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Expanding lipidomics coverage: effective ultra performance liquid chromatography-high resolution mass spectrometer methods for detection and quantitation of cardiolipin, phosphatidylglycerol, and lysyl-phosphatidylglycerol

Eric D. Tague¹, Brittini M. Woodall^{1,4}, John R. Harp², Abigail T. Farmer¹, Elizabeth M. Fozo², Shawn R. Campagna^{1,3}

¹Department of Chemistry, University of Tennessee, Knoxville, TN 37996, USA

²Department of Microbiology, University of Tennessee, Knoxville, TN 37996, USA

³Biological and Small Mass Spectrometry Core, University of Tennessee, Knoxville, TN 37996, USA

⁴Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, TN 37996, USA

Abstract

Introduction—Lipidomics can reveal global alterations in a broad class of molecules whose functions are innately linked to physiology. Monitoring changes in the phospholipid composition of biological membranes in response to stressors can aid the development of targeted therapies. However, exact quantitation of cardiolipins is not a straightforward task due to low ionization efficiencies and poor chromatographic separation of these compounds.

Objective—The aim of this study was to develop a quantitative method for the detection of cardiolipins and other phospholipids using both a targeted and untargeted analyses with a Q-Exactive.

Methods—HILIC chromatography and high-resolution mass spectrometry with parallel reaction monitoring was used to measure changes in lipid concentration. Internal standards and fragmentation techniques allowed for the reliable quantitation of lipid species including: lysyl-phosphatidylglycerol, phosphatidylglycerol, and cardiolipin.

Shawn R. Campagna campagna@utk.edu.

Author contributions EDT, SRC, and EMF designed the project. EDT and BMW designed and conducted targeted analysis. EDT and ATF designed and conducted the untargeted analysis. JRH grew and extracted lipids from the bacteria. EDT, BMW, JRH, SRC, and EMF wrote the manuscript. All authors read and approved the manuscript. Mass spectrometric analyses were performed at the University of Tennessee, Knoxville Biological and Small Molecule Mass Spectrometry Core with the assistance of Dr. Hector F. Castro.

Compliance with ethical standards

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Results—The untargeted analysis was capable to detecting 6 different phospholipid classes as well as free fatty acids. The targeted analysis quantified up to 23 cardiolipins, 10 phosphatidylglycerols and 10 lysyl-phosphatidylglycerols with detection limits as low as 50 nM. Biological validation with *Enterococcus faecalis* demonstrates sensitivity in monitoring the incorporation of exogenously supplied free fats into membrane phospholipids. When supplemented with oleic acid, the amount of free oleic acid in the membrane was 100 times greater and the concentration of polyunsaturated cardiolipin increased to over 3.5 μ M compared to controls.

Conclusions—This lipidomics method is capable of targeted quantitation for challenging biologically relevant cardiolipins as well as broad, untargeted lipid profiling.

Keywords

Lipidomics; Cardiolipin; Q Exactive Plus; Orbitrap; Parallel reaction monitoring; *Enterococcus faecalis*

1 Introduction

Lipids play an essential role for multiple cellular functions including energy acquisition, maintenance of cellular physiology, cellular mobility, and cellular transport (Dowhan 1997). Biological membranes contain several types of phospholipids, and the phospholipid composition can vary drastically depending on genetics or environmental factors (Cronan 2003). Membrane phospholipids consist of polar head groups and multiple non-polar tails which form an amphipathic lipid bilayer, creating a barrier between internal cellular components and the extracellular environment. The head groups of membrane phospholipids are highly variable and tend to combine with tails of varying length and degrees of unsaturation, creating a large number of possible combinations. Although the numbers of possible lipids are vast, bacteria have limited their membranes to use only three or four different head groups (Oliver et al. 2014). Bacterial membranes are primarily composed of various combinations of cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), or phosphatidylserine (PS). Depending on the bacterium, lipids with lyso or amino acid modifications may also be present (Sohlenkamp and Geiger 2016). Membrane fluidity can be altered by adjusting the fatty acid tails (adding degrees of unsaturation) or changing ratios of lipid classes, providing a quick way to respond to external stressors (Beney and Gervais 2001). The ability to alter membrane composition has been used to preserve viability when bacteria are challenged by antibiotics (Saito et al. 2014) or extreme temperatures (Fozo and Rucks 2016).

To date, most research has focused on glycerol phospholipids, which have two positions containing fatty acids varying in lengths from 14 to 18 carbons with the possibility of zero, one, or two degrees of unsaturation. The low number of biologically relevant tails, combined with their high abundance in biological samples, allows this set of lipids to be easily measured (Oliver et al. 2014). Over 90% of laboratory cultured bacteria consist mainly of two-tailed lipids such as PG or PE (Renner and Weibel 2011). Cardiolipins (CL) are essentially two PG molecules connected by a shared glycerol molecule resulting in a four

tailed lipid (Romantsov et al. 2009). These four-tailed lipids are not as abundant in biological specimens as two-tailed lipids (Dowhan 1997; Renner and Weibel 2011). Doubling the number of tails doubles the number of possible biologically relevant combinations. Thus, combining their low abundance in biological membranes with the high number of possible CL tail combinations leads to difficulty in measurement.

Current lipidomics analysis includes various mass spectrometric techniques such as direct injection of extracted lipid sample, separation of lipid sample components based on chromatography followed by tandem mass-spectrometry, and analysis of lipids and imaging of tissues utilizing MALDI (Beate and Jürgen 2009; Watson 2006). Although there are many mass spectrometry experiments that allow the detection of common phospholipids (Schwalbe-Herrmann et al. 2010) there are only a handful of methods that explicitly measure cardiolipins (Bird et al. 2011; Hines et al. 2017; Minkler and Hoppel 2010; Rashid et al. 2017). An even smaller number of methods quantify cardiolipin (Scherer et al. 2010; Valianpour et al. 2002).

There is often a compromise between coverage and the ability to measure specific compounds in UPLC-HRMS analyses due to limitations of the mass analyzer. However, this method demonstrates the practicality of using a single extraction method for both targeted and untargeted lipid analysis. The untargeted analysis uses an Orbitrap mass spectrometer operating in full scan mode to provide relative quantitation of the six most common phospholipid head groups, the lysyl derivatives, and free fatty acids that make up the tails of bacterial phospholipids. Accounting for all possible combinations between head group and fatty acid tails, this method has the potential to measure hundreds of identifiable lipids along with the opportunity to search the unidentified spectral features for molecules with an unknown structure. The targeted approach uses a hybrid quadrupole Orbitrap mass spectrometer, allowing for the quantification of specific cardiolipin, phosphatidylglycerol, and lysyl-phosphatidylglycerol compounds within a sample. The method was validated by analyzing relative lipidome changes as well as measuring exact quantities of three lipid classes in *Enterococcus faecalis* samples supplemented with two unsaturated exogenous fatty acids.

2 Experimental section

Protocols for lipid extraction, liquid chromatography-mass spectrometry analysis, and data processing are included in the supplementary material.

2.1 Untargeted standards

Phospholipid standards were used to verify retention time and head group ionization potential. The standards assessed were as follows: phosphatidylinositol with two 16:0 tails (PI 32:0), phosphatidic acid with one 18:0 and one 18:1 tail (PA 36:1), phosphatidylglycerol with two 18:0 tails (PG 36:0), phosphatidylcholine with two 18:0 tails (PC 36:0), phosphatidylserine with one 16:0 tail and one 18:2 tail (PS 34:2), phosphatidylethanolamine with two 16:0 tails (PE 32:0), and lysyl-phosphatidylglycerol with two 18:1 tails (LPG 36:2). All standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and one representative compound was chosen from each head group class. The fatty acid standards:

cis-9-octadecanoic acid (oleic acid), *cis* 9,12 octadecadienoic acid (linoleic acid), octadecanoic acid (stearic acid), and hexadecenoic acid (palmitic acid) were purchased from Sigma Aldrich.

2.2 Targeted standards

Representative standards from three head group classes; cardiolipin (CL), phosphatidylglycerol (PL), and lysyl-phosphatidylglycerol (LPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) Even though this method was targeted, exact matched compounds were not available for all species of interest, therefore one compound from each of the three classes was used to make external calibration curves. Cardiolipin (CL 72:4) with four 18:1 tails, phosphatidylglycerol (PG 36:0) with two 18:0 tails, and lysyl-phosphatidylglycerol (LPG 36:2) with two 18:1 tails were chosen as external standards. Since HILIC chromatography was used, it was hypothesized that there would be minimal retention time variation between molecules within the same head group regardless of tail configurations. Internal standards were chosen based on lipids that were not present or had very limited presence in the biological samples. Cardiolipin with four 14:0 tails (CL 56:0) and phosphatidylglycerol with two 18:0 tails (PG 36:0) were chosen as the internal standards. It should be noted that based on gas chromatography fatty acid methyl ester analyses, a small portion (< 5%) of fatty acid tails in *E. faecalis* membranes are tetradecanoic acid (C14:0) (Harp et al. 2016; Saito et al. 2018). There were no non-naturally abundant LPG standards available for purchase, therefore external calibration curves were used to quantify LPGs. Data for all targeted standards are presented in Table 1. Calibration and test samples were spiked with an internal standard to account for any loss of signal due to ionization efficiency or suppression.

2.3. Extraction of lipids from biological samples

Enterococcus faecalis OG1RF cultures were grown in brain heart infusion (BHI) medium at 37 °C. Overnight cultures were diluted into fresh BHI to an optical density (OD₆₀₀) of 0.01 and supplemented with either 10 µg/mL of linoleic acid or 20 µg/mL oleic acid and allowed to grow until an OD₆₀₀ of 0.3. Ethanol (0.1%) was used as the solvent control.

Enterococcus faecalis supplemented with the previously mentioned fatty acids were harvested in 15 mL aliquots at mid log phase (OD₆₀₀ ~ 0.3). The cells were washed twice with 1X PBS via centrifugation, resuspended in 1 mL of 1X PBS, and treated with 100µg/mL of lysozyme. After 20 min of incubation at 37 °C, the lysozyme treated cells were transferred to plastic screw top microfuge tubes containing 0.5 g of glass beads (106 µm). The cells were then homogenized using a mini bead-beater (Biospec Products, Bartlesville, OK) for two, one min intervals. The homogenized cells were then transferred to a 15 mL polypropylene conical vial containing a mixture of chloroform: methanol 2:1 (v/v) (Bligh and Dyer 1959; Iverson et al. 2001). Samples were briefly vortexed and then centrifuged for 5 min at 3500 rpm. Layers formed containing the glass beads at the bottom and the cell debris in the middle. The two phases were carefully transferred to a clean 15 mL polypropylene conical vial containing 1.5 mL of 0.9% NaCl, leaving behind the cell debris and glass beads. After briefly vortexing, samples were centrifuged, and the lower lipid containing organic phase was transferred to a glass screw top vial. Lipid extracts were dried

under nitrogen gas and suspended in 260 μL of 1:1 methanol: chloroform and 40 μL of internal standard before being transferred to autosampler vials with PTFE septa.

2.4 Liquid chromatography–mass spectrometry parameters

Samples and standards were stored at $-20\text{ }^{\circ}\text{C}$ until they could be analyzed. A Dionex UltiMate 3000RS ultra high performance liquid chromatography (UHPLC) (Sunnyvale, CA) system was used in conjunction with a Kinetex HILIC column ($100\text{ }\text{\AA}$ $2.6\text{ }\mu\text{m}$ $150\text{ mm} \times 2.1\text{ mm}$, Phenomenex, Torrance, CA) to separate lipid compounds for both the targeted and untargeted analysis. A $10\text{ }\mu\text{L}$ injection of sample was injected using a Dionex UltiMate 3000 RS autosampler kept at $4\text{ }^{\circ}\text{C}$. The syringe and fluidics were washed twice with $150\text{ }\mu\text{L}$ of 50:50 (v/v) water:2-propanol. All chromatographic conditions were controlled by Chromeleon Xpress version 6.80 and all mass spectrometric conditions were controlled by Xcalibur version 4.0. Mass calibration was performed every 24 h in positive and negative mode using calibration solutions from Thermo Scientific (Waltham, MA).

2.5 Untargeted lipid analysis

The untargeted method used 10 mM ammonium formate adjusted to a pH of 3.0 for mobile phase A, and 97:3 acetonitrile: water (v/v) adjusted to a pH of 3.0 with 10 mM ammonium formate for mobile phase B. Gradient: $t = 0\text{ min}$ 0% solvent A, 100% solvent B; $t = 1\text{ min}$ 0% solvent A, 100% solvent B; $t = 15\text{ min}$ 19% solvent A, 81% solvent B; $t = 15.1\text{ min}$ 52% solvent A, 48% solvent B; $t = 25\text{ min}$ 52% solvent A, 48% solvent B; $t = 25.1\text{ min}$ 0% solvent A, 100% solvent B; $t = 35\text{ min}$ 0% solvent A, 100% solvent B. The flow rate was set to 0.200 mL/min .

An Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA) was used to detect the different phospholipids and fatty acids. Eluent from the UHPLC was introduced using electrospray ionization (ESI) utilized under the following settings: sheath gas—25 (arbitrary units); auxiliary gas—10 (arbitrary units); spray voltage—4 kV; capillary temperature— $350\text{ }^{\circ}\text{C}$; resolution—140,000. Ions between 100 and $1500\text{ }m/z$ were recorded in both positive and negative mode in separate scan events. In addition to full scan data, the all ion fragmentation (AIF) was collected throughout the run using a normalized collisional energy of 30 eV with a stepped energy of 50%.

2.5.1 Targeted phospholipid analysis—The targeted analysis used 5 mM ammonium acetate adjusted to a pH of 5.8 for mobile phase A, and 97:3 acetonitrile:water adjusted to a pH of 5.8 with a final concentration of 5 mM ammonium acetate. Gradient: $t = 0\text{ min}$ 3% solvent A, 97% solvent B; $t = 1\text{ min}$ 3% solvent A, 97% solvent B; $t = 1.2\text{ min}$ 5% solvent A, 95% solvent B; $t = 4\text{ min}$ 5% solvent A, 95% solvent B; $t = 4.2\text{ min}$ 10% solvent A, 90% solvent B; $t = 7.6\text{ min}$ 10% solvent A, 90% solvent B; $t = 8.1\text{ min}$ 30% solvent A, 70% solvent B; $t = 10.9\text{ min}$ 30% solvent A, 70% solvent B; $t = 11\text{ min}$ 50% solvent A, 50% solvent B; $t = 18\text{ min}$ 50% solvent A, 50% solvent B. The flow rate for separation was 0.200 mL/min . Following the 18 min separation, the gradient was set to 3% solvent A, 97% solvent B at a flow rate of 0.500 mL/min for 12 min to re-equilibrate the column.

Eluent from the UHPLC was ionized using an electrospray ionization (ESI) source operated under the following settings: sheath gas 30 (arbitrary units); aux gas 8 (arbitrary units); sweep gas 3 (arbitrary units); spray voltage 3 kV; capillary temperature 300 °C. A Q-Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA) was used to collect data for all targeted data. All ions were detected in negative mode using parallel reaction monitoring (PRM), which requires a list of parent m/z s in order to isolate and subsequently collect a full scan of the fragments produced. Parameters for the mass spectrometer were as follows: resolution—140,000; automatic gain control (AGC)— 3×10^6 ions; maximum IT time—100 ms; isolation window—0.4 m/z ; normalized collision energy 35 eV.

2.6 Targeted compound list

Phosphatidylglycerol (PG) and lysyl-phosphatidylglycerol (LPG) compounds were detected as the $[M-H]^-$ ion and the cardiolipin (CL) compounds were detected as $[M-2H]^{2-}$ ions. In total, 23 unique CL, 10 PG, and 10 LPG masses were targeted for quantitation. This list only included the most abundant fatty acids bound to each head group: C16:0, C16:1, C18:0, C18:1, and C18:2, based on bacterial profiles (Harp et al. 2016; Saito et al. 2018). A complete list of all the lipids included in the analysis is provided in Table S1. The m/z listed in the table corresponds to the parent mass isolated and eventually fragmented. However, quantitation was performed by selecting a specific fragment that is common to the head group not the specific lipid. A dehydrated glycerol phosphate (GP) fragment with a m/z of 152.9958 was used for all PG and CL compounds, and a lysine fragment with a m/z of 145.0948 was used for LPG (Fig. 1). In addition, the PRM analysis method has the advantage of determining the relative concentrations of specific fatty acid tails attached to the parent ion.

2.7 Calibration curve preparation

Standards for calibration curves were dissolved in 1:1 methanol:chloroform using masses of purchased solid standards. A mixed stock of CL 72:4, PG 36:0, and LPG 36:2 was prepared at 75 μ M and stored at -20 °C. Serial dilutions from the stock were made at the following concentrations: 50, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, and 0.001 μ M. Internal standards of CL 56:0 and PG 16:0 were made by dissolving the solid standard in solvent to make a mixed working stock of 50 μ M and added to each calibration standard at a final concentration of 8.3 μ M.

3. Data analysis

3.1. Untargeted

Data generated from the Xcalibur software was converted from the *.raw* file extension to the *.mzML* using msconvert, a tool in ProteoWizard (Holman et al. 2014; Kessner et al. 2008). All peak area integration was performed within MAVEN (Clasquin et al. 2012), using a 5 ppm mass window and exact m/z based on chemical formula and preferred ionization mode. Phospholipids with the head groups of PA, PE, PI, PS, PG, LPG, and fatty acids were detected as $[M-H]^-$ and PC were detected as $[M+H]^+$.

3.2. Targeted

Xcalibur's Quan Browser (Thermo Scientific, Waltham, MA) was used to integrate areas under the curve for fragment masses that corresponded to exact m/z of the specific ion of interest and were also matched to retention time range of phospholipid classes. Cardiolipin molecules elute at 4.5 min, phosphatidylglycerol elutes at 2 min, and lysyl-phosphatidylglycerol elutes at 10 min. Calibration plots were made by plotting the log of the ratios of the area of the analyte to the area of the internal standard " $\log(A_a/A_{is})$ " vs. the log of the ratios of concentration of the analyte to the concentration of the internal standard " $\log([C_a]/[C_{is}])$ ". Biological data was analyzed using the same protocol and the exact concentration of the lipid was interpolated using the regression line for the corresponding standard. Each calibration level was analyzed in replicates of six and standard error was calculated.

4 Results and discussion

4.1 Untargeted data

Operating the instrument in dual polarity full scan mode, along with all ion fragmentation (AIF) allowed for the collection of information about the parent ion and the fragments produced. Although the hydrophobic extraction preferentially selects for compounds with lipid-like moieties, the mass spectrometer is only constrained by its m/z range. This allows for analysis of experiments that are exploratory in nature or of unknown sample composition. The analysis leads to an initial understanding of the lipid profiles in samples, which has not been previously studied. Additionally, global lipidome changes can be measured between a treatment group and a control. HILIC chromatography is concentrated on hydrophobicity; therefore, any phospholipid with the same polar head group (PE, PC, PG, etc.) will have the same retention time (RT) without regard to the composition of aliphatic tails. A typical experiment requires a standard to be analyzed using the exact chromatographic and mass spectrometric conditions for each compound that is to be identified, but this is not the case for this untargeted method. Identification for exact lipid species can be made by matching two factors: exact mass and retention time of the head group. Each phospholipid head group only needs to have one representative standard analyzed, saving instrument time and cost. This makes it possible to identify any possible tail combination for all head groups, without having multiple standards. This can be very advantageous if the compounds desired are not easily purchased or synthesized.

As a proof of concept, six phospholipid standards, one from each of the most abundant head groups, were analyzed by the untargeted lipidomics methods and were detected in either positive or negative mode (Table 2). There was complete temporal resolution between groups except for phosphatidylinositol (5.08 min) and phosphatidic acid (4.99 min), but because a high-resolution mass analyzer was used, there was a low possibility of lipid misidentification. The lysine conjugated PG was also detected at 12.3 min. Even though no additional amino acid (AA) modified phospholipid standards were analyzed, it is indicative that this HILIC-MS method is capable of detecting many other AA modified lipids. It has been shown that these compounds serve an important role in creating surface membrane charge sites, thus providing some form of antibiotic tolerance (Kilelee et al. 2010; Nishi et

al. 2004) and reduces attraction of host generated antimicrobial peptides (Joo and Otto 2015). Since free fatty acids (FFA) are the building blocks of phospholipids it would be beneficial to detect those as well (Hamilton and Kamp, 1999; Klausner et al. 1980). These compounds were not retained by the column, which was expected, but four biologically relevant FFA were capable of being detected (oleic acid, linoleic acid, stearic acid, and palmitic acid).

This method has shown its ability to discriminate seven different phospholipids and four fatty acids; it is reasonable to assume that other lipids that do not contain phosphorus or have a sugar moiety as a head group could be detected in this approach. Photosynthetic organisms have sulfolipids, such as sulfoquinovosyl diacylglycerols (SQDG), and can replace phospholipids with sulfur containing lipids under phosphorus limited conditions (Sprott et al. 2003; Van Mooy et al. 2009). Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are the major lipid classes present in plants and have roles in photosynthesis (Chapman et al. 1985; Dörmann and Benning, 2002).

Being able to precisely measure a large set of compounds within and across a lipid class in a 30-minute analysis is very valuable. However, it is possible to accurately detect thousands of m/z that may not match any known lipid. Matching the retention time for each m/z can help identify the head group; and AIF can be used to identify fragments that are present, aiding in piecing the molecule back together. The standard for PS (34:2) elutes at 10.7 min and several fragments that match the structure can be identified. Based upon theoretical fragmentation data from LipidMaps (Fahy et al. 2009), it is possible to identify at least 5 fragments of the parent ion, including the 16:0 (255.2333 m/z) and 18:2 (279.2334 m/z) fatty acid tail fragments (Fig. 2). This added feature can be useful for structure elucidation in the case of an unidentified ion.

4.2 Targeted data

In efforts to compliment the untargeted approach, the design and optimization of a targeted approach was used to quantify a reduced number of lipids. Although this exact method only focuses on three lipid classes (CL, PG, and LPG) present in a specific bacterium (Rashid et al. 2017; Tran et al. 2013), the fundamentals can be applied to any set of lipids that desire a more precise measurement.

The above-mentioned untargeted method uses 10 mM ammonium formate at pH 3, which provides excellent detection of all six common phospholipid classes and several other lipid species; however, CL do not ionize well with these conditions. Herein, we have created a method to quantify CL without using ion-pairing agents, while still being able to quantify two other lipid classes, PG and LPG. It was determined that raising the pH to 5.8 and changing the buffer concentration to 5 mM ammonium acetate resulted in reliable detection in cardiolipin standards. This is supported by experimentation showing pK_{a1} and pK_{a2} have values of 2.15 and 3.15, respectively (Olofsson and Sparr 2013), thus shifting equilibrium towards the doubly deprotonated form at a pH of 5.8. Again, due to the HILIC nature of the chromatography, separation can be achieved based on headgroup regardless of tail composition. There are discrete regions in the chromatogram where PG, CL and LPG's elute (Supplementary Fig. 1). In contrast to the AIF that is used in the untargeted method, parallel

reaction monitoring (PRM) is used to isolate and fragment individual lipid m/z . High resolution data of the all the fragments originating from the parent mass is obtained. This provides an extra level of confirmation to identify the exact structural moieties present in a mixed FA lipid while reducing noise, thus lowering the detection limit. The $[M+1-H]^-$ isotope peak becomes significant (i.e. $> 30\%$ relative intensity to the $[M-H]^-$ peak) for molecules with 30 or more carbons, such as the CLs. The PRM method utilized an isolation window of 0.4 m/z that only allowed for the selection of the $[M-H]^-$ ions for each CL species, thus the $^{13}C_1$ isotopologue was not measured. The contribution from this isotopologue could be sufficiently large that using both peaks would lower the detection limit for this class of molecules.

To make the targeted method as quantitative as possible, a calibration curve was made by using a single external standard each for lipid class and adding an internal standard that is not naturally found in biological samples. Since standards for each specific lipid of interest are not available, this methodology for quantitation accounts for any changes in ionization efficiency between the chosen external standard and the detected lipid. When calibration curves were plotted, a high correlation of determination value (R^2) was achieved for all lipid classes (Supplementary Fig. 2 and Supplementary Table 2). Less than 2% variation was seen in the internal standard across all points in the calibration curve (Table 1). The detection limit of 41.6 nM was observed for LPG, while the detection limits for PG and CL were 416 nM. Linear range for CL and PG spanned three orders of magnitude and LPG was linear over four orders of magnitude. Signal for the standards below these concentrations was either not detected, or not above the noise.

4.3 Biological validation

To show the application of this lipidomics approach, *Enterococcus faecalis* (*E. faecalis*) OG1RF cells were supplemented with exogenous fatty acids and subjected to lipid analysis using both the untargeted and targeted approaches. Treated and control samples were grown and lipids were extracted as mentioned in the earlier section. It has been shown that when *E. faecalis* cells are grown in the presence of host unsaturated fatty acids (oleic acid or linoleic acid, Supplementary Fig. 3), a tolerance to daptomycin is observed, along with alterations in membrane fatty acid profiles as measured through GC-FAME (Saito et al. 2014). Applying our approach on this bacterial system shows the advantages of performing both analyses on a single sample. Several studies have been published measuring lipid changes in *E. faecalis* using thin layer chromatography (TLC) (Bao et al. 2012; Mishra et al. 2012; Tran et al. 2013), and GC-FAME (Harp et al. 2016; Saito et al. 2014, 2018). TLC methods suffer from the ability to only identify different classes of lipids and has limited use for quantitation. GC-FAME allows for quantitation of individual fatty acids but is hindered by the non-specific esterification of phospholipids resulting in loss of information about head groups. One group explicitly measured individual phospholipids (LPG and PG) in these bacterium (Rashid et al. 2017). A triple quadrupole was used to develop a method to measure relative concentrations of PG and LPG, and a quadrupole time of flight mass spectrometer to measure CL. Although a LC-MS method was presented in the manuscript, they were unable to detect any CL in the biological sample. Supplementary Table 3

compares what lipids were detected in *E.faecalis* by Rashid and coworkers to what was measured in this study.

The untargeted analysis also facilitated the detection of two free fatty acids (FFA), 9 lysyl-phosphatidylglycerols (LPG), and 12 phosphatidylglycerols (PG). The level of metabolite identification is level 3 unless an exact standard was analyzed (Supplementary Table 4) (Sumner et al. 2007). The heat map in Fig. 3 shows the log₂ fold change in the normalized areas for each lipid detected (exact ratios shown in Supplementary Table 5). Each column represents the ratio of the samples supplemented with exogenous fatty acids compared to the control samples. As expected, a significant difference is seen in the relative amounts of FFAs detected in the corresponding supplemented samples (e.g. oleic acid is more abundant in the samples when supplemented with oleic acid, and likewise for linoleic acid). Smaller, positive, significant fold changes are seen in the oleic acid samples for LPG 34:2, LPG 36:2, and LPG 36:4, showing that some amount of oleic acid may be incorporated into the membrane as LPG. An opposite trend is seen when looking at the linoleic acid samples; a decrease is seen in monosaturated LPGs such as 36:1, 32:1 34:1. These compounds have one degree of unsaturation in either of the two fatty acid tails, but this method is lacking the ability to determine exactly which fatty acid tail harbors the double bond. Regardless of the fatty acid that is supplemented, there is a global decrease in all of the PGs detected when compared to the controls. The one thing this analysis cannot provide is the ability to compare the fold change of one lipid to the fold change to another lipid (e.g. the fold change for LPG 36:4 vs the fold change for PG 36:1).

While the untargeted method provides the ability to quickly examine how multiple lipids species (within and across head groups) are changing compared to others, information about exact concentrations is lacking. This limitation can be overcome by using this targeted method, thus gaining more information about lipid species identified as significant from the untargeted method. The targeted analysis (n = 6 per group) was able to quantify 9 phosphatidylglycerols (PG), 8 cardiolipins (CL) and 10 lysyl-phosphatidylglycerols (LPG) (Table 3 and Supplementary Table 6), level of identification is listed in the supporting material. If a value is listed as < LOQ, a peak was identified but its concentration was lower than the limit of quantitation, and if a ND is listed, no peak was detected. This method was capable of quantifying cardiolipins which has been difficult to do with previous methods. When the cells were provided linoleic acid, the polyunsaturated CL (72:7 and 72:8) concentrations were 1.46 and 2.69 μ M respectively and were not detected in the oleic acid and control samples. Within the PRM data, it can be verified that the CL 72:7 compound's major tail fragment is a C18:2 (*m/z* 279.2231), and a minor component of C18:1 (*m/z* 281.2486). As expected, the CL 72:8 fragmentation data showed only C18:2 tail fragments (Supplementary Fig. 4). It is clear that the quantity of PG lipids with C16 tails (saturated or unsaturated) was very low when given either of the fats, which was also seen in the untargeted data. The targeted approach showed the most abundant PG in the control samples is PG 34:1 and has a concentration of 11.21 μ M, which is two times higher than the next most abundant PG. This information is not obtainable from the untargeted method alone. This increases the understanding of how the membrane composition is changing within a head group, but not necessarily across treatments.

5 Conclusion

The data shows that using a high-resolution mass spectrometer in full scan mode allows for the detection of many different lipid classes with the ability to discriminate within each class, and a more holistic understanding of how lipid profiles change. It was possible to detect six major phospholipids, free fatty acids, and lysine-based lipids, but there is reason to believe that many more lipid classes could be detected using this analysis. By adjusting the pH of the mobile phase, it was possible to confidently detect cardiolipins, which are notoriously difficult to measure. With the implementation of representative standards (internal and external), and parallel reaction monitoring, it was possible to quantitate cardiolipin, phosphatidylglycerol, and lysyl-phosphatidylglycerol to the nanomolar range. Biological applications were shown by using a single extraction method to measure global lipid changes and quantify eight different cardiolipin species in *E.faecalis*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Bao Y, Sakinc T, Laverde D, Wobser D, Benachour A, Theilacker C, et al. (2012). Role of mprF1 and mprF2 in the pathogenicity of Enterococcus faecalis. PLoS ONE, 7, e38458.
- Beate F, & Jürgen S. (2009). Application of MALDI-TOF mass spectrometry in lipidomics. European Journal of Lipid Science and Technology, 111, 83–98.
- Beney L, & Gervais P. (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. Applied Microbiology and Biotechnology, 57, 34–42. [PubMed: 11693931]
- Bird SS, Marur VR, Sniatynski MJ, Greenberg HK, & Kristal BS (2011). Lipidomics profiling by high-resolution LC-MS and high-energy collisional dissociation fragmentation: Focus on characterization of mitochondrial cardiolipins and monolysocardiolipins. Analytical Chemistry, 83, 940–949. [PubMed: 21192696]
- Bligh EG, & Dyer WJ (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 911–917. [PubMed: 13671378]
- Chapman DJ, De-Felice J, & Barber J. (1985). Characteristics of chloroplast thylakoid lipid composition associated with resistance to triazine herbicides. Planta, 166, 280–285. [PubMed: 24241445]
- Clasquin MF, Eugene M, & Rabinowitz DJ (2012). LC-MS data processing with MAVEN: A metabolomic analysis and visualization engine. Current Protocols in Bioinformatics, 37, 14.11.1–14.11.23.
- Cronan JE (2003). Bacterial membrane lipids: Where do we stand? Annual Review of Microbiology, 57, 203–224.
- Dörmann P, & Benning C. (2002). Galactolipids rule in seed plants. Trends in Plant Science, 7, 112–118. [PubMed: 11906834]
- Dowhan W. (1997). Molecular basis for membrane phospholipid diversity: Why are there so many lipids? Annual Review of Biochemistry, 66, 199–232.

- Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CH, Shimizu T, et al. (2009). Update of the LIPID MAPS comprehensive classification system for lipids. *Journal of Lipid Research*, 50, S9–S14. [PubMed: 19098281]
- Fozo EM, & Rucks EA (2016) Chapter two—The making and taking of lipids: The role of bacterial lipid synthesis and the harnessing of host lipids in bacterial pathogenesis In Poole RK (Ed.), *Advances in microbial physiology* (pp. 51–155) Academic Press.
- Hamilton JA, & Kamp F. (1999). How are free fatty acids transported in membranes? Is it by proteins or by free diffusion through the lipids? *Diabetes*, 48, 2255–2269. [PubMed: 10580412]
- Harp JR, Saito HE, Bourdon AK, Reyes J, Arias CA, Campagna SR, et al. (2016). Exogenous fatty acids protect *Enterococcus faecalis* from daptomycin-induced membrane stress independently of the response regulator LiaR. *Applied and Environmental Microbiology*, 82, 4410–4420. [PubMed: 27208105]
- Hines KM, Waalkes A, Penewit K, Holmes EA, Salipante J, Werth BJ, et al. (2017). Characterization of the mechanisms of daptomycin resistance among gram-positive bacterial pathogens by multidimensional lipidomics. *mSphere*, 2, e00492.
- Holman JD, Tabb DL, & Mallick P. (2014). Employing proteowizard to convert raw mass spectrometry data. *Current Protocols in Bioinformatics*, 46, 13241–13249.
- Iverson SJ, Lang SL, & Cooper MH (2001). Comparison of the bligh and dyer and folch methods for total lipid determination in a broad range of marine tissue. *Lipids*, 36, 1283–1287. [PubMed: 11795862]
- Joo H-S, & Otto M. (2015). Mechanisms of resistance to antimicrobial peptides in staphylococci. *Biochimica et Biophysica Acta (BBA)—Biomembranes*, 1848, 3055–3061. [PubMed: 25701233]
- Kessner D, Chambers M, Burke R, Agus D, & Mallick P. (2008). ProteoWizard: Open source software for rapid proteomics tools development. *Bioinformatics*, 24, 2534–2536. [PubMed: 18606607]
- Kilelee E, Pokorny A, Yeaman MR, & Bayer AS (2010). Lysyl-phosphatidylglycerol attenuates membrane perturbation rather than surface association of the cationic antimicrobial peptide 6 W-RP-1 in a model membrane system: implications for daptomycin resistance. *Antimicrobial Agents and Chemo-therapy*, 54, 4476–4479.
- Klausner RD, Kleinfeld AM, Hoover RL, & Karnovsky MJ (1980). Lipid domains in membranes. Evidence derived from structural perturbations induced by free fatty acids and lifetime heterogeneity analysis. *Journal of Biological Chemistry*, 255, 1286–1295. [PubMed: 7354027]
- Minkler PE, & Hoppel CL (2010). Separation and characterization of cardiolipin molecular species by reverse-phase ion pair high-performance liquid chromatography-mass spectrometry. *Journal of Lipid Research*, 51, 856–865. [PubMed: 19965604]
- Mishra NN, Bayer AS, Tran TT, Shamoo Y, Mileyskovskaya E, Dowhan W, et al. (2012). Daptomycin resistance in enterococci is associated with distinct alterations of cell membrane phospholipid content. *PLoS ONE*, 7, e43958.
- Nishi H, Komatsuzawa H, Fujiwara T, McCallum N, & Sugai M. (2004). Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in *Staphylococcus aureus*. *Antimicrobial Agents and Chemo-therapy*, 48, 4800–4807.
- Oliver PM, Crooks JA, Leidl M, Yoon EJ, Saghatelian A, & Weibel DB (2014). Localization of anionic phospholipids in *Escherichia coli* cells. *Journal of Bacteriology*, 196, 3386–3398. [PubMed: 25002539]
- Olofsson G, & Sparr E. (2013). Ionization constants pKa of cardiolipin. *PLoS ONE*, 8, e73040.
- Rashid R, Cazenave-Gassiot A, Gao IH, Nair ZJ, Kumar JK, Gao L, et al. (2017). Comprehensive analysis of phospholipids and glycolipids in the opportunistic pathogen *Enterococcus faecalis*. *PLoS ONE*, 12, e0175886.
- Renner LD, & Weibel DB (2011). Cardiolipin microdomains localize to negatively curved regions of *Escherichia coli* membranes. *Proceedings of the National Academy of Sciences*, 108, 6264–6269.
- Romantsov T, Guan Z, & Wood JM (2009). Cardiolipin and the osmotic stress responses of bacteria. *Biochimica et Biophysica Acta (BBA)—Biomembranes*, 1788, 2092–2100. [PubMed: 19539601]

- Saito HE, Harp JR, & Fozo EM (2014). Incorporation of exogenous fatty acids protects *Enterococcus faecalis* from membrane-damaging agents. *Applied and Environmental Microbiology*, 80, 6527–6538. [PubMed: 25128342]
- Saito HE, Harp JR, & Fozo EM (2018). *Enterococcus faecalis* responds to individual exogenous fatty acids independently of their degree of saturation or chain length. *Applied and Environmental Microbiology*, 84, e01633.
- Scherer M, Schmitz G, & Liebisch G. (2010). Simultaneous quantification of cardiolipin, bis(monoacylglycero)phosphate and their precursors by hydrophilic interaction LC–MS/MS including correction of isotopic overlap. *Analytical Chemistry*, 82, 8794–8799. [PubMed: 20945919]
- Schwalbe-Herrmann M, Willmann J, & Leibfritz D. (2010). Separation of phospholipid classes by hydrophilic interaction chromatography detected by electrospray ionization mass spectrometry. *Journal of Chromatography A*, 1217, 5179–5183. [PubMed: 20598701]
- Sohlenkamp C, & Geiger O. (2016). Bacterial membrane lipids: Diversity in structures and pathways. *FEMS Microbiology Reviews*, 40, 133–159. [PubMed: 25862689]
- Sprott GD, Larocque S, Cadotte N, Dicaire CJ, McGee M, & Brisson JR (2003). Novel polar lipids of halophilic eubacterium *Planococcus H8* and archaeon *Haloferax volcanii*. *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, 1633, 179–188. [PubMed: 14499737]
- Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, et al. (2007). Proposed minimum reporting standards for chemical analysis. *Metabolomics*, 3, 211–221. [PubMed: 24039616]
- Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, et al. (2013). Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. *mBio*, 4, e00281.
- Valianpour F, Wanders RJA, Barth PG, Overmars H, & van Gennip AH (2002). Quantitative and compositional study of cardiolipin in platelets by electrospray ionization mass spectrometry: Application for the identification of Barth syndrome patients. *Clinical Chemistry*, 48, 1390–1397. [PubMed: 12194913]
- Van Mooy BAS, Fredricks HF, Pedler BE, Dyhrman ST, Karl DM, Koblížek M, et al. (2009). Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. *Nature*, 458, 69. [PubMed: 19182781]
- Watson AD (2006). Thematic review series: Systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: A global approach to lipid analysis in biological systems. *Journal of Lipid Research*, 47, 2101–2111. [PubMed: 16902246]

Fig. 1.
Structure of fragments used to quantitate lipids cardiolipin, phosphatidylglycerol, and lysyl-phosphatidylglycerol

Fig. 2.

All ion fragmentation of PS (34:2) standard. A: $[M-H]^-$, B: loss of serine, C: loss of serine and C18:2 fatty acid as ketene, D: loss of serine and C18:2 fatty acid as neutral specie, E: C18:2 fatty acid, F: C16:0 fatty acid

Heat map of lipid compounds detected using the untargeted analysis. All fold changes are compared to control samples and then $\log_2$ transformed. An orange color represents a fold change > 1 , and a blue color represents a fold change < 1 . Asterisks depict the level of confidence based on a Student's T test

Table 1

Details for lipid standards used for the targeted analysis

Compound class	Formula	Tails	RT (min)	Abbr.	Species	Transition	Standard type	IS variation
Cardiolipin	C ₈₁ H ₁₅₀ O ₁₇ P ₂	18:1	4.50	CL (72:4)	[M-2H] ²⁻	619.4162 → 152.9958	External	1.92%
Cardiolipin	C ₆₅ H ₁₂₆ O ₁₇ P ₂	14:0	4.50	CL (54:0)	[M-2H] ²⁻	727.5101 → 152.9958	Internal	
Phosphatidylglycerol	C ₄₂ H ₈₃ O ₁₀ P	18:0	2.00	PG (36:0)	[M-H] ⁻	777.5651 → 52.9958	External	2.20%
Phosphatidylglycerol	C ₂₂ H ₄₃ O ₁₀ P	8:0	2.00	PG (16:0)	[M-H] ⁻	497.2521 → 152.9958	Internal	
Lysyl phosphatidylglycerol	C ₄₈ H ₉₁ N ₂ O ₁₁ P	18:1	10.20	LPG (36:2)	[M-H] ⁻	901.6288 > 145.0948	External	N/A

Table 2

Details for lipid standards used for the untargeted analysis

Lipid class	Abbr.	Formula	Species detected	RT (min)	Exact m/z	Detected m/z	Error (ppm)
Phosphatidylinositol	PI (32:0)	$C_{41}H_{79}O_{13}P$	$[M-H]^-$	5.08	809.5180	809.5209	3.58
Phosphatidic acid	PA (36:1)	$C_{39}H_{75}O_8P$	$[M-H]^-$	4.99	701.5121	701.5146	3.56
Phosphatidylcholine	PC (36:0)	$C_{44}H_{88}NO_8P$	$[M+H]^+$	11.91	790.6326	790.6315	1.39
Phosphatidylserine	PS (34:2)	$C_{40}H_{74}NO_{10}P$	$[M-H]^-$	10.79	758.4972	758.4992	2.64
Phosphatidylethanolamine	PE (32:0)	$C_{37}H_{74}NO_8P$	$[M-H]^-$	9.17	690.5074	690.5093	2.75
Phosphatidylglycerol	PG (36:0)	$C_{42}H_{82}O_{10}P$	$[M-H]^-$	3.43	777.5665	777.5659	0.77
Lysyl-phosphatidylglycerol	LPG (36:2)	$C_{48}H_{91}NA_{11}P$	$[M-H]^-$	12.3	901.6282	901.6307	2.77
Oleic acid	OA	$C_{18}H_{34}O_2$	$[M-H]^-$	1.59	281.2481	281.2491	3.56
Linoleic acid	LA	$C_{18}H_{32}O_2$	$[M-H]^-$	1.66	279.2324	279.2334	3.58
Palmitic acid	P	$C_{16}H_{32}O_2$	$[M-H]^-$	1.74	255.2324	255.2334	3.92
Stearic acid	S	$C_{18}H_{36}O_2$	$[M-H]^-$	1.53	283.2637	283.2648	3.88

Table 3

Quantitative lipid data for biological samples

Lipid	Concentration (μM)		
	Control	Oleic acid	Linoleic acid
PG (32:0)	1.79 ± 0.23	< LOD	< LOD
PG (32:1)	4.98 ± 0.92	< LOD	< LOD
PG (34:0)	1.28 ± 0.12	< LOD	< LOD
PG (34:1)	11.21 ± 1.76	< LOD	< LOD
PG (34:2)	0.97 ± 0.12	< LOD	1.25 ± 0.18
PG (36:1)	< LOD	1.21 ± 0.25	< LOD
PG (36:2)	1.87 ± 0.44	18.34 ± 2.2	< LOD
PG (36:3)	ND	< LOD	0.79 ± 0.15
PG (36:4)	< LOD	< LOD	8.14 ± 1.57
CL (66:0)	ND	ND	0.75 ± 0.14
CL (70:0)	0.77 ± 0.12	ND	1.71 ± 0.31
CL (70:6)	ND	ND	0.90 ± 0.16
CL (72:0)	3.81 ± 0.53	< LOD	< LOD
CL (72:3)	< LOD	1.04 ± 0.31	ND
CL (72:4)	< LOD	2.68 ± 0.7	ND
CL (72:7)	ND	ND	1.46 ± 0.35
CL (72:8)	ND	ND	2.69 ± 0.68
LPG (32:0)	0.53 ± 0.18	< LOD	< LOD
LPG (32:1)	0.53 ± 0.19	0.19 ± 0.03	0.04 ± 0.03
LPG (34:0)	0.46 ± 0.2	0.05 ± 0.01	< LOD
LPG (34:1)	2.97 ± 0.69	0.57 ± 0.11	0.08 ± 0.12
LPG (34:2)	0.22 ± 0.04	0.38 ± 0.04	0.67 ± 1.16
LPG (36:0)	< LOD	0.05 ± 0.01	ND
LPG (36:1)	0.17 ± 0.02	1.57 ± 0.2	< LOD
LPG (36:2)	0.39 ± 0.08	12.8 ± 1.46	0.13 ± 0.19
LPG (36:3)	< LOD	0.07 ± 0.01	0.49 ± 0.92
LPG (36:4)	0.05 ± 0.03	< LOD	2.46 ± 4.48

The first column represents the lipid species detected, and the second, third, and fourth columns represent the three different exogenous fats that were added. The data is an average of six replicates and the standard error