Such detection has been problematic because the multiple subspecies of CL and its damage product monolysocardiolipin (MLCL) are at or below the limits of detection in many methods. Thus, measuring CLs in mitochondrial samples has often required dedicated targeted methods.

Detection of the changes to CLs can be extremely challenging in the context of a profiling method. CL MS detection methods generally consist of shotgun methods[12, 14, 18, 19], by which as many as 27 CL species were identified in mouse heart mitochondria. However, this technique was unable to characterize all CL species fully and was often hindered by ion suppression caused by the lack of LC separation before MS detection. Targeted LC-MS techniques have also been developed for CL analysis that use chromatographic modifiers, such as acidified triethylamine[20], to better resolve these species. Such techniques resulted in the detection of 6 unique CL molecules in rat liver mitochondria. Piperidine[21] has also been used to increase the low ionization efficiency of these compounds in diverse mixtures. Although these methods can all achieve CL detection, identification and quantitation, their targeted nature means that other essential mitochondrial lipids in the samples, which also contribute to mitochondrial structure and function, are not detected.

The current report presents a HR LC-MS with HCD fragmentation profiling method for lipidomics that is broadly applicable to many biological matrices. The method is presented here in the context of general mitochondrial lipidomics with a specific focus on the characterization and relative quantitation of the CLs, which highlights the sensitivity and robustness of the analytical methodology. It was then applied to a large rat study in which the effects of nutritional macronutrient diversity (in the form of diets that differed in fat and carbohydrate composition) on mitochondrial lipid composition were assessed. Twenty eight unique CL molecules and two MLCL species, known to be possible biomarkers of CL-related diseases[22], were characterized and the unique relationship between these species and dietary fat content quantified across all samples in the analysis.

MATERIALS AND METHODS

Chemicals

LC-MS grade acetonitrile (ACN), methanol (MeOH), and isopropanol (IPA), as well as HPLC grade dichloromethane (DCM) and dimethyl sulfoxide (DMSO), were purchased from Fisher Scientific (Pittsburg, PA) and Ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO). A detailed list all lipid standards purchased as well as their abbreviations and sources are in Tables S1 and S2 of the supplemental information.

Preparation of Lipid Standards

Stock lipid samples were prepared by dissolving lipid standards in DCM:MeOH (2:1 v/v) at concentrations ranging from 1-10 mg/mL and were stored at −20°C. The internal standard (IS) mixture spiked into each sample prior to extraction consisted of 50 μg/mL of CL (T18:1), PC (17:0/17:0), PG (14:0/14:0), PC (20:0) and PS (16:0) (Table S1, panel A). A second set of standards with varying concentrations, used to asses method linearity, limit of detection (LOD) and extraction efficiency, are shown in Table S1, panel B. The biological standard (BIOSTD) used to assess the method's ability to separate and detect multiple lipid species, as well as the HCD fragmentations of those species, contained the 37 standards in Table S2, all at a working concentration of 5 μg/mL.

Rat Diet Experiments and Liver Mitochondria Isolation

Male Fisher 344 x Brown Norway F_1 (FBNF1) rats (n=8), aged 7-9 weeks, were fed *ad libitum* one of 24 isocaloric diets that differed in fat and carbohydrate composition. This approach yielded a total of 192 animals in the study. Body weights and food composition were measured twice a week and rats were sacrificed after 8 weeks. Details of their husbandry and diets are not critical for the current report and will be presented elsewhere (Greenberg, Stavrovskaya, Baranov, Kristal, manuscripts in preparation).

After the animals were sacrificed, each rat liver was harvested and the mitochondria isolated by the standard differential centrifugation method, using sucrose-based buffers, as described and as used previously in our laboratory.[23] Mitochondrial protein concentration was determined by the Lowry method using BSA as a standard[24]. Sample aliquots containing 1 mg of protein from mitochondria were washed in 160 mM KCl and frozen as dry pellets at −80°C before analysis. Secondary gradient purification was not performed, so some contamination with non-mitochondrial lipids is likely.

Mitochondria Preparation and Lipid Extraction

Immediately before extraction, each aliquot of mitochondria (containing 1 mg of protein) was dissolved in 40 μL DMSO and the membranes were disrupted by sonication. A mitochondrial pool sample was created by combining 8 μL samples from the sonicated mitochondria of each rat. The pool samples were processed for quality control (QC), lipid identification studies and lipid extraction efficiency experiments at the same time as the dietary samples.

Lipids were extracted according to the method of Bligh and Dyer[25], substituting DCM for chloroform.[26] First, 30 μL of IS was added to each 30 μL sample, followed by 190 μL of MeOH. Samples were then vortexed for 20 seconds. Next, 380 μL of DCM was added, the sample was again vortexed for 20 seconds and 120 μL of water was added to induce phase separation. The samples were then vortexed for 10 seconds and allowed to equilibrate at room temperature for 10 minutes before centrifugation at 8000 *g* for 10 minutes at 10 °C. A total of 370 μL of the lower lipid-rich DCM layer was then collected and the solvent evaporated to dryness under vacuum. Samples were reconstituted in 300 μL of ACN/IPA/

H_2O (65:30:5 v/v/v) containing PG (17:0/17:0) at a concentration of 5 μg/mL before LC-MS analysis. Ten μL of sample was injected onto the LC-MS system.

LC-MS Conditions and Experiments

Lipid extracts were separated on an Ascentis Express C_{18} 2.1 × 150 mm 2.7μm column (Sigma-Aldrich, St. Louis, MO) connected to a Thermo Fisher Scientific autosampler and Accela quaternary HPLC pump (Thermo Fisher, San Jose, CA). A binary solvent system was used, in which mobile phase A consisted of ACN:H_2O (60:40), 10 mM Ammonium formate, 0.1% formic acid and mobile phase B of IPA:ACN (90:10), 10 mM Ammonium formate, 0.1% formic acid. Separations were done over a 30 minute period following the conditions set by Hu and colleagues[4]. A flow rate of 260 μL/min was used for the analysis and the column and sample tray were held at 45 °C and 4 °C, respectively. The same LC conditions and buffers were used for all MS experiments, and the scan range was between *m/z* 120-2000.

The HPLC system was coupled to an Exactive benchtop orbitrap mass spectrometer (Thermo Fisher, San Jose, CA) equipped with a heated electrospray ionization (HESI) probe. The spray voltage was set to 3.5 kV, whereas the heated capillary and the HESI probe were held at 250 °C and 350 °C, respectively. The sheath gas flow was set to 25 units and the auxiliary gas set to 15 units. These conditions were held constant for both positive and negative ionization mode acquisitions. The instrument was tuned by direct infusion of PG (17:0/17:0) in both positive and negative mode and external mass calibration was performed before each sample sequence acquisition, approximately every five days.

For full scan only experiments, the MS was run in high resolution mode, corresponding to a resolution of 60k and a 2 Hz scan speed. Mitochondria were profiled by injecting each sample once, in randomized order, with 17 pool samples, 17 blanks and 5 standard mixture samples spread throughout the analysis. This sample sequence yielded a total of 231 injections, each of which was run separately in positive and negative modes. Lipid extraction efficiencies were done by comparing pool samples spiked with the IS mixture before extraction to those spiked with the IS mixture after extraction. Each extraction was performed in triplicate and 4 injections of each sample were done. These experiments were also run in both positive and negative mode.

HCD experiments were performed by alternating between full scan acquisitions and HCD scan acquisitions, both run at 2 Hz. Three different HCD energies, 30, 60 and 100 eV, were used in separate experiments in both positive and negative mode as described in the text. For lipid identification studies, HCD experiments were run on the pool, BIOSTDand blank samples only.

Data Analysis and Lipid Identification

All LC-MS files from the diet studies were analyzed using the MS label free differential analysis software package SIEVE v 1.3 (ThemroFisher and Vast Scientific, Cambridge, MA). Non-differential SIEVE profiling experiments were run on all 231 LC-MS full scan chromatograms using the small molecule analysis setting. The chromatograms were time-aligned, referencing a pool sample acquired in the middle of our sequence, and 10k frames were built off of this same reference file. The framing parameters in these experiments were set at 0.01 Daltons for the *m/z* window and 1.00 minute for the RT window; 1000 was used at the intensity threshold. The frames built off the reference were then applied to all samples in the experiment and the resultant information, which corresponded to *m/z*, RT, and intensity, were then used to differentiate between the diets via statistical analysis and to identify unknown lipid species in the samples.

For identification, the frame *m/z* values were used to do batch searches on both the Metlin database[27] and the human metabolome database[28] (HMDB), which is an experimentally confirmed database of over 7900 metabolites and 3500 lipids. Both sites allow the user to search via ionization mode, either positive or negative, and multiple M+adduct possibilities are calculated based on exact mass. The frame *m/z* and RT information was then used to do extracted ion chromatograms (XICs) in the HCD experiments of the pool, using QualBrowser software (Thermo Fisher, San Jose, CA), both to confirm the SIEVE analysis and use the HCD fragmentations to supplement exact match database searches for lipid identification and characterization.

RESULTS AND DISCUSSION

Overview

The long-range goal of the work presented here was to facilitate the broad profiling of the lipophilic constituents of a biological system by using a single LC-MS technique that (i) did not require sample pre-fractionation; (ii) enabled robust and reproducible qualitative and quantitative analysis of the lipids within multiple categories (i.e., glycerolipids vs glycerophospholipids) as well as across classes within categories (i.e., phosphocholine, phosphoserine, etc.); (iii) uses HCD fragmentation for lipid characterization; and (iv) has sensitivity approaching that of methods that focus on single lipids. The performance characteristics of this profiling method are discussed with emphasis on the specific case of mitochondrial CL.

LC-MS Method Assessment

Because the ionizable moieties on lipids are quite diverse, an LC-MS method must be efficient in both positive and negative ionization modes to achieve comprehensive coverage. This aspect of the method was assessed using a standard mixture (BIOSTD) that, by design, incorporated more lipid classes than were expected to be required for mitochondrial analysis. This approach facilitates long-range applicability of the method to other biological samples such as plasma or tissue.

Two representative full scan total ion current (TIC) chromatograms from the BIOSTD analysis are shown in Figure 1 (Panel A, negative ion mode; Panel B, positive ion mode).

The lipid categories and subclasses surveyed each class's ionization polarity and adduct preference, and its occurrence (or lack thereof) in mitochondria is shown in Table 1. As indicated in both Figure 1 and Table 1, there are certain lipid classes that are only detectable in a single ionization mode, such as glycerolipids that are only observed under positive ESI conditions as $[M+NH_4]$ + species, or phosphoethanolamines that are only detected under negative ESI mode as [M-H]-. To ignore one of these groups would limit the depth of the analysis. This simple experiment demonstrated how each lipid class would ionize and where they would elute in the chromatogram, which is essential information for identifying unknown lipids during mitochondrial analysis. Because many lipid species are isobaric, the knowledge that separation is being achieved by both lipid head group polarity and acyl side chain length can be beneficial for characterization.

Using this LC-MS method, the five CL compounds in the BIOSTD (Table S2)—CL $(14:1)_3(15:1)$, CL $(15:0)_3(16:1)$, CL $(18:1)_4$, CL $(22:1)_3(14:1)$ and CL $(24:1)_3(14:1)$—eluted between 18 and 26 minutes or 75 - 97% B in the gradient. The CLs were detected in both positive and negative ion mode, as $[M+NH_4]$ + or [M-H] - ions, respectively. The BIOSTD was injected a total of 5 times over the course of a 5 day period. During this time, the CL standards showed retention time (RT) coefficients of variance (CV) of less than 0.5% when compared within a single ionization mode or across all 10 injections between both modes.

The integration of XIC areas had CVs of less than 13% when compared within either of the ionization modes as normalized to the total CL signal from the five CLs in the BIOSTD sample (This separation can be seen in the Supplemental Information, Figure S1).

Aliquots of serially diluted CL standard CL $(14:1)_4$ were spiked into mitochondria pool samples before lipid extraction to assess the relative limits of detection for CL in mitochondria. For both positive and negative analysis of the mitochondrial matrix, the relative LOD, defined as a signal-to-noise (S/N) ratio approaching 3, for both positive and negative analysis in the mitochondria matrix was 5 pmols of material injected on the column (0.9 μM in the solution injected), over 3 orders of magnitude tested and an R^2 of 0.097. This LOD is directly comparable to the concentrations reported by Houtkooper et al. when these authors established a diagnostic test for Barth Syndrome using a triple quadrupole mass spectrometer for LC-MS quantitation.[29] In comparison, the method used here is not limited to CL analysis, and can detect and characterize multiple categories of lipid species, while achieving the same relative LOD as a more specific CL detection method.

Cardiolipin Characterization

The exact molecular composition of the CL molecules found in mitochondria can vary greatly. This diversity reflects changes to acyl side chain length, the degree of unsaturation and location of the FA moieties on the diphosphate backbone. The biological implications of this diversity (e.g., susceptibility to peroxidation, contribution to membrane fluidity) highlight the importance of fully characterizing CL populations. Identification of unknown CLs in mitochondrial samples was achieved by first sorting the full scan exact mass measurements determined by SIEVE, between m/z 1200-1600 and at RTs after 18 minutes or 75% B as determined by both the BIOSTD and POOL analyses to be the region in which CL species are detected. Values falling into both of these categories were then searched through online databases for hits matching within 0.01 Daltons. Both the Metlin[27] and Human Metabolome Database[28] (HMDB) were searched for all lipid identifications, not just those that were CL specific. It was found that these databases were at times complimentary to each other, and it was necessary to search both to increase the absolute number of possible hits as well as the number of high probability hits. Both databases allow the user to search by ionization mode, in a batch setting, where possible exact mass adducts, such as [M $+NH_4$] +, are calculated and included as possible identification options. This aspect was important because the LC-MS method not only yielded [M-H] or [M+H] ions, but also multiple variations depending on the lipid species.

Given that each CL molecule contains 4 FA side chains, each database search could yield multiple possible CL structural variations for a given exact mass. The HCD capabilities of the Exactive were used to fragment all ions in the spectrum and reconstruct the molecule. This ability eliminates the majority of the remaining false positives and further refines the list. An example of how HCD fragmentation at different energies was used to differentially fragment a CL molecule into its diacylphosphate and FA groups for a more complete molecular characterization is shown in Figure 2. The molecule in this figure is CL $(18:1)_2(18:2)_2$, in which each phosphate head group contains a mixture of the FA side chains. The FA and diacylphosphate fragmentation *m/z* values labeled are calculated based on negative ionization, HCD fragmentation and detection of the molecule. Possible FA and diacylphosphate group fragment exact masses were calculated based on the databases searched; XICs of these masses were used to search results from mitochondrial pool samples. Observation of a peak at a given RT that matched a full MS measurement indicates a CL species that contained a certain fragment and that information was used to eliminate nonsensical database hits.

For example, when searching for structures that correspond to an unknown mass of 1451.9960 m/z, obtained from LC-MS analysis of a mitochondrial pool sample in negative ion mode, a hit within 0.2 ppm of the exact mass was determined for a CL containing two 18:1 FA chains and two 18:2 FA chains. The structure of this CL species can be oriented such as in Figure 2, where the FA chains are mixed on the phosphate head groups; an equally valid alternative structure has the identical FA chains conserved together on each head group. The two different structures, although having the same exact mass, would yield different diacyl fragmentations masses at 60 eV in an LC-MS HCD experiment. Figure 3 shows XICs for both the full scan exact mass measurement of the CL $(18:1)_2(18:2)_2$ parent ion and all 3 possible diacylphosphate fragmentations that can arise, labeled as CL (18:1)(18:2), CL $(18:2)_2$ and CL $(18:1)_2$ in panels B, C and D, respectively. From this chromatogram, it is clear that although all 3 diacylphosphate fragments are present in the mitochondrial pool, only the CL (18:1)(18:2) fragment can be chromatographically aligned with the parent ion molecule, therefore representing the CL orientation detected to be identical to that found in Figure 2. The observation of the other diacylphosphate fragments in the chromatogram indicates that other mitochondrial CL species contain these groups; this information can be used for their subsequent characterization.

Figure 4 shows how fragmentation at 100 eV further confirms the characterization of ion 1451.9960 m/z as CL (18:1)2(18:2)2 (Figure 2) by aligning both fragments for FA 18:1 and FA 18:2 under the parent ion peak at 22.93 minutes. The HCD scan mass spectrum at 100 eV and RT of 22.93 minutes seen in Figure 4 shows not only both FA peaks and their corresponding M+1 isotope, but also that these ions are observed at an equal intensity. This result is exactly what was predicted for the fragmentation of a CL molecule containing equal molar ratios of these FA side chains.

Thus, the previous example highlights the utility of this method of HR exact mass LC-MS measurements in combination with differential HCD fragmentation. The technique should be readily applicable to other lipid classes and categories found not just in mitochondria but various biological tissues and fluids for a more complete lipidomic profiling analysis.

Cardiolipin Relative Quantitation

We next evaluated the semi-quantitative aspects of this platform. Larger scale profiling studies (i.e. > 200 samples) often require LC-MS chromatograms to be acquired over several days or weeks, a time frame that complicates the analysis. This complexity is a result not only of the large number of chromatograms and ions that must be assessed, but also of the inherent shifts in chromatographic retention and MS accuracy that will occur over the course of the evaluation. To minimize these known sources of error, quantification in this study was achieved using the label-free MS differential analysis platform SIEVE v 1.3.

SIEVE initially time-aligns the LC-MS chromatograms using a non-linear correction algorithm based on full scan MS data only; this process eliminates the need for spiked internal standard landmarks. After alignment, SIEVE builds intensity-based features, termed frames, that define a given retention time and *m/z* window in the chromatogram. SIEVE builds these frames by first identifying all *m/z* peaks above a user-defined intensity threshold. It then organizes these peaks by intensity, such that frame 1 is the most intense peak in the analysis, and eliminates any *m/z* peaks that overlap with respect to RT. The framing parameters of *m/z*, RT, and intensity threshold as well as total number of frames SIEVE should build are set by the user based on each individual LC-MS system and study. This frame information (RT, *m/z* and intensity) is then used for all lipid analyses across the dataset. Frame RT and *m/z* values are used to characterize the unknown lipids using the method just described, whereas the intensity values are the basis of relative quantitation for each characterized lipid across all samples in the analysis.

Diet-Dependent Modulation of CL and MLCL in Rat Liver Mitochondria

After evaluating the qualitative and semi-quantitative performance characteristics of the LC-MS method, it was applied to a lipidomic profiling analysis of rat liver mitochondrial samples from a nutrition study conducted in the authors' laboratory. This study is a component of the NIH Genes and Environment Initiative (GEI) and tests a hypothesis that intra-class shifts of fats and carbohydrates in the diet will affect the physiological function and biochemical fingerprint of mitochondria. These changes are hypothesized to serve as a pathogenic link between environmental stresses, in this case diet, and the role mitochondria play in long-term pathobiological outcomes. This study consisted of 24 different isocaloric diets, with 8 rats held for eight weeks on each diet for a total sample set of 192 rats/cohort. The diets were comprised of six different fat groups with the major constituent of each being either saturated fats (SFA), trans fats (Trans), monounsaturated fats (MUFAs), or one of 3 groups of polyunsaturated fats (PUFAs), which vary in the ω-6/ω-3 PUFA ratios. Each fat group was combined with one of 4 carbohydrate groups that varied based on sucrose content, from high to low. The fat, carbohydrate and protein percentages in all diets were held consistent at 5, 66, and 20 (w/w), equivalent to 12, 68, and 21 (kcal %).

All 192 rat liver mitochondrial samples were assessed using the LC-MS method outlined above. From the negative ion profiling analysis, 28 unique CL molecules, varying by FA chain length and degree of unsaturation, were characterized and quantified. Table 2 organizes these compounds from lowest to highest percent composition of the total CL signal observed in the 17 pools. The pool samples also (i) served as method QC for the 5 day analysis; (ii) were used to generate the data needed for CL identification, and (iii) were used to determine the mass accuracy (within 1.5 ppm after correction based on CL normalization) and quantitative precision of the platform (error consistently below 8 % for the more abundant CL species). Certain CL compounds in Table 2 are associated with CV values that extend to around 15%, a result that is expected in cases where the platform LOD is being approached. In addition to its quantitative abilities, the high mass accuracy capabilities of the Exactive proved useful for characterization by not only by facilitating unambiguous CL identifications, but also eliminating possible misidentifications (see supplemental information for additional details).

We next considered the source of the quantitative variation observed. Examination of the 5 IS compounds (Supplemental Information, Table S1A), showed excellent correlation for standards 2 through 5 (GPCho(17:0/17:0), GPGro (14:0/14:0), lysoGPCho (20:0) and PSer (16:0)) and the larger error values observed for standard 1, $(CL\ (18:4)_4)$, likely represents signal confounding with endogenous lipid found in the sample. Per design, we attempted to correct all CL measurements to its IS to compensate for loss during extraction, etc. Surprisingly, this substantially increased CVs, suggesting that by far the primary source of error was due to an aspect of CL either orthogonal to or upstream of the point of entry for the standards. Since there is no upstream point in the workflow, this suggests that the problem likely resides in the efficiency of extraction from the sample itself. We tested this component by normalizing to the total level of CLs. This normalization reduced median variation to ~5% for all CL species (median CVs were 18% before correction). Thus, despite the presence of strong organic solvents and sonication, it appears that the majority of the observed variation, ~13%, (or two-thirds of the total variance) lies in the extraction efficiency of the CLs, with the remaining 5% (one-third of total) within the analytical platform. From this study, the extraction was determined to be 50% ± 4%. This inefficient number may be attributable to the large amount of CL species that are protein-bound and therefore lost in the protein crash of the liquid-liquid extraction technique. As a result, total CL signal normalization better presents the overall precision in the analytical platform as a whole. This total signal normalization is essentially equivalent to standardization to an

internal standard, and thus includes the LC-MS analysis and data reduction steps as remaining sources of variation to the overall method.

In addition to the 28 CL compounds identified, 2 MLCL species, MLCL $(18:2)_3$ and MLCL $(18:2)_2(18:1)$, were also characterized in the rat liver mitochondrial samples and pools. MLCL, as shown in Figure 5, is an intermediate in CL metabolism as well as a potential byproduct of lipid peroxidation damage (following removal of an FA chain by phospholipase A_2).[30-33] A substantial (45-115 fold) increase in MLCLs is a specific marker for Barth's Disease, a disorder caused by mutations in *tafazzin*—a gene whose product is required to complete cardiolipin sysnthesis—and that results in abnormal mitochondria, especially in cardiac and skeletal muscle.[22] The detection of MLCLs in more physiologically normal conditions, however, can be quite complicated because they are generally found at concentrations lower than that of the other lipids present[22, 29]. Using the comprehensive lipidomic LC-MS profiling approach developed here, however, they were not only detected but also fragmented, characterized and quantified across all samples in the diet-dependent study, despite each MLCL species representing 0.0015% of the total lipophilic signal. This percentage is based on signal comparison of the average of each MLCL species across the 17 pool samples to the top 10, 000 frames quantified.

The MLCL relative quantitation across all rats in the study shows a trend linking the amount of MLCL $(18:2)_3$ present in mitochondria and the major fat component of the diet. The greatest relative percentage of mitochondrial MLCL $(18:2)_3$ was found in liver mitochondria from rats maintained on diets containing trans fat as the major constituent (Figure 6), a finding that may reflect impaired CL maturation or increased steady-state oxidative stress in the liver mitochondria of animals fed these diets. Other dietary studies have shown that high trans fat intake can lead to a higher incidence of coronary heart disease[34] in addition to raising the individuals risk of certain metabolic diseases such as liver dysfunction[35] and diabetes[36]. The data here, although preliminary, link the observation of MLCL increases in mitochondria to the type of fat available as structural components (trans fats may disturb the integrity of the electron transport chain) and for use in energy production. Further experiments are required to develop a more solid biological interpretation; however, the ability to measure and characterize these less abundant but nevertheless important CL and MLCL species is a major step in the right direction.

CONCLUSION

The intention of this study was to establish a method to separate, detect and characterize the multiple classes of mitochondrial lipids, including CL. CL is difficult to assay in this regard, but the ability perform such assays will be central for mitochondrial lipidomics studies. The dynamic range of the method was pushed by its ability to precisely measure and characterize the lower intensity species CL and MLCL as well as higher abundant species, representing a 4 order of magnitude range. The breadth of coverage makes this approach a powerful tool for comprehensive LC-MS lipidomic analysis. Although the focus in the current report was CL and MLCL detection, characterization and relative quantitation, the HR LC-MS method can be applied to other biological samples, such as plasma or tissue. The ability to useHCD scans to fragment all ions in the chromatogram with mass accuracies consistently below 3ppm allows the data to be retroactively searched and analyzed to characterize classes of lipids that may not have been of initial interest in the study. The method will next be applied to other lipidomic studies where lipid quantitative comparisons and characterization are both necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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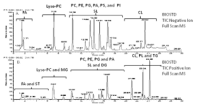

Figure 1.

Panels A and B show the LC-MS separation of 37 lipid species (BIOSTD) performed by negative and positive ionization modes, respectively. Sections of the chromatograms are labeled with the BIOSTD lipid categories, shown in Table S1, indicating a region where each will elute using the LC-MS method.

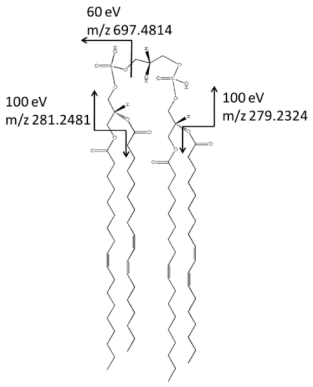

Figure 2.

shows CL(18:1)(18:2)(18:1)(18:2) and its diagnostic HCD fragmentation. At 60 eV the diacylphophate fragment yields an ion of *m/z* 697.4814 corresponding to the FA 18:1 and FA 18:2 chains being mixed on the CL backbone. At 100 eV the fragmentation is due to the free FA groups of *m/z* 281.2481 and *m/z* 279.2324 for the 18:1 and 18:2 fragments, respectively.

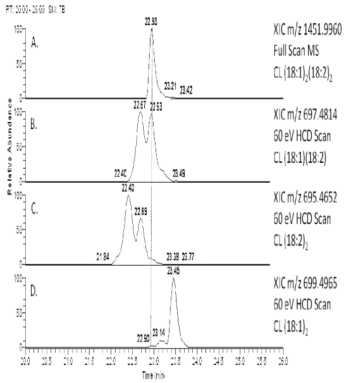

Figure 3.

Panel A represents the XIC chromatogram from the full scan MS data of the parent ion, CL (18:1)(18:2)(18:1)(18:2), with a mass of *m/z* 1451.9960, run in negative ion mode. Panels B, C and D correspond to the 3 possible diacylphosphate fragments that can arise depending on the structural orientation of the 4 FA groups. The peak at 22.93 minutes in panel A chromatographically aligns with the peak at 22.93 minutes in panel B, suggesting that at 60 eV the diacylphosphate fragment observed corresponds to a mixture of FA 18:1 and FA 18:2 species. The other diacylphosphate fragments correspond to other CL species present in the mitochondrial pool sample.

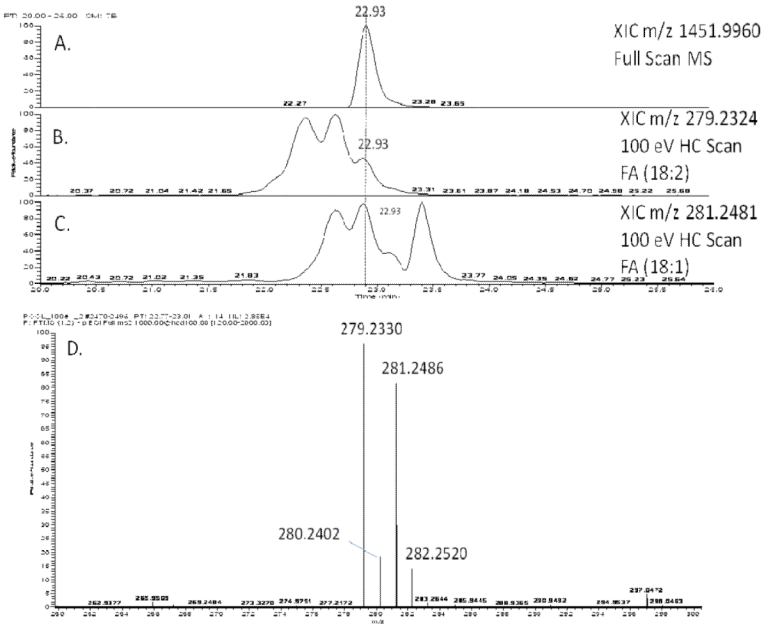

Figure 4.

Panel A shows the XIC chromatogram from the full scan MS acquisition of the parent ion, CL (18:1)(18:2)(18:1)(18:2), with a mass of *m/z* 1451.9960, run in negative ion mode. Panels B and C show the XIC chromatograms for FA 18:2 and 18:1 obtained from the HCD scans run at 100 eV in negative ion mode. The three peaks at 22.93 minutes are aligned to represent the parent ion and its two FA groups arising from HCD fragmentation at 100 eV. The mass spectrum in panel D is taken from RT 22.93 minutes during an 100 eV HCD scan. The equal intensity, of both FA 18:1 and FA 18:2 ions in the spectrum indicate equal molar ratios of those FA groups in the parent ion structure.

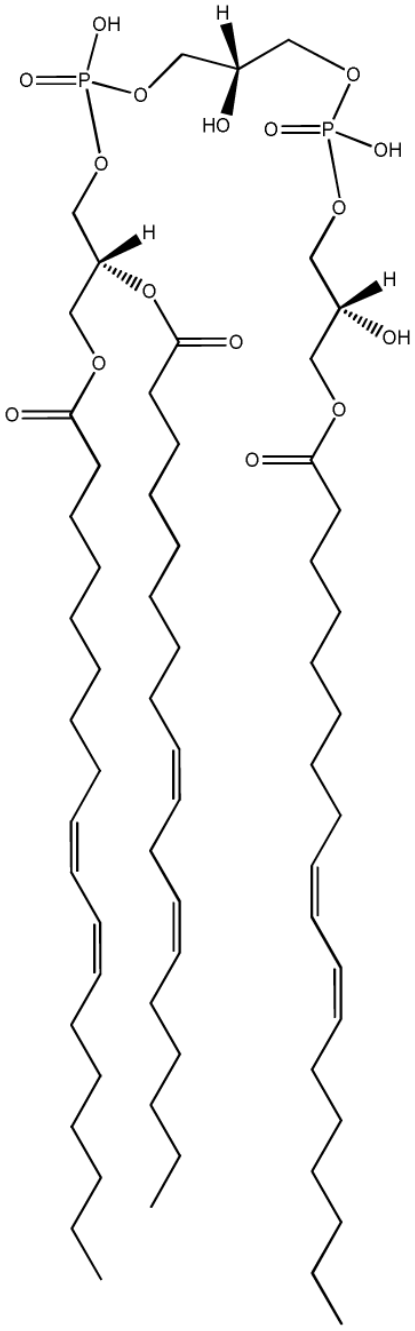

Figure 5.
shows the compound MLCL $(18:2)_3$, which has the same backbone structure as CL with merely 3 FA side chains as opposed to 4.

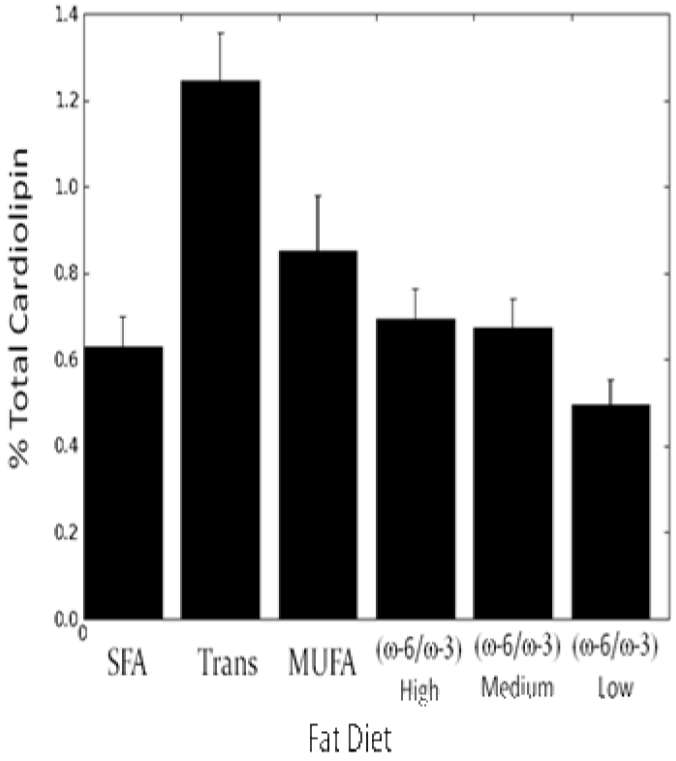

Figure 6.

shows the bar graph representing the amount of MLCL $(18:3)_3$, shown as a percent of the total CL detected, found in mitochondrial samples of animals held on each fat diet. In this analysis, the 4 carbohydrate diets did not affect the amount of MLCL found; the trend observed was solely dictated by the major fat constituent consumed, with Trans fat diets resulting in the largest amount of MLCL. Bars show means +/− s.e.m. (standard error of the mean)

Table 1

is a breakdown of the lipid class's surveyed in the BIOSTD using the LC-MS system developed. Each classes ionization and adduct preference are defined by the major adduct observed upon the analysis of standards any preliminary observations of these classes in unknown mitochondrial samples are indicated

Lipid Class	− Ionization	+ Ionization	Found in Mitochondria
Glycerophospholipids (GP)			
Phosphocholine (PC)	[M+FormAcid]−	[M+H]+	+
LysoPhosphocholine (Lyso-PC)	[M+FormAcid]−	[M+H]+	+
Phosphoethanolamine (PE)	[M-H]−		+
Phosphoserine (PS)	[M-H]−		+
Phosphoinositol (PI)	[M-H]−		
Phosphoglycerol (PG)	[M-H]−	[M+NH4]+	
Phosphatidic Acid (PA)	[M-H]−	[M+NH4]+	
Cardiolipin (CL)	[M-H]−	[M+NH4]+	+
Fatty Acyls (FA)	[M-H]−	[M+NH4]+	+
Sphingolipids (SL)	[M+FormAcid]−	[M+H]+	+
Sterol Lipids (ST)		[M+NH4]+	+
Glycerolipids (GL)			
Monoacylglycerol (MG)		[M+NH4]+	
Diacylglycerol (DG)		[M+NH4]+	
Triacylglycerol (TG)		[M+NH4]+	
Prenol Lipids (PL)		[M+H]+ / [M+NH4]+	+

Table 2

shows the 28 unique CL species detected and characterized in the mitochondrial pool samples using LC-MS run in negative ion mode, from lowest to highest percentage of the total CL signal observed in the POOL. The mass errors for these measurements were less than 1.5 ppm, whereas measurement precision showed less than 15% error (median of 5%) over the course of the 5 day analysis when normalized to total CL signal, as explained in the text

	MZ	Error (ppm)	CL Identification	% Total Composition	CV (n=17) before correction	CV (n=17) after correction
1	1343.9033	−0.47	CL (14:0)(16:1)(16:1)(18:2)	0.3	20.9	7.9
2	1517.9498	−0.67	CL (18:2)(18:3)(20:4)(22:6)	0.5	24.2	9.4
3	1523.9979	0.05	CL (18:1)(18:2)(20:3)(22:6)	0.9	15.4	9.8
4	1454.0137	0.15	CL (18:1)(18:1)(18:1)(18:2)	1.0	18.6	11.5
5	1427.9995	1.16	CL (16:0)(18:1)(18:1)(18:2)	1.0	19.6	9.4
6	1521.9832	0.59	CL (18:2)(18:2)(20:3)(22:6)	1.1	17.0	12.1
7	1371.9351	−0.14	CL (14:0)(16:0)(18:2)(18:2)	1.1	18.7	4.7
8	1519.9663	−0.09	CL (18:2)(18:2)(20:4)(22:6)	1.1	16.8	3.1
9	1369.9203	0.46	CL (14:0)(16:1)(18:2)(18:2)	1.1	19.3	4.3
10	1399.9669	0.26	CL (16:0)(16:1)(18:1)(18:2)	1.7	18.1	5.7
11	1478.0125	−0.68	CL (18:1)(18:2)(18:2)(20:2)	2.3	17.6	6.6
12	1495.9685	1.37	CL (18:2)(18:2)(18:2)(22:6)	2.5	16.2	7.3
13	1456.0300	0.60	CL (18:1)(18:1)(18:1)(18:1)	2.8	21.8	15.5
14	1499.9957	−1.42	CL (18:1)(18:1)(18:2)(22:6)	3.1	17.9	7.4
15	1395.9351	−0.14	CL (14:0)(16:0)(18:2)(20:4)	3.3	18.7	3.2
16	1497.9828	0.36	CL (18:2)18:2)(20:3)(20:4)	3.8	17.4	2.9
17	1469.9496	−0.86	CL (16:1)(18:2)(18:2)(22:6)	3.9	21.3	8.0
18	1397.9503	−0.45	CL(16:1)(16:1)(18:1)(18:2)	3.9	18.3	2.6
19	1475.9974	−0.28	CL (18:2)(18:2)(18:2)(20:2)	4.0	17.5	8.3
20	1425.9827	0.29	CL (16:1)(18:1)(18:1)(18:2)	4.5	18.4	4.1
21	1445.9490	−1.30	CL (18:2)(18:2)(18:2)(18:3)	5.3	21.7	6.6
22	1451.9984	0.39	CL (18:1)(18:1)(18:2)(18:2)	5.5	19.0	4.6
23	1421.9526	1.25	CL(18:2)(18:2)(18:2)(16:1)	6.2	17.5	2.6
24	1473.9819	−0.22	CL (18:2)(18:2)(18:2)(20:3)	6.8	17.5	2.8
25	1471.9646	−1.26	CL (16:0)(18:2)(20:4)(20:4)	7.0	19.9	4.2
26	1423.9673	0.94	CL(18:2)(18:2)(18:1)(16:1)	7.9	19.3	3.3

	MZ	Error (ppm)	CL Identification	% Total Composition	CV (n=17) before correction	CV (n=17) after correction
27	1447.9674	0.66	CL (18:2)(18:2)(18:2)(18:2)	8.0	16.9	4.2
28	1449.9816	−0.47	CL (18:2)(18:2)(18:2)(18:1)	9.5	18.0	4.3