Journal of Visualized Experiments

Quantitative Analysis of the Cellular Lipidome of the Yeast Saccharomyces Cerevisiae Using Liquid Chromatography Coupled with Tandem Mass Spectrometry

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60616R2
Full Title:	Quantitative Analysis of the Cellular Lipidome of the Yeast Saccharomyces Cerevisiae Using Liquid Chromatography Coupled with Tandem Mass Spectrometry
Section/Category:	JoVE Biochemistry
Keywords:	Lipids, cellular lipidome, unesterified (free) fatty acids, glycerophospholipids, neutral lipids, sphingolipids, ceramides, cardiolipins, lipid extraction, lipidomics, liquid chromatography, mass spectrometry
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Montreal, Quebec, Canada

December 6, 2019

Phillip Steindel, Ph.D.
Review Editor
JoVE

Dear Dr. Steindel:

Please find attached a revised version of our manuscript, entitled "Quantitative Analysis of the Cellular Lipidome of the Yeast Saccharomyces Cerevisiae Using Liquid Chromatography Coupled with Tandem Mass Spectrometry" (manuscript reference number JoVE60616R1), which we would like to be considered for publication in the *JoVE*.

We have addressed each of the editorial and peer review comments and revised the manuscript accordingly.

We are grateful to both Reviewers for constructive criticisms and suggestions for changes to be made in the text, all of which contributed to a substantial improvement of the original manuscript. We would also like to thank you for giving us the opportunity to address these criticisms and for handling our manuscript.

Yours sincerely,

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TITLE:

Quantitative Analysis of the Cellular Lipidome of *Saccharomyces Cerevisiae* Using Liquid Chromatography Coupled with Tandem Mass Spectrometry

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KEYWORDS:

lipids, cellular lipidome, unesterified (free) fatty acids, glycerophospholipids, neutral lipids, sphingolipids, ceramides, cardiolipins, lipid extraction, lipidomics, liquid chromatography, mass spectrometry

SUMMARY:

We present a protocol using liquid chromatography coupled with tandem mass spectrometry to identify and quantify major cellular lipids in *Saccharomyces cerevisiae*. The described method for a quantitative assessment of major lipid classes within a yeast cell is versatile, robust, and sensitive.

ABSTRACT:

Lipids are structurally diverse amphipathic molecules that are insoluble in water. Lipids are essential contributors to the organization and function of biological membranes, energy storage and production, cellular signaling, vesicular transport of proteins, organelle biogenesis, and regulated cell death. Because the budding yeast *Saccharomyces cerevisiae* is a unicellular eukaryote amenable to thorough molecular analyses, its use as a model organism helped uncover mechanisms linking lipid metabolism and intracellular transport to complex biological processes within eukaryotic cells. The availability of a versatile analytical method for the robust, sensitive, and accurate quantitative assessment of major classes of lipids within a yeast cell is crucial for getting deep insights into these mechanisms. Here we present a protocol to use liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for the quantitative analysis of major cellular lipids of S. cerevisiae. The LC-MS/MS method described is versatile and robust. It enables the identification and quantification of numerous species (including different isobaric or isomeric forms) within each of the 10 lipid classes. This method is sensitive and allows

identification and quantitation of some lipid species at concentrations as low as 0.2 pmol/µL. The method has been successfully applied to assessing lipidomes of whole yeast cells and their purified organelles. The use of alternative mobile phase additives for electrospray ionization mass spectrometry in this method can increase the efficiency of ionization for some lipid species and can be therefore used to improve their identification and quantitation.

INTRODUCTION:

A body of evidence indicates that lipids, one of the major classes of biomolecules, play essential roles in many vital processes within a eukaryotic cell. These processes include the assembly of lipid bilayers that constitute the plasma membrane and membranes surrounding cellular organelles, transport of small molecules across cell membranes, response to changes in the extracellular environment and intracellular signal transduction, generation and storage of energy, import and export of proteins confined to different organelles, vesicular trafficking of proteins within the endomembrane system and protein secretion, and several modes of regulated cell death[1-10].

The budding yeast *S. cerevisiae*, a unicellular eukaryotic organism, has been successfully used to uncover some of the mechanisms underlying the essential roles of lipids in these vital cellular processes[4-20]. *S. cerevisiae* is a valuable model organism for uncovering these mechanisms because it is amenable to comprehensive biochemical, genetic, cell biological, chemical biological, system biological, and microfluidic dissection analyses[21-25]. Further progress in understanding mechanisms through which lipid metabolism and intracellular transport contribute to these vital cellular processes requires sensitive mass spectrometry technologies for the quantitative characterization of the cellular lipidome, understanding the lipidome molecular complexity, and integrating quantitative lipidomics into a multidisciplinary platform of systems biology[1-3, 26-30].

Current methods for the mass spectrometry-assisted quantitative lipidomics of yeast cells and cells of other eukaryotic organisms are not sufficiently versatile, robust, or sensitive. Moreover, these currently used methods are unable to differentiate various isobaric or isomeric lipid species from each other. Here we describe a versatile, robust, and sensitive method that allows use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for quantitative analysis of major cellular lipids of *S. cerevisiae*.

PROTOCOL:

1. Preparation of sterile media for culturing yeast

1.1. Prepare 90 mL of a complete YP medium that contains 1% (w/v) yeast extract and 2% (w/v) bactopeptone.

1.2. Prepare 90 mL of a synthetic minimal YNB medium containing 0.67% (w/v) yeast nitrogen base without amino acids, 20 mg/L *L*-histidine, 30 mg/L *L*-leucine, 30 mg/L *L*-lysine, and 20 mg/L uracil.

1.3. Divide 90 mL of the complete YP medium equally into two 250 mL Erlenmeyer flasks (i.e., 45 mL each).

1.4. Divide 90 mL of the synthetic minimal YNB medium into two 250 mL Erlenmeyer flasks (i.e., 45 mL each).

1.5. Autoclave the flasks with YP and YNB media at 15 psi/121 °C for 45 min prior to use.

2. Yeast strain

2.1. Use the wild type strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*).

3. Culturing yeast in the complete YP medium with glucose

3.1. Autoclave a 20% (w/v) stock solution of glucose at 15 psi/121 °C for 45 min prior to use.

3.2. Add 5 mL of the sterile 20% (w/v) stock solution of glucose to each of the two Erlenmeyer flasks containing the sterile YP medium for a final concentration of 2% glucose (w/v).

3.3. Use a microbiological loop to inoculate cells of the wild type strain BY4742 into each of the two Erlenmeyer flasks containing the YP medium with glucose.

3.4. Culture the cells overnight at 30 °C with rotational shaking at 200 rpm.

3.5. Take an aliquot of yeast culture. Use a hemocytometer to determine the total number of yeast cells per mL of culture.

4. Transferring yeast to and culturing them in the synthetic minimal YNB medium with glucose

4.1. Add 5 mL of the sterile 20% (w/v) stock solution of glucose to each of the two Erlenmeyer flasks containing the sterile YNB medium to a final concentration of 2% glucose (w/v).

4.2. Use a sterile pipette to transfer a volume of the overnight yeast culture that contains the total number of 5.0×10^7 cells into each of the two Erlenmeyer flasks containing the YNB medium with glucose.

4.3. Culture the cells for at least 24 h (or more, if the experiment requires) at 30 °C with rotational shaking at 200 rpm.

5. Preparation of reagents, labware and equipment for lipid extraction

5.1. Prepare the following: 1) high grade (>99.9%) chloroform; 2) high grade (>99.9%) methanol; 3) 28% (v/v) ammonium hydroxide solution in nano-pure water; 4) glass beads (acid-washed, 425–600 µM); 5) a vortex with appropriate adapter; 6) 15 mL high-speed glass centrifuge tubes

with polytetrafluoroethylene lined caps; 7) 17:1 and 2:1 mixtures of chloroform and methanol; 8) a chloroform/methanol (2:1) mixture with 0.1% ammonium hydroxide (v/v); 9) ABC solution (155 mM ammonium bicarbonate, pH = 8.0); 10) a mixture of internal lipid standards prepared in a 2:1 mixture of chloroform and methanol as indicated in **Table 1**; and 11) 2 mL glass sample vials with polytetrafluoroethylene lined caps for the extraction of cellular lipids.

6. Preparation of reagents, labware, and equipment for LC

6.1. Prepare the following: 1) acetonitrile/2-propanol/nano-pure water (65:35:5) mixture; 2) a vortex with appropriate adapter; 3) an ultrasonic sonicator; 4) glass vials with inserts for a wellplate; 5) an LC system equipped with a binary pump, degasser, and an autosampler; 6) a C18 reverse-phase column (2.1 mm; 75 mm; pore size 130 Å; pH range of 1–11) coupled to a pre-column system; 7) mixture A: acetonitrile/water (60:40); and 8) mixture B: isopropanol/acetonitrile (90:10).

7. Lipid extraction from yeast cells

7.1. Take an aliquot of yeast culture. Use a hemocytometer or measure OD_{600} to determine the total number of yeast cells per mL of culture.

7.2. Take a volume of yeast culture that contains a total number of 5.0×10^7 cells (3.3 units OD_{600}). Place this volume of culture into a prechilled 1.5 mL microcentrifuge tube.

7.3. Harvest the cells by centrifugation at 16,000 x *g* for 1 min at 4 °C. Discard the supernatant.

7.4. Add 1.5 mL of ice-cold nano-pure water and wash the cells by centrifugation at 16,000 x *g* for 1 min at 4 °C. Discard the supernatant.

7.5. Add 1.5 mL of ice-cold ABC solution and wash the cells by centrifugation at 16,000 x *g* for 1 min at 4 °C. Discard the supernatant. The cell pellet can be stored at -80 °C prior to lipid extraction.

7.6. To begin the lipid extraction, thaw the cell pellet on ice.

7.7. Resuspend the cell pellet in 200 µL of ice-cold nano-pure water. Transfer the cell suspension to a 15 mL high-strength glass screw top centrifuge tube with a polytetrafluoroethylene lined cap. Add the following to this tube: 1) 25 µL of the mixture of internal lipid standards prepared in chloroform/methanol (2:1) mixture; 2) 100 µL of 425–600 µM acid-washed glass beads; and 3) 600 µL of chloroform/methanol (17:1) mixture.

7.8. Vortex the tube at high speed for 5 min at room temperature (RT) to disrupt the cells.

7.9. Vortex the tube at low speed for 1 h at RT to facilitate the extraction of lipids.

7.10. Incubate the sample for 15 min on ice to promote protein precipitation and the separation of the aqueous and organic phases from each other.

7.11. Centrifuge the tube in a clinical centrifuge at 3,000 x *g* for 5 min at RT. This centrifugation step allows to separate the upper aqueous phase from the lower organic phase, which contains all lipid classes.

7.12. Use a borosilicate glass pipette to transfer the lower organic phase (~400 µL) to another 15 mL high-strength glass screw top centrifuge tube with a polytetrafluoroethylene lined cap. Do not disrupt the glass beads or upper aqueous phase during such transfer. Keep the lower organic phase under the flow of nitrogen gas.

7.13. Add 300 µL of chloroform-methanol (2:1) mixture to the remaining upper aqueous phase to allow the extraction of sphingolipids and PA, PS, PI, and CL. Vortex the tube vigorously for 5 min at RT.

7.14. Centrifuge the tube in a clinical centrifuge at 3,000 x *g* for 5 min at RT.

7.15. Use a borosilicate glass pipette to transfer the lower organic phase (~ 200 µL) formed after centrifugation to the organic phase collected at step 7.13.

7.16. Use the flow of nitrogen gas to evaporate the solvent in the combined organic phases. Close the tubes containing the lipid film under the flow of nitrogen gas. Store these tubes at -80 °C.

8. Separation of extracted lipids by LC

8.1. Add 500 µL of acetonitrile/2-propanol/nano-pure water (65:35:5) mixture to a tube containing the lipid film obtained at step 7.16. Vortex the tube 3x for 10 s at RT.

8.2. Subject the content of the tube to ultrasonic sonication for 15 min. Vortex the tube 3x for 10 s at RT.

8.3. Take 100 µL of a sample from the tube and add it to a glass vial with an insert used for a wellplate. Eliminate air bubbles in the insert before pacing it into the wellplate.

8.4. Use an LC system to separate different lipid species on a reverse-phase C18 column CSH coupled to a pre-column system (see **Table of Materials**). During the separation, maintain the column at 55 °C and at a flow rate of 0.3 mL/min. Keep the sample in the wellplate at RT.

8.5. Use the mobile phases that consist of mixture A (acetonitrile/water [60:40 (v/v)]) and mixture B (isopropanol/acetonitrile [90:10 (v/v)]). For a positive mode of the detection of parent ions created using the electrospray ionization (ESI) ion source, the ESI (+) mode, the mobile phases A and B contain ammonium formate at the final concentration of 10 mM. For a negative mode of parent ions detection, the ESI (-) mode, the mobile phases A and B contain ammonium

acetate at the final concentration of 10 mM.

8.6. Use a sample volume of 10 µL for the injection into both the ESI (+) and ESI (-) mode.

8.7. Separate different lipid species by LC using the following LC gradient: 0–1 min 10% (phase B); 1–4 min 60% (phase B); 4–10 min 68% (phase B); 10–21 97% (phase B); 21–24 min 97% (phase B); 24–33 min 10% (phase B).

8.8. Run extraction blanks as the first sample, between every four samples, and as the last sample. Subtract the background to normalize data.

8.9. A representative total ion chromatogram from LC/MS data of lipids extracted from cells of the wild type strain BY4742 is shown in **Figure 1**.

9. Mass spectrometric analysis of lipids separated by LC

9.1. Use a mass spectrometer equipped with a HESI (heated electrospray ionization) ion source to analyze lipids that were separated by LC. Use the settings provided in **Table 2**.

9.2. Use the Fourier transform analyzer to detect parent ions (MS1) at a resolution of 60,000 and within the mass range of 150–2,000 Da.

9.3. Use the settings provided in **Table 3** to detect secondary ions (MS2).

10. Identification and quantitation of different lipid classes and species by processing of raw data from LC-MS/MS

10.1. See the **Table of Materials** for software to carry out the identification and quantitation of different lipids from raw LC-MS/MS files. This software uses the largest lipid database, containing more than 1.5 million lipid ion precursors (MS1) and their predicted fragment ions (MS2). The software also uses MS1 peaks for lipid quantitation and MS2 for lipid identification. A representative chromatogram of two isomeric phosphatidylserine forms (34:0) that have the same m/z value but different retention times, as well as their MS1 and MS2 spectra, are shown in **Figure 2**, **Figure 3**, and **Figure 4**, respectively.

10.2. Search LC-MS raw files containing full-scan MS1 data and data-dependent MS2 data for free (unesterified) fatty acids (FFA), cardiolipin (CL), phytoceramide (PHC), phytosphingosine (PHS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and triacylglycerol (TAG) lipid classes using an m/z tolerance of 5 ppm for precursor ions and 10 ppm for product ions. Other search parameters are shown in **Table 4**. Follow the instructions provided in the software user manual. The identities of internal lipid standards and lipid species with unusual fatty acid composition need to be verified manually.

10.3. To identify and quantitate different lipid classes and species with the help of freely available open-source alternatives for the Lipid Search software, use the Lipid Data Analyzer (http://genome.tugraz.at/lda2/lda_download.shtml), MZmine 2 (http://mzmine.github.io/), or XCMS (https://bioconductor.org/packages/release/bioc/html/xcms.html) software to process raw data from LC-MS/MS.

REPRESENTATIVE RESULTS:

Our method for a quantitative assessment of major cellular lipids within a yeast cell with the help of LC-MS/MS was versatile and robust. It allowed us to identify and quantify 10 different lipid classes in *S. cerevisiae* cells cultured in the synthetic minimal YNB medium initially containing 2% glucose. These lipid classes include free (unesterified) fatty acids (FFA), CL, phytoceramide (PHC), phytosphingosine (PHS), PC, PE, PG, PI, PS, and TAG (**Supplemental Table 1**). Numerous molecular species of each of these classes were identified and quantified using this LC-MS/MS method (**Supplemental Table 1**).

Our LC-MS/MS method was also sensitive. Indeed, it enabled the identification and quantitation of molecular species of lipids at concentrations as low as 0.165 pmol/µL (see data for phytoceramide in **Table 5**). This limit of quantitation differs for different lipid classes within a wide range of concentrations (**Table 5**).

Importantly, our method allowed identification and quantification of different isobaric or isomeric forms of lipids. Isobaric forms of lipids are lipid species with the same nominal mass (ie., sum of the masses of the most abundant isotopes) but differing exact masses[31]. Isomeric forms of lipids are lipid species with the same molecular formula but with different chemical structure[31]. For example, the use of our LC-MS/MS method distinguished between PHC (16:0_26:0) and the isobaric lipid species PC (16:0_10:0): although they have the same nominal mass value of 650, their exact masses are 650.6457 and 650.4755, respectively. Moreover, this LC-MS/MS method distinguished between two pairs of isomeric lipid species with the same molecular formula but different chemical structure: 1) PC (18:0_18:1) and PC (20:0_16:1), with molecular formula (C44H87N1O8P1) and exact mass (788.6163); and 2) PE (16:0_16:0) and PE (14:0_18:0), with the molecular formula (C37H75N1O8P1) and exact mass (692.5224).

Our LC-MS/MS method can be used to increase the efficiency of ionization for lipids of all classes, thus improving the identification and quantitation of major cellular lipids. Such improvement can be achieved by using alternative mobile phase additives for the ESI MS (**Table 6**). These alternative phases include ammonium formate, ammonium formate with formic acid, ammonium acetate, ammonium acetate with acetic acid, and ammonium acetate with formic acid. Each of these alternative mobile phase additives can be used for both the normal-phase and reverse-phase LC columns (**Table 6**).

Another advantage of our LC-MS/MS method consists in the ability to use two different methods for the fragmentation of precursor ions (MS1) of lipids into MS2 products. These two methods are high-energy collisional dissociation (HCD) and collision-induced dissociation (CID)[32]. We found that the CID method is beneficial if used in combination with the ammonium acetate

mobile phase additive for the ESI (-) mode of MS, as under these conditions it allows an increase in the efficiency of MS1 lipid ion fragmentation into MS2 products for PHC, CL, FFA, PE, PG, PI, and PS (**Table 7**). In contrast, the HCD method is favorable if used in combination with the ammonium formate mobile phase additive for the ESI (+) mode of MS, as under these conditions it enables an increase in the efficiency of MS1 lipid ion fragmentation into MS2 products for PC, PHS and TAG (**Table 8**).

FIGURE AND TABLE LEGENDS:

Figure 1: The total ion chromatogram (TIC) from liquid chromatography/mass spectrometry (LC/MS) data of lipids that were extracted from cells of the wild type strain BY4742. The TIC of lipids separated by LC on a reverse-phase column CSH C18 and detected by MS of parent ions that were created using the negative electrospray ionization mode.

Figure 2: A chromatogram of two isomeric phosphatidylserine forms (34:0) that have the same m/z value (M-H) of 762.5294 but different retention times of 7.65 min and 8.49 min. The lipids were extracted from cells of the wild type strain BY4742 and separated by liquid chromatography on a reverse-phase column CSH C18.

Figure 3: MS1 spectra of two isomeric phosphatidylserine species (34:0) that have the same m/z value (M-H) of 762.5294 but different retention times of 7.65 min and 8.49 min. The lipids were extracted from cells of the wild type strain BY4742, separated by liquid chromatography on a reverse-phase column CSH C18 (as shown in **Figure 2**) and detected by mass spectrometry of parent (MS1) ions that were created using the negative electrospray ionization mode. (**A**) The MS1 spectrum of a phosphatidylserine form (34:0) with the m/z value (M-H) of 762.5294 and the retention time of 7.65 min. (**B**) The MS1 spectrum of a phosphatidylserine form (34:0) with the m/z value (M-H) of 762.5294 and the retention time of 8.49 min.

Figure 4: MS2 spectra of two isomeric phosphatidylserine species (34:0) that have the same m/z value (M-H) of 762.5294 but different retention times of 7.65 min and 8.49 min. The lipids were extracted from cells of the wild type strain BY4742, separated by liquid chromatography on a reverse-phase column CSH C18 (as shown in **Figure 2**) and detected by mass spectrometry of MS1 ions (as shown in **Figure 3**). Secondary ions (MS2) were then detected by mass spectrometry. (**A**) The MS2 spectrum of a phosphatidylserine form (34:0) with the m/z value (M-H) of 762.5294 and the retention time of 7.65 min. The loss of a serine moiety produced an ion with the m/z value (M-H) of 675.6149. (**B**) The MS2 spectrum of a phosphatidylserine form (34:0) with the m/z value (M-H) of 762.5294 and the retention time of 8.49 min. The loss of a serine moiety produced an ion with the m/z value (M-H) of 675.8843.

Table 1: The composition of a mixture of internal lipid standards. Internal lipid standards were prepared in chloroform/methanol (2:1) mixture. Detection mode refers to a positive or negative mode of parent ions detection using an Orbitrap Velos Mass Spectrometer equipped with electrospray ionization (ESI) ion source. The calculated m/z values are for the adducts of lipids.

Table 2: The Orbitrap Velos Mass Spectrometer's settings used to analyze lipids that were

separated by LC. Abbreviations: FTMS + p = Fourier transform-based mass spectrometry in the ESI (+) mode; HESI = heated electrospray ionization.

Table 3: The Orbitrap Velos Mass Spectrometer's settings used to detect secondary ions (MS2). Abbreviation: FTMS + C = Fourier transform-based mass spectrometry in the ESI (+) mode.

Table 4: The search parameters used to identify different lipid classes and species with the help of the Lipid Identification software "Lipid Search" (V 4.1). Abbreviations: CER = ceramide; CL = cardiolipin; FFA = free (unesterified) fatty acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PI = phosphatidylinositol; PS = phosphatidylserine; TAG = triacylglycerol.

Table 5: The lowest concentrations of molecular species of different lipid classes that can be identified and quantitated with the help of our LC-MS/MS method. An estimate of the lowest quantifiable concentration for each lipid class is based on the MS peak areas for its internal standard (these MS peak areas and lipid standard concentrations are displayed in bold) and its representative molecular form present at the lowest detectable concentration. Data are presented as mean values of two independent experiments, for each of which three technical replicates were performed.

Table 6: The effects of alternative mobile phase additives for the ESI MS on the efficiencies of ionization for lipids of different classes. Different lipids were separated from each other by reverse-phase liquid chromatography. Commercial lipid standards that belong to different classes of lipids are named in **Table 1**. The ESI (-) or ESI (+) mode of ionization was used for MS in the presence of different mobile phase additives. The percentage of ionization for each lipid standard is shown as a mean value from three technical replicates. For each lipid, the ionization percentage was calculated based on the MS peak area. A value of the highest ionization efficiency for each lipid is displayed in bold. Abbreviations: AmF = ammonium formate; AmF/FA = ammonium formate with formic acid; AmAc = ammonium acetate; AmAc/AA = ammonium acetate with acetic acid; AmAc/FA = ammonium acetate with formic acid; CL = cardiolipin; FFA = free (unesterified) fatty acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PHC = phytoceramide; PHS = phytosphingosine; PI = phosphatidylinositol; PS = phosphatidylserine; TAG = triacylglycerol.

Table 7: The effect of the collision-induced dissociation (CID) method on the efficiency of precursor ions (MS1) fragmentation. Commercial lipid standards that belong to different classes of lipids are named in **Table 1**. The ESI (-) or ESI (+) mode of ionization was used for MS, in the presence of an ammonium formate (AmF) or ammonium acetate (AmAc) mobile phase additive. The percentage of MS1 lipid ions that were fragmented into MS2 products is shown as a mean value from three technical replicates. For each lipid, the ionization percentage was calculated based on the MS2 peak area. A value of the highest percentage of MS1 lipid ions that were fragmented is displayed in bold for each lipid (compare with the data presented in **Table 8**). This value is the highest if the efficiency of MS2 product ion formation is the highest. Abbreviations: CL = cardiolipin; FFA = free (unesterified) fatty acid; PC = phosphatidylcholine; PE =

phosphatidylethanolamine; PG = phosphatidylglycerol; PHC = phytoceramide; PHS = phytosphingosine; PI = phosphatidylinositol; PS = phosphatidylserine; TAG = triacylglycerol.

Table 8: The effect of the high-energy collisional dissociation (HCD) method on the efficiency of precursor ions (MS1) fragmentation. Commercial lipid standards that belong to different classes of lipids are named in **Table 1**. The ESI (-) or ESI (+) mode of ionization was used for MS, in the presence of an ammonium formate (AmF) or ammonium acetate (AmAc) mobile phase additive. The percentage of MS1 lipid ions that were fragmented into MS2 products is shown as a mean value from three technical replicates. For each lipid, the ionization percentage was calculated based on the MS2 peak area. A value of the highest percentage of MS1 lipid ions that were fragmented is displayed in bold for each lipid (compare with the data presented in **Table 7**). This value is the highest if the efficiency of MS2 product ion formation is the highest. Other abbreviations: CL = cardiolipin; FFA = free (unesterified) fatty acid; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PHC = phytoceramide; PHS = phytosphingosine; PI = phosphatidylinositol; PS = phosphatidylserine; TAG = triacylglycerol.

Supplemental Table 1: A list of molecular species of 10 different lipid classes identified and quantified in *S. cerevisiae* cells with the help of our LC-MS/MS method. These lipid classes included free (unesterified) fatty acids (FFA), cardiolipin (CL), phytoceramide (PHC), phytosphingosine (PHS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and triacylglycerol (TAG). *S. cerevisiae* cells were cultured in the synthetic minimal YNB medium initially containing 2% glucose. Aliquots of yeast cells for lipid extraction and LC-MS/MS analysis of extracted lipids were recovered on days 1, 2, 3, 4, 6, 8, and 10 of culturing. All lipid species of each lipid class that were identified in yeast cells recovered on different days of culturing are shown. Some of these lipid species were present only in chronologically young yeast cells recovered on days 1–4 of culturing, others only in chronologically old yeast cells recovered on days 6–10 of culturing, whereas some were present in both chronologically young and old yeast cells. The highest MS peak area for each lipid species of each lipid class identified on a certain day of culturing is shown. Lipid standards of different lipid classes that were used for quantitation of other species within this lipid class are displayed in red color. The calculated m/z values are for the adducts of lipids. Abbreviation: ESI (-) = the ESI (-) mode of MS; ESI (+) = the ESI (+) mode of MS. Data are presented as mean values of two independent experiments, for each of which three technical replicates were performed.

DISCUSSION:

The following precautions are important for the successful implementation of the protocol described here:

1. Chloroform and methanol are toxic. They efficiently extract various substances from surfaces, including laboratory plasticware and your skin. Therefore, handle these organic solvents with caution by avoiding the use of plastics in steps that involve contact with chloroform and/or methanol, using borosilicate glass pipettes for these steps, and rinsing these pipettes with chloroform and methanol before use.

2. During lipid extraction by methanol/chloroform (17:1) mixture, use a borosilicate glass pipette to transfer the lower organic phase (~400 µL) to a 15 mL high-strength glass screw top centrifuge tube with a polytetrafluoroethylene lined cap. Do not disrupt the glass beads or upper aqueous phase during such transfer. Keep the lower organic phase under the flow of nitrogen gas.

3. During sample preparation for LC-MS/MS, it is important to eliminate all air bubbles in the glass vials before inserting the vials into a wellplate.

4. To achieve a complete solubilization of ammonium formate and ammonium acetate in mobile phases A and B prior to lipid separation by LC, dissolve each salt in 500 µL of nano-pure water before mixing the solution with the mobile phase and sonicating for 20 min.

5. Do not store the lipid film formed after solvent evaporation for a long period of time prior to running. We store this film at -80 °C for no more than a week before dissolving it in the acetonitrile/2-propanol/nano-pure water (65:35:5) mixture and then subjecting the lipids to LC-MS/MS.

The LC-MS/MS method described here is a versatile, robust, and sensitive technique for a quantitative assessment of many lipid species comprising the cellular lipidome of yeast or any other eukaryotic organism. The method enables the identification and quantification of different isobaric or isomeric lipid species, allows to use alternative mobile phase additives for the ESI MS to promote lipid ionization and to make lipid identification and quantification more efficient, and can use both HCD and CID methods for the fragmentation or activation of MS1 lipid ions.

We use this LC-MS/MS method to study age-related changes in the cellular and organellar lipidomes during chronological aging of the budding yeast *S. cerevisiae*. We also employ this method to investigate how many aging-delaying genetic, dietary, and pharmacological interventions influence lipid composition of the entire *S. cerevisiae* cell and its various organelles. Because of its versatility, robustness, and sensitivity, this LC-MS/MS method can be successfully used for the quantitative assessment of the cellular and organellar lipidomes in eukaryotic organisms across phyla.

ACKNOWLEDGMENTS:
We are grateful to current and former members of the Titorenko laboratory for discussions. We acknowledge the Centre for Biological Applications of Mass Spectrometry and the Centre for Structural and Functional Genomics (both at Concordia University) for outstanding services. This study was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada (RGPIN 2014-04482) and Concordia University Chair Fund (CC0113). K.M. was supported by the Concordia University Merit Award.

DISCLOSURES:
The authors declare that they have no competing financial interests.

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Figure 1

Click here to access/download;Figure;Figure 1_NEW.psd

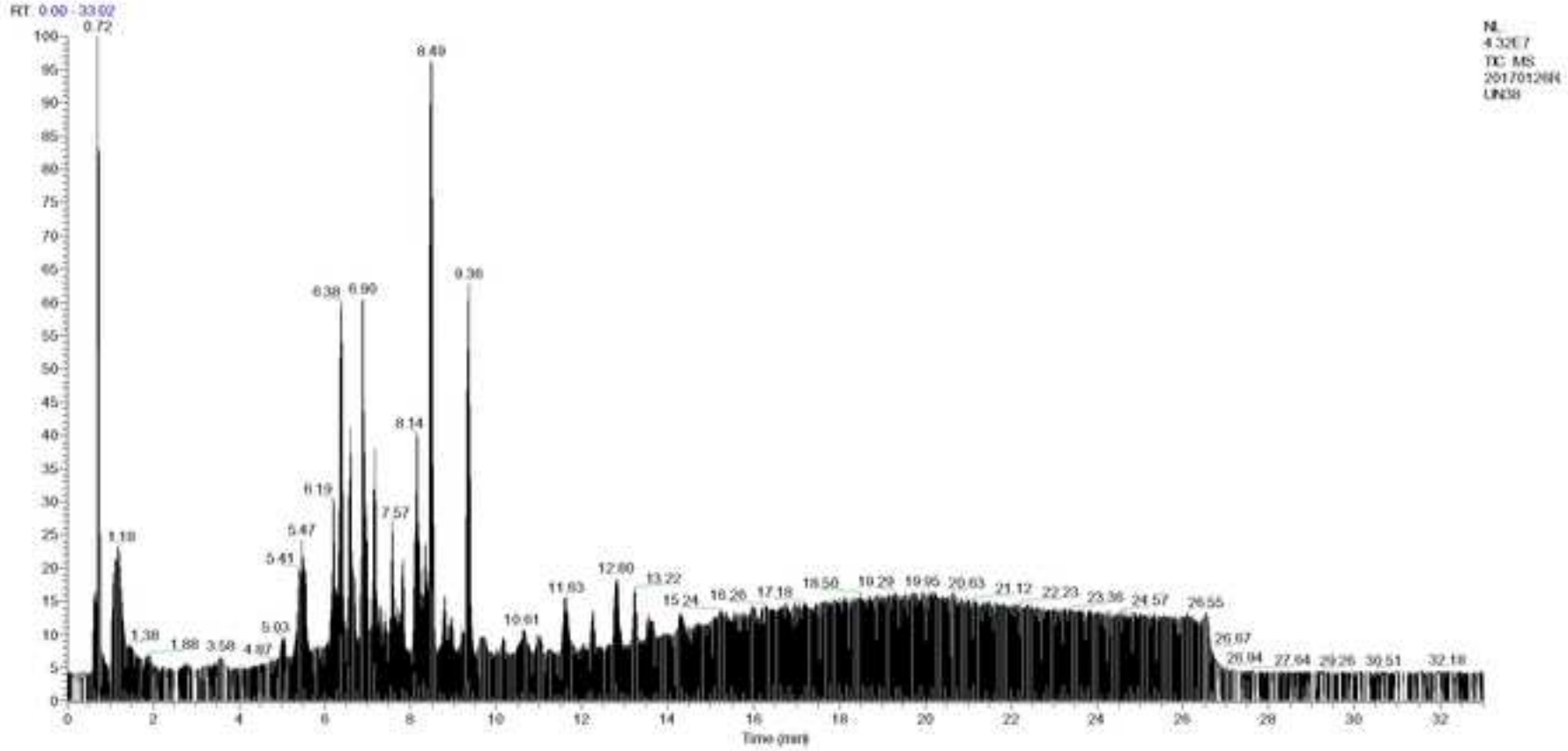

Figure 2

Click here to access/download;Figure;Figure 2_NEW.psd

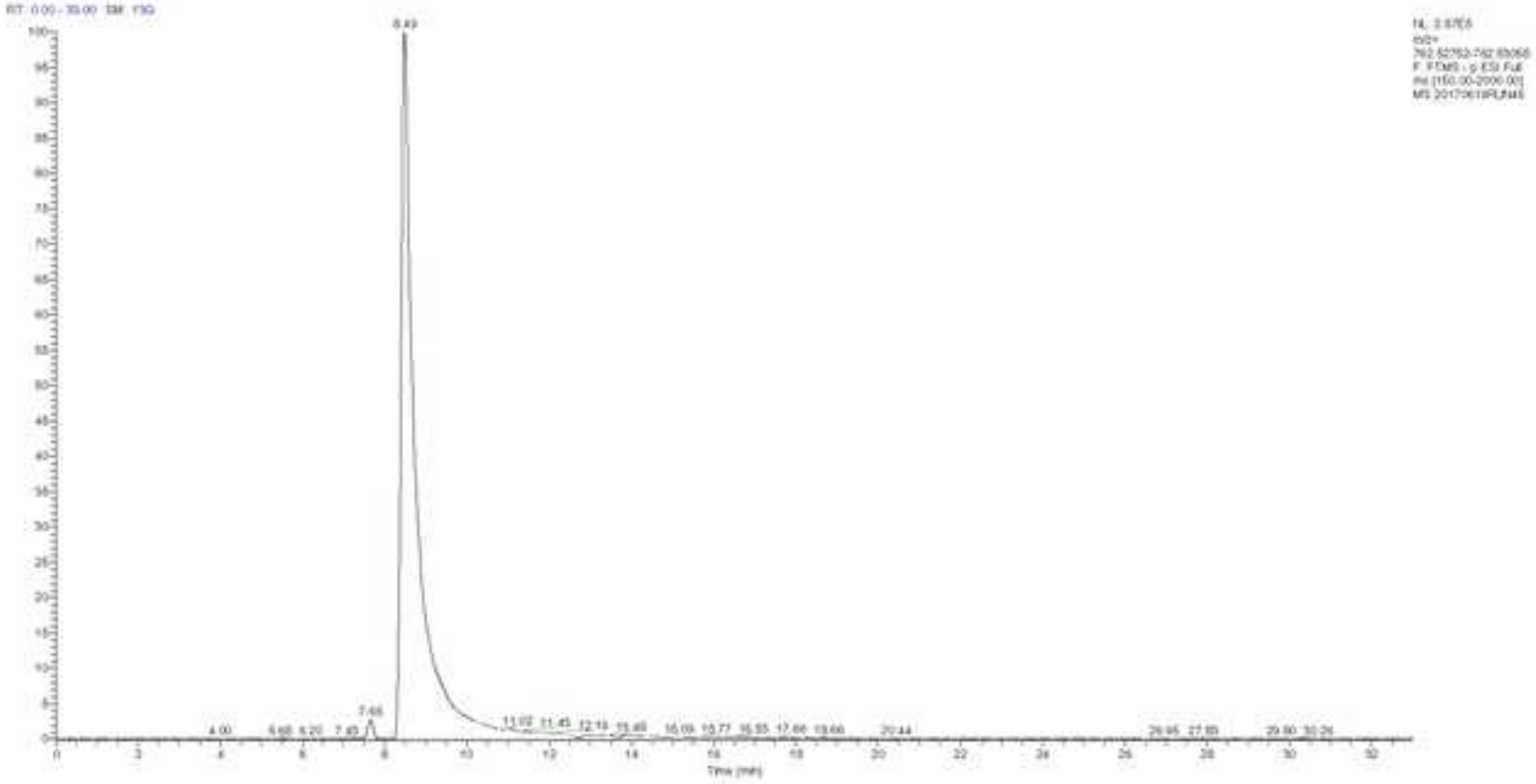

Figure 3

Click here to access/download;Figure;Figure 3_NEW.psd

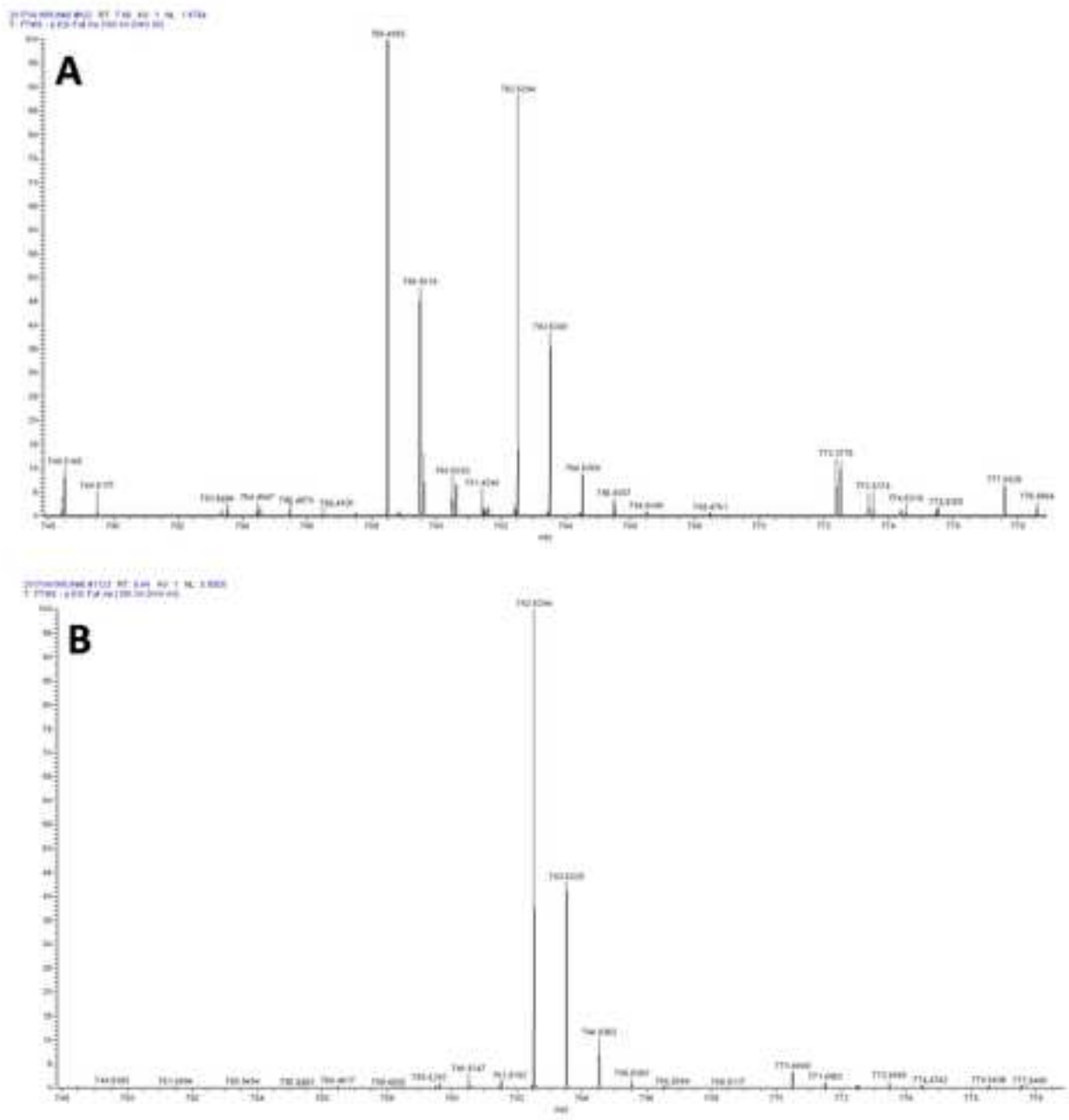

Figure 4

Click here to access/download;Figure;Figure 4_NEW.psd

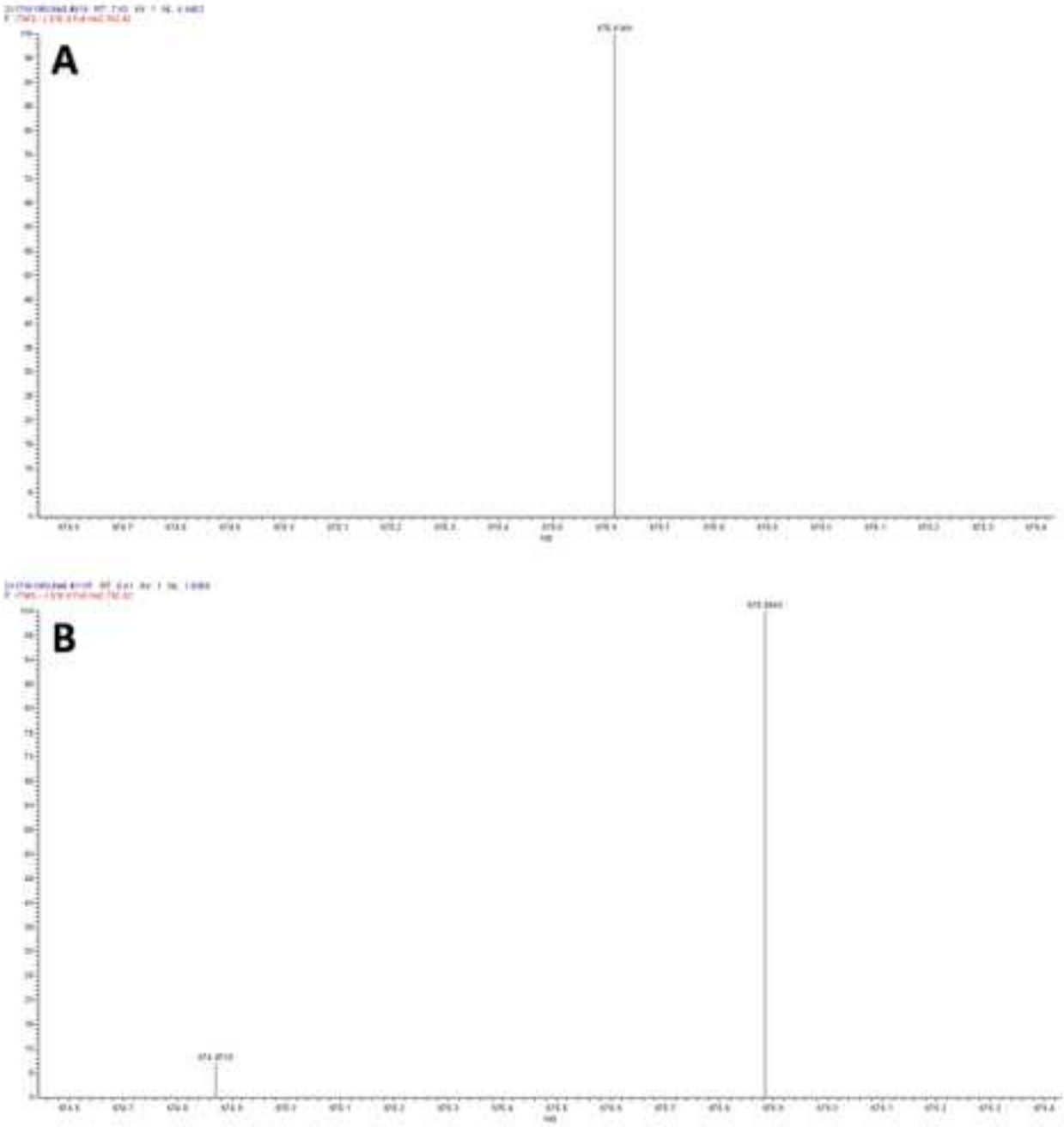

Table 1

Detection mode	Lipid class	Hydrophobic tail composition
Negative	Ceramide	18:1_17:0
Negative	Cardiolipin	14:0_14:0_14:0_14:0
Negative	Free fatty acid	19:0
Negative	Phosphatidylethanolamine	15:0_15:0
Negative	Phosphatidylglycerol	15:0_15:0
Negative	Phosphatidylinositol	17:0_20:4
Negative	Phosphatidylserine	17:0_17:0
Positive	Phosphatidylcholine	13:0_13:0
Positive	Phytosphingosine	16:1
Positive	Triacylglycerol	28:1_10:1_10:1

Calculated m/z value	Concentration (pmoles/µl)
550.5204675	226
1239.839755	196
297.2799035	837
662.4766305	377
693.4712115	349
871.5342065	3
762.5290605	318
650.4755335	385
272.2584055	225
818.7232155	367

Table 2

FTMS + p resolution	60000
Mass range (dalton)	150-2000
Ion source type	HESI
Capillary temperature (°C)	300
Source heater temperature (°C)	300
Sheath gas flow	10
Aux gas flow	5
Positive polarity voltage (kV)	3
Negative polarity voltage (kV)	3
Source current (µA)	100

Table 3

Instrument polarity	Positive
Activation type	High-energy-induced-collision-dissoc
Minimal signal required	5000
Isolation width	2
Normalized collision energy	55
Default charge state	2
Activation time	0.1
FTMS + C resolution	
5 most intense peaks were selected for ms/ms	

	Negative
ciation	Collision-induced-dissociation
	5000
	2
	35
	2
	10
7500	

Table 4

Identification	
Database	
Peak detection	
Search option	
Search type	
Experiment type	
Precursor tolerance	
	Hig
Product tolerance	
Quantitation	
Execute quantitation	
m/z tolerance	
Tolerance type	
Filter	
Top rank filter	
Main node filter	
m-score threshold	
c-score threshold	
FFA priority	
ID quality filter	
Lipid Class	
High-energy-induced-collision-dissociation [ESI (+) mode]	
Collision-induced-dissociation [ESI (-) mode]	
Ions	
High-energy-induced-collision-dissociation [ESI (+) mode]	
Collision-induced-dissociation [ESI (-) mode]	

Orbitrap
Recall isotope (ON)
Product search Orbitrap
Product
LC-MS
10 ppm
h-energy-induced-collision-dissociation [ESI (+) mode]: 20 ppm
Collision-induced-dissociation [ESI (-) mode]: 0.5 Daltons

ON
-5.0; +5.0
ppm

ON
Main isomer peak
5
2
ON
A: Lipid class & all fatty acids are completely identified
B: Lipid class & some fatty acids are identified
C: Lipid class or FA are identified
D: Lipid identified by other fragment ions (H2O, etc.)

PC, TAG
CER, CL, FFA, PE, PG, PI, PS

+ H; + NH4; + Na
- H; - 2H; - HCOO

Table 5

Detection mode	Lipid class	Hydrophobic tail composition
Negative	Ceramide	18:1_17:0
Negative	Cardiolipin	14:0_14:0_14:0_14:0
Negative	Free fatty acid	19:0
Negative	Phosphatidylethanolamine	15:0_15:0
Negative	Phosphatidylglycerol	15:0_15:0
Negative	Phosphatidylinositol	17:0_20:4
Negative	Phosphatidylserine	17:0_17:0
Positive	Phosphatidylcholine	13:0_13:0
Positive	Phytosphingosine	16:1
Positive	Triacylglycerol	28:1_10:1_10:1

Calculated m/z value	pmol/µL	Lipid std MS peak	Lipid std conc (pmol/µL)
550.5204675	226	**3.36E+07**	**2.26E+02**
1239.839755	196	**2.18E+07**	**1.96E+02**
297.2799035	837	**6.71E+07**	**8.37E+02**
662.4766305	377	**3.30E+07**	**3.77E+02**
693.4712115	349	**6.89E+07**	**3.49E+02**
871.5342065	3	**2.94E+05**	**3.00E+00**
762.5290605	318	**1.98E+06**	**3.18E+02**
650.4755335	385	**2.13E+08**	**3.85E+02**
272.2584055	225	**1.26E+06**	**2.25E+02**
818.7232155	367	**1.27E+08**	**3.67E+02**

Minimum MS peak detected	Lowest concentration detected (pmol/µL)
2.46E+04	1.65E-01
4.16E+04	3.74E-01
6.64E+04	8.28E-01
6.04E+04	6.90E-01
2.24E+05	1.13E+00
4.68E+04	4.78E-01
2.94E+04	4.72E+00
1.09E+05	1.97E-01
3.11E+05	5.55E+01
1.07E+07	3.09E+01

Table 6

Lipid standard	ESI mode	AmF	AmF/FA	AmAc	AmAc/AA	AmAc/FA
PHC	-	74	75.2	**85.8**	77	74.8
CL	-	72.9	75.1	**78.3**	68.4	74
FFA	-	77.1	77.2	75.5	**84**	72.9
PE	-	**98**	96	95	91.5	86
PG	-	64	45.1	**75.9**	60.5	55
PI	-	79.3	76	**82.5**	80.3	77
PS	-	73.7	61.6	**85.8**	81.4	73.7
PC	+	**93.5**	86.9	69.3	60.5	62.7
PHS	+	**89.1**	79.2	78.1	73.7	69.3
TAG	+	**92.4**	88	86.9	80.3	84.7

Table 7

Lipid standard	ESI mode	AmF	AmAc
PHC	-	75	**83.8**
CL	-	70.9	**79.3**
FFA	-	76.1	**74.5**
PE	-	98	**95**
PG	-	64	**75.9**
PI	-	79.3	**82.5**
PS	-	71.5	**84.8**
PC	+	52.3	60.5
PHS	+	78.4	75.2
TAG	+	65.7	69.7

Table 8

Lipid standard	ESI mode	AmF	AmAc
PHC	-	68.4	65.4
CL	-	74.3	75.2
FFA	-	84.2	81.2
PE	-	85.1	73.1
PG	-	68.4	67.1
PI	-	58.7	55.8
PS	-	67.4	68.5
PC	+	**92.5**	65.3
PHS	+	**87.1**	75.1
TAG	+	**91.4**	84.9

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
15 mL High-speed glass centrifuge tubes with Teflon lined caps	PYREX	05-550	
2 mL Glass sample vials with Teflon lined caps	Fisher Scientific	60180A-SV9-1P	
2-Propanol	Fisher Scientific	A461-500	
Acetonitrile	Fisher Scientific	A9554	
Agilent 1100 series LC system	Agilent Technologies	G1312A	
Agilent1100 Wellplate	Agilent Technologies	G1367A	
Ammonium acetate	Fisher Scientific	A11450	
Ammonium bicarbonate	Sigma	9830	
Ammonium formate	Fisher Scientific	A11550	
Ammonium hydroxide	Fisher Scientific	A470-250	
Bactopeptone	Fisher Scientific	BP1420-2	
Cardiolipin	Avanti Polar Lipids	750332	
Centra CL2 clinical centrifuge	Thermo Scientific	004260F	
Ceramide	Avanti Polar Lipids	860517	
Chloroform	Fisher Scientific	C297-4	
CSH C18 VanGuard	Waters	186006944	Pre-column system
Free fatty acid (19:0)	Matreya	1028	
Glass beads (acid-washed, 425-600 µM)	Sigma-Aldrich	G8772	
Glucose	Fisher Scientific	D16-10	
Hemacytometer	Fisher Scientific	267110	
L -histidine	Sigma	H8125	
Lipid Search software (V4.1)	Fisher Scientific	V4.1	LC-MS/MS analysis softwar
L -leucine	Sigma	L8912	
L -lysine	Sigma	L5501	
Methanol	Fisher Scientific	A4564	
Phosphatidylcholine	Avanti Polar Lipids	850340	
Phosphatidylethanolamine	Avanti Polar Lipids	850704	
Phosphatidylglycerol	Avanti Polar Lipids	840446	
Phosphatidylinositol	Avanti Polar Lipids	LM1502	
Phosphatidylserine	Avanti Polar Lipids	840028	

Reverse-phase column CSH C18	Waters	186006102
Sphingosine	Avanti Polar Lipids	860669
Thermo Orbitrap Velos MS	Fisher Scientific	ETD-10600
Tricylglycerol	Larodan, Malmo	TAG Mixed FA
Ultrasonic sonicator	Fisher Scientific	15337416
Uracil	Sigma	U0750
Vortex	Fisher Scientific	2215365
Yeast extract	Fisher Scientific	BP1422-2
Yeast nitrogen base without amino acids	Fisher Scientific	DF0919-15-3
Yeast strain BY4742	Dharmacon	YSC1049

e

December 6, 2019

Phillip Steindel, Ph.D.
Review Editor
JoVE

Dear Dr. Steindel:

Please find attached a revised version of our manuscript, entitled "Quantitative Analysis of the Cellular Lipidome of the Yeast Saccharomyces Cerevisiae Using Liquid Chromatography Coupled with Tandem Mass Spectrometry" (manuscript reference number JoVE60616R1), which we would like to be considered for publication in the *JoVE*.

We have addressed each of the editorial and peer review comments and revised the manuscript accordingly. Provided below is a detailed description of the revisions we have made.

Editorial comments:

General:

1. Table 5 will either be too tall or too wide to fit on a page; would it be possible to include it as supplemental material instead?
Our response:
The data presented in table 5 of the original manuscript can be found in supplemental table 1 of the revised manuscript.

Reviewer #1:

Major Concerns:
Only 10 lipid classes are detected which are not representing the whole cellular yeast lipidome as claimed in the summary. Please rephrase.
Our response:
We rephrased this statement in the summary, abstract, introduction, representative results and discussion sections of the revised manuscript.

Line 46 and 287:
How was the sensitivity of the method tested?
Is 0.2 pmol/ul the lower limit of quantification or the lower limit of detection? Is this concentration limit the same for every lipid class investigated?
Please add corresponding data supporting this statement.
Our response:
The sensitivity of the method was assessed, as shown in table 5 and as described in the legend to this table. Specifically, an estimate of the lowest quantifiable concentration for each lipid class was based on the MS peak areas for its internal standard (these MS peak areas and lipid standard concentrations are displayed in red in Table 5) and its representative molecular form present at the lowest detectable concentration. 0.165 pmol/µl the lower limit of quantification for phytoceramide,

as shown in table 5. This limit of quantitation differs for different lipid classes within a wide range of concentrations (see table 5).

Line 273:
LipidXplorer works mainly with direct infusion data and LipidView is not freely available. Rather extend this list with Lipid Data Analyzer, mzMine, XCMS, etc.
Our response:
The revised manuscript provides the names and the URLs of these freely available open-source alternatives for the commercially available Lipid Search software from Thermo Fisher.

Line 293-294: To separate both isobaric species the resolution is more than efficient and they would also give specific fragments. Please rephrase and concentrate on separation of isomeric species.
Our response:
Indeed, the resolution of an Orbitrap mass spectrometer is more than sufficient to separate the isobaric lipid species PHC (16:0/26:0) and PC (16:0/10:0) without their prior separation by liquid chromatography. However, the liquid chromatography step is an indispensable part of our LC-MS/MS method for the identification and quantitation of many lipid species (some of which are isobaric, whereas most of which are not isobaric). Thus, our LC-MS/MS method is not only more efficient in identifying and quantifying non-isobaric lipid species than MS alone but can also distinguish between isobaric lipid species (akin to MS alone). Moreover, the use of a liquid chromatography step enhances the ionization efficiencies of these two isobaric lipid species, thus improving their detection. Taking into consideration these essential factors, we believe that it is important to emphasize in the text that our LC-MS/MS method can be used for the identification and quantitation of different isobaric forms of lipids.

Figure 1: just show the chromatogram on MS1 level. Choose other lipid species (separation of isomeric species: chromatogram and MS1/2 spectrum). FFA and PS seem rather random and don't show the quality of the method.
Our response:
Only the total MS1 ion chromatogram from liquid chromatography/mass spectrometry data is shown in figure 1 of the revised manuscript. A representative chromatogram of two isomeric phosphatidylserine forms (34:0) that have the same m/z value but different retention times, as well as their MS1 and MS2 spectra, are shown in figures 2, 3 and 4 (respectively) of the revised manuscript.

Reviewer #3:

Major Concern:
The outline of the paper is a bit confusing. On the one hand, the authors want to present a quantitative method for yeast lipidome analysis. They show the peak areas for some lipid species, but they do not provide real quantitative data (except some lower limits of quantitation for selected lipid species). On the other hand, they investigate the effect of mobile phase additives on the ionization efficiency for various lipid classes. The authors should reorganize the paper, remove the mobile phase additives part and focus on quantitative lipid data, such as for example the amount of TAG (total TAG and/or single TAG species) in nmol/1 million cells.

Our response:
Our manuscript describes a protocol for using a versatile, robust and sensitive LC-MS/MS method to identify and quantify major cellular lipids within a yeast cell. We show that this method can identify many molecular species of 10 different lipid classes in yeast cultured in a minimal synthetic medium with 2% glucose (Supplemental Table 1). For each of these lipid classes, we provide the lowest concentration that can be quantitated with the help of our LC-MS/MS method (Table 5).

To quantitate individual lipid classes and species in nmol/1 million cells (as the Reviewer suggested), at least three (or more) internal lipid standards per class would be required; these lipid standards would need to differ from each other in the length of fatty acids and in the extent of their saturation. However, we regularly use this LC-MS/MS method to quantitatively compare the cellular and organellar lipidomes of yeast treated with various aging-delaying chemicals/diets to those of yeast remained untreated (or the cellular and organellar lipidomes of yeast carrying aging-delaying mutations to those of wild-type yeast); please see https://www.ncbi.nlm.nih.gov/pubmed/31645900 as an example. In these comparative studies, we follow the convention (see below) of expressing the concentration of a lipid class in mol% of all lipid classes but not in nmol/1 million cells. One internal lipid standard per class is enough to have a proper normalization for this type of quantitative comparison experiments, which are often conducted by many researchers studying effects of various dietary, pharmacological or genetic interventions on diverse biological processes.

It needs to be emphasized that both papers on the global analysis of the yeast lipidome by quantitative shotgun mass spectrometry mentioned by the Reviewer (i.e. https://www.pnas.org/content/106/7/2136 and https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0035063) follow the convention of expressing the concentration of a lipid class in mol% of all lipid classes but not in nmol/1 million cells.

We believe that it is important to keep in our manuscript a description of how alternative mobile phase additives can be used to increase the efficiency with which each class of lipids can be ionized. This is because such a description provides evidence of a significant advantage provided by our LC-MS/MS method. Indeed, lipid classes whose chemical properties facilitate their conversion into anions during ESI undergo more efficient ionization when ammonium acetate is added to a mobile phase for the ESI MS (Table 6). Moreover, lipid classes whose chemical properties facilitate their conversion into cations during ESI undergo more efficient ionization when ammonium formate is added to a mobile phase for the ESI MS (Table 6). Please note that, because we have optimized alternative mobile phase additives for the ESI MS (Table 6), a single sample does not need to be run several times with different additives. In contrast, a sample needs to be run twice. The first run is performed with a mobile phase additive that most efficiently increases the extent of lipid conversion into anions, whereas the second run is carried out using a mobile phase additive that is most proficient in increasing the extent of lipid conversion into cations.

Major Concern:
The authors claim several times (title, line 77, line 279) to present a method for quantitative assessment of the entire yeast lipidome, which is - politely spoken - very optimistic. They reduce their ambitions already in line 280, when they admit that they could find 10 different lipid classes.

Ejsing et al (ref. 26) describe 19 different lipid classes in yeast and Klose et al. [PLoS ONE, 7(4), e35063. http://doi.org/10.1371/journal.pone.0035063] describe 18 different lipid classes in yeast in 2012. Although some lipid classes might be quite low abundant and maybe not present under the described growth conditions, some of the classes like ergosterol esters, free ergosterol and diacylglycerol can easily be detected even with thin layer chromatography. The authors should adopt their claims to the data presented and also discuss, why they did not analyze some of the quite prominent yeast lipid classes. They should also compare their findings with the lipid species found in the mentioned literature.

Our response:

We rephrased the statement about the entire yeast lipidome in the summary, abstract, introduction, representative results and discussion sections of the revised manuscript.

We employ this LC-MS/MS method to reproductively identify and quantitate 10 lipid classes when we use a single extraction method and a single pair of mobile phases to separate lipids by liquid chromatography on a reverse-phase column. To identify and quantitate lysoglycerophospholipids, diacylglycerols, inositolphosphorylceramides, free ergosterol and ergosterol esters, cellular lipids would need to be extracted using a different method and extracted lipids would need to be separated by liquid chromatography performed under different conditions.

Again, our LC-MS/MS method can be successfully used for the quantitative comparison experiments aimed at understanding how various dietary, pharmacological or genetic interventions influence the abundance of 10 lipid classes, thereby affecting diverse biological processes. If lysoglycerophospholipids, diacylglycerols, inositolphosphorylceramides, free ergosterol and ergosteryl esters need to be identified and quantitated, different methods of lipid extraction and separation by liquid chromatography need to be used.

It needs to be emphasized that both papers on the global analysis of the yeast lipidome by quantitative mass spectrometry mentioned by the Reviewer (i.e. https://www.pnas.org/content/106/7/2136 and https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0035063) employ a "shotgun" ESI/MS method of lipidomics directly from crude extracts of biological samples without prior chromatographic separation. The use of this method causes a gradual decline in the sensitivity of the mass analyzer. The technical service of restoring the sensitivity of the mass analyzer is expensive and may not be affordable for every laboratory or mass spectrometry center. In contrast, our LC-MS/MS method does not affect the sensitivity of the mass analyzer and, thus, is a valuable alternative to shotgun lipidomics.

Minor Concern:

Line 178 - 180: What is the difference between these the steps 7.9 and 7.10 beside the assumption of the authors, that the cells break within the first 5 minutes of vortexing and the lipids need another 60 minutes to be extracted under the same conditions? Why not just: Vortex vigorously at room temperature for 65 minutes???

Our response:

In the revised manuscript, these steps are described as follows: "7.9. Vortex the tube at high speed for 5 min at room temperature to disrupt the cells. 7.10. Vortex the tube at low speed for 1 h at room temperature to facilitate the extraction of lipids."

Minor Concern:
Regarding the lipid date, the authors should stick to the common lipid nomenclature, as for example published by Liebisch (Liebisch et al, J Lipid Res. 2013 Jun; 54(6): 1523-1530. doi: 10.1194/jlr.M033506). According to the nomenclature, the authors should only note the fatty acids in the lipids separated by "/", when the position of the fatty acid is known. When just the type of fatty acid is defined, but not the position, the fatty acids are separated by an "_"; e.g. TAG 18:0_18:1_20:0. It is very important to stick to one common lipid nomenclature in order to guarantee precise discussions in the field of lipidomic.
Our response:
We made the requested changes in the text and tables of the revised manuscript.

Minor Concern:
The data analysis (line 266 - 276) is a crucial step for qualitative and quantitative lipidome analysis. It seems to be quite risky to just mention the user manual of a software (line 271). The authors should point out whether they just trusted in the software parameters or if they manually verified some of the result outputs. I have the impression that some of the lipid species with quite unusual fatty acid compositions (as for example TAG (30:0/10:1/10:1), TAG (18:1/10:1/24:1)) are just "calculated" lipids with the identical formula and therefore the identical mass as some more "usual" or "expected" lipids. Possible wrong interpretations of lipid species such as the **PC 8:0/8:0** versus **Lyso-PC16:0** may be caused by incomplete databases combined with a lack of reasonable control.
Our response:
We manually verified the identities of all of the internal lipid standards we used, as well as the identities of the following lipid species FFA (30:0), PHC (16:0_26:0), PHC (18:0_26:0), PHC (16:0_30:0), PE (26:0_16:1), PE (26:0_18:1), PI (26:0_16:1), PI (26:0_18:1), TAG (30:1_10:1_10:1), TAG (30:0_10:1_10:1), TAG (18:1_10:1_24:1) and TAG (18:0_10:1_24:0). We revised the text accordingly.

We don't have the commercial lipid standards of PC 8:0_8:0 and Lyso-PC 16:0 needed to manually verify that the lipid identified as PC 8:0_8:0 by the software is not Lyso-PC16:0. We therefore removed PC 8:0_8:0 from the list of lipid species presented in supplemental table 1 of the revised manuscript.

Minor Concern:
Regarding the PC species in table 5, I am really surprised that the authors could not find the **PC** 34:2 at m/z 758, which I thought is one of the main PC species in this yeast strain. I am also wondering, about the PC 8:0/8:0 and PC 8:0/10:0. The authors list these species two times in table 5, but with different m/z values respectively. And if I calculate correct, a PC 8:0/8:0 has the formula C24H48O8N1P1, resulting in a mass of 510 for the proton adduct. The m/z values listed by the authors (m/z 494 and 496) might also fit to Lyso-PC 16:0 and Lyso-PC 16:1 species. According to this, the so called "PC 8:0/10:0" with m/z 522 is more likely a Lyso-PC 18:1 and Lyso-PC18:0 with m/z 524. Additional data about retention times would also be helpful in this case.
Our response:
The data presented in the original table 5 (which is supplemental table 1 in the revised manuscript) are coming from 2 independent experiments, for each of which 3 technical replicates were performed. In all of them, we were able to detect PC 34:2 with the m/z value (M+H) of 758.569431.

However, the shape of the MS1 peak (which we used for quantitation) varied in all these experiments. We therefore didn't include PC 34:2 in supplemental table 1 of the revised manuscript.

As noted above, we don't have the commercial lipid standards of PC 8:0_8:0, PC 8:0_10:0, Lyso-PC 16:0, Lyso-PC 16:1, Lyso-PC18:0 and Lyso-PC 18:1 needed to manually verify that the lipids identified as PC 8:0_8:0 and PC 8:0_10:0 by the software are not any of the above Lyso-PC species. We therefore removed PC 8:0_8:0 and PC 8:0_10:0 from the list of lipid species presented in supplemental table 1 of the revised manuscript.

Minor Concern:
In general, I am very surprised by the small number of different TAG species (listed in table 5). Especially when cultivating the yeast for several days, the cells reach their stationary phase with their energy storage (= TAGs) filled. There are so many more lipid TAG species described and just the combination of the main fatty acid species C16:0, C16:1, C18:0 and C18:0 allow the formation of much more TAG species. The authors should consider two possible reasons for their low TAG yield: On the one hand they harvest the yeast cells by centrifuging at 16.000 x g (line 161). The cells might burst at such high g values, which might cause the floating of the lipid droplets and therefore the loss of a significant amount of the lipids. On the other hand, cell disruption by just vortexing (line 178 - 180) is maybe not efficient enough. The authors should at least provide more specific information on the shaking frequency.
Our response:
In the revised manuscript, the vortexing steps used for cell disruption and lipid extraction are described as follows: "7.9. Vortex the tube at high speed for 5 min at room temperature to disrupt the cells. 7.10. Vortex the tube at low speed for 1 h at room temperature to facilitate the extraction of lipids."

Minor Concern:
It would be very interesting to see the MS/MS spectra that were used to identify these lipid species. For example, the TAG (30:0/10:1/10:1) with m/z 848.77 (as ammonium-adduct) could theoretically also consist of the way more common fatty acids 16:0/16:1/18:1. If the authors have clear evidence, that yeast TAGs consist of such strange fatty acids (at least in yeast), they should point this out. It might be very interesting for the yeast community.
Our response:
Because the revised manuscript already contains 10 tables and 4 figures, we believe that providing even more data will complicate its comprehension by readers.

Minor Concern:
Regarding the PI species listed in table 5: I would really be interested in the MS/MS data of the PI 27:0/16:1 as it seems that this is the only PI species with an odd numbered fatty acid. Is this a typo or do the authors have maybe hints that there are more lipid species with odd numbered fatty acid chains?
Our response:
PI 27:0/16:1 was a typo, which was corrected in supplemental table 1 of the revised manuscript.

Minor Concern:
Line 289-299
The authors should provide LC, MS and MS/MS data for the separation of these lipid species to demonstrate the possibilities of their method.
Our response:
A representative chromatogram of two isomeric phosphatidylserine forms (34:0) that have the same m/z value but different retention times, as well as their MS1 and MS2 spectra, are shown in figures 2, 3 and 4 (respectively) of the revised manuscript.

Minor Concern:
The authors should provide bar charts showing the quantified amounts of the lipid species and classes; and for each timepoint they investigated. It would be beneficial for the yeast lipid community to see the amounts and maybe the changes during the aging process. Additionally, the lipid profiles of yeast cell organelles (as mentioned in line 469) would be of great interest for yeast lipidomic research.
Our response:
Because the revised manuscript already contains 10 tables and 4 figures, we believe that providing even more data will complicate its comprehension by readers. Indeed, our manuscript describes a protocol of an LC-MS/MS method for lipidomic analysis. However, our manuscript is not a research article aimed at understanding the temporal dynamics of 10 different lipid classes during several consecutive steps of yeast chronological aging. Our manuscript is also not a research article that investigates an aging-associated changes in 10 different lipid classes present in various yeast organelles.

Minor Concern:
Figure 1: This figure is not very instructive, the authors should remove it and add some more specific figures showing the potential of their method (as mentioned above).
Our response:
Only the total MS1 ion chromatogram from LC/MS data is shown in figure 1 of the revised manuscript. A representative chromatogram of two isomeric phosphatidylserine forms (34:0) that have the same m/z value but different retention times, as well as their MS1 and MS2 spectra, are shown in figures 2, 3 and 4 (respectively) of the revised manuscript.

Minor Concern:
Table 1: Moles is not the unit for a concentration, but for the amount of a substance. Use correct units like moles per liter (mol/L). The calculated masses in the table are obviously the masses for the adduct ions. For example, a TAG (28:1/10:1/10:1) [=TAG 48:3] has the formula C51H92O6; therefore, the nominal mass is 800; the m/z value of m/z 818 might be the ammonium-adduct. The authors should be more precise and describe the different adducts in the table.
Our response:
Table 1 and its legend were revised as requested by the Reviewer.

Minor Concern:
Table 3: Why is the isolation width 2 (selected mass +/- 1)? Could the specificity of MS/MS spectra be increased if the isolation width is reduced to 1 or even 0.7?
Our response:
The isolation width is 2 because a selection window of 2 Da is the default selection window for the Orbitrap Velos Mass Spectrometer.

Minor Concern:
Table 4: If the authors list ions in the table, they should also write the charges of the ions.
Our response:
The charges of the H^+, H^-, NH_4^+, Na^+ and $HCOO^-$ ions are provided in table 4 of the revised manuscript.

Minor Concern:
Table 5: The calculated masses in the table are obviously the masses for the adduct ions. For example, a TAG (28:1/10:1/10:1) [=TAG 48:3] has the formula C51H92O6; therefore, the nominal mass is 800; the m/z value of m/z 818 might be the ammonium-adduct. The authors should be more precise and describe the different adducts in the table.

The authors claim the good separation of different lipid species therefore it would be interesting for the reader to see retention times of the different lipid species. I encourage the authors to add these values to table 5.

Regarding the Peak area data: For proper quantitation it is normally recommended to use standards concentrations in the same concentration range as the analyte. Especially for the Cardiolipin data, the peak area of the standard is in the range of E+07, only 4 out of the 23 identified CL species have peak areas in the range of E+06, all the other peak areas are 2 or more orders of magnitude smaller than the standard. At PHC, only 2 out of 24 analytes have peak areas in the same order of magnitude as the standard, most of them 2 or even 3 orders of magnitude below. At PC the peak area of the standard is in the range of E+08, only two PC analytes are in the range of E+07, all the other PC analytes have a peak are 2 ore more orders of magnitude smaller than the standard. I really doubt that a proper quantification of those PC species is possible under these conditions. The peak areas of the 2 PG species are also 2 orders of magnitude smaller than the standard. If the authors want to provide quantitative data, they should put some effort in a reasonable adaption of their lipid standard concentrations.
Our response:
Table 5 of the original manuscript is supplemental table 1 of the revised one. Its legend was revised regarding the calculated m/z values as requested by the Reviewer. This figure is already overcrowded to show any additional data, such as retention times of the different lipid species; besides, a chromatogram of two isomeric PS forms (34:0) is shown in figure 2 of the revised manuscript. As for the peak area data, our titration experiments revealed that the quantitation range for each of the 10 lipid classes covers at least 3 orders of magnitude.

Minor Concern:
Table 7: Information about normal phase LC is missing in the manuscript: No column mentioned in table 11; As there is also no reversed phase chromatography mentioned in the method-section of the

paper, the authors should either include this method if necessary, or otherwise remove this part completely.
Our response:
Table 7 and its discussion in the text have been removed from the manuscript.

Minor Concern:
Table 9: Line 406: [...effect of the induced-collision dissociation (CID) method...]: The abbreviation CID stands for collision-induced dissociation; the authors should not change the order of the words.
Our response:
"The induced-collision dissociation" had been changed to "the collision-induced dissociation" in the revised manuscript."

Minor Concern:
Table 11: For better clarity, the authors should split the items in the table into subgroups for chemicals, lab material, hardware equipment and software.
Our response:
This table was revised as requested by the Reviewer.

We are grateful to both Reviewers for constructive criticisms and suggestions for changes to be made in the text, all of which contributed to a substantial improvement of the original manuscript. We would also like to thank you for allowing us to address these criticisms and for handling our manuscript.

Yours sincerely,

Vladimir

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Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Table 1_NEW.xlsx

Lipid Class	Hydrophobic tail composition	Addudct-	MS Pea
FFA	19:0	M-H	6.71
FFA	10:0	M-H	6.64
FFA	12:0	M-H	2.60
FFA	16:1	M-H	1.68
FFA	16:0	M-H	1.84
FFA	18:1	M-H	2.98
FFA	18:0	M-H	7.54
FFA	20:1	M-H	5.83
FFA	20:0	M-H	2.41
FFA	22:0	M-H	8.20
FFA	24:0	M-H	1.23
FFA	26:0	M-H	2.65
FFA	30:0	M-H	9.86

ak Area	Calculated m/z value	Lipid Class	Hydr
E+07	297.2799	CL	
E+04	171.1391	CL	
E+06	199.1704	CL	
E+07	253.2173	CL	
E+06	255.233	CL	
E+07	281.2486	CL	
E+06	283.2643	CL	
E+05	309.2799	CL	
E+06	311.2956	CL	
E+05	339.3269	CL	
E+06	367.3582	CL	
E+07	395.3895	CL	
E+04	451.4521	CL	
		CL	
		CL	
		CL	
		CL	
		CL	
		CL	
		CL	
		CL	
		CL	
		CL	
		CL	

·ophobic tail composition	Addudct-	MS Peak Area	Calculated m/z
14:0_14:0_14:0_14:0	M-H	2.18E+07	1239.84
14:0_14:0_14:0_12:0	M-H	5.92E+05	1211.808
10:0_16:1_16:1_16:1	M-H	7.58E+04	1261.824
12:0_18:1_12:0_16:1	M-H	5.34E+04	1263.84
12:0_16:1_16:1_16:1	M-H	3.33E+05	1289.855
16:1_16:1_16:0_12:0	M-H	2.07E+05	1291.871
18:1_16:0_12:0_14:0	M-H	4.16E+04	1293.887
18:1_16:1_12:0_16:1	M-H	6.36E+05	1317.887
12:0_16:0_18:1_16:1	M-H	2.32E+05	1319.902
16:1_16:1_16:1_16:1	M-H	8.78E+05	1343.902
12:0_18:1_16:1_18:1	M-H	5.20E+05	1345.918
18:1_18:1_16:0_12:0	M-H	1.33E+05	1347.934
8:0_20:0_16:0_20:0	M-H	1.77E+05	1351.965
16:1_16:1_18:1_16:1	M-H	2.86E+06	1371.934
18:1_16:0_16:1_16:1	M-H	9.82E+05	1373.949
16:0_16:1_18:1_16:0	M-H	1.14E+05	1375.965
10:0_16:0_20:0_20:1	M-H	4.66E+05	1377.981
18:1_16:1_16:1_18:1	M-H	3.93E+06	1399.965
18:1_16:1_16:0_18:1	M-H	4.09E+05	1401.981
18:1_16:0_16:0_18:1	M-H	6.33E+04	1403.996
18:1_16:1_18:1_18:1	M-H	2.14E+06	1427.996
18:1_18:1_16:0_18:1	M-H	1.32E+05	1430.012
18:1_18:1_18:1_18:1	M-H	2.00E+06	1456.028
22:1_18:0_14:0_18:1	M-H	1.91E+05	1460.059

value	Lipid Class	Hydrophobic tail composition
	PHC	d18:1_17:0
	PHC	12:0_4:0
	PHC	18:1_4:0
	PHC	18:0_4:0
	PHC	16:0_12:0
	PHC	16:0_16:0
	PHC	14:1_16:0
	PHC	14:0_18:0
	PHC	18:0_16:0
	PHC	18:1_18:0
	PHC	20:0_16:0
	PHC	18:0_18:0
	PHC	18:0_20:0
	PHC	20:1_18:0
	PHC	16:0_26:0
	PHC	18:0_22:0
	PHC	18:0_24:0
	PHC	18:0_26:0
	PHC	18:0_24:0
	PHC	18:0_26:1
	PHC	18:0_26:0
	PHC	20:0_26:0
	PHC	18:0_26:0
	PHC	16:0_30:0
	PHC	20:0_26:0

Addudct-	MS Peak Area	Calculated m/z value
M-H	3.36E+07	550.5205
M-H	1.84E+05	286.2380
M-H	3.86E+04	368.3170
M-H	2.46E+04	370.3327
M-H	8.39E+04	454.4266
M-H	1.53E+05	510.4892
M-H	2.93E+05	480.4422
M-H	8.28E+04	510.4892
M-H	3.98E+07	538.5205
M-H	2.54E+04	564.5361
M-H	4.51E+05	566.5518
M-H	3.31E+05	566.5518
M-H	1.58E+05	594.5831
M-H	4.84E+05	592.5674
M-H	8.81E+04	650.6457
M-H	4.35E+04	622.6144
M-H	5.54E+04	650.6457
M-H	5.18E+05	678.6770
M-H	3.27E+05	650.6457
M-H	5.44E+04	676.6613
M-H	3.33E+06	678.6770
M-H	4.52E+04	706.7083
M-H	1.03E+07	678.6770
M-H	5.95E+05	706.7083
M-H	1.16E+06	706.7083

Lipid Class	Hydrophobic tail composition	Adduct+
PHS	16:1	M+H
PHS	16:0	M+H
PHS	18:1	M+H
PHS	18:0	M+H
PHS	18:0	M+H
PHS	20:0	M+H
PHS	22:1	M+H
PHS	22:1	M+H
PHS	22:0	M+H

MS Peak Area	Calculated m/z value	Lipid Class
1.26E+06	272.2584	PC
4.64E+07	274.2741	PC
3.11E+05	300.2897	PC
5.43E+05	302.3054	PC
1.13E+06	318.3003	PC
3.71E+06	346.3316	PC
4.55E+06	356.3523	PC
3.13E+05	372.3472	PC
2.16E+07	374.3629	PC
		PC
		PC
		PC
		PC

Hydrophobic tail composition	Adduct+	MS Peak Area	Calculated m/z value
16:0_10:0	M+H	2.13E+08	650.4755
16:1_10:0	M+H	4.85E+05	648.4599
16:1_12:0	M+H	1.58E+06	676.4912
16:0_12:0	M+H	2.23E+06	678.5068
16:1_14:0	M+H	3.33E+06	704.5225
16:0_14:0	M+H	9.26E+05	706.5381
16:0_16:1	M+H	6.91E+07	732.5538
16:0_16:0	M+H	5.29E+06	734.5694
16:0_18:1	M+H	3.37E+07	760.5851
18:0_16:0	M+H	1.56E+06	762.6007
18:0_18:1	M+H	6.38E+06	788.6164
18:0_24:1	M+H	3.10E+06	872.7103
20:0_24:1	M+H	9.28E+05	900.7416

Lipid Class	Hydrophobic tail composition
PE	15:0_15:0
PE	16:1_10:0
PE	16:0_10:0
PE	16:1_12:0
PE	16:0_12:0
PE	16:1_14:1
PE	18:1_12:0
PE	16:1_16:1
PE	16:0_16:1
PE	16:0_16:0
PE	16:1_18:1
PE	18:0_16:1
PE	18:0_16:0
PE	18:1_18:1
PE	18:0_18:1
PE	18:0_18:0
PE	20:1_18:1
PE	20:0_18:1
PE	26:0_16:1
PE	26:0_18:1

Addudct-	MS Peak Area	Calculated m/z value
M-H	3.30E+07	662.4766
M-H	6.04E+04	604.3984
M-H	1.08E+05	606.414
M-H	1.89E+05	632.4297
M-H	2.62E+05	634.4453
M-H	1.33E+05	658.4453
M-H	9.71E+04	660.461
M-H	8.66E+06	686.4766
M-H	1.65E+07	688.4923
M-H	3.18E+05	690.5079
M-H	1.36E+07	714.5079
M-H	2.74E+06	716.5236
M-H	1.40E+05	718.5392
M-H	2.05E+06	742.5392
M-H	1.52E+06	744.5549
M-H	6.33E+04	746.5705
M-H	2.67E+05	770.5705
M-H	1.95E+05	772.5862
M-H	2.53E+05	828.6488
M-H	1.68E+05	856.6801

Lipid Class	Hydrophobic tail composition	Addudct-	MS Pea
PG	15:0_15:0	M-H	6.89
PG	16:0_16:1	M-H	2.24
PG	16:0_18:1	M-H	6.40

ak Area	Calculated m/z value	Lipid Class	Hydr
E+07	693.4712	PI	
E+05	719.4869	PI	
E+05	747.5182	PI	
		PI	
		PI	
		PI	
		PI	
		PI	
		PI	
		PI	
		PI	
		PI	
		PI	

·ophobic tail composition	Addudct-	MS Peak Area	Calculated m/z
17:0_20:4	M-H	2.94E+05	871.5342
16:0_10:0	M-H	4.14E+05	725.4247
16:0_12:0	M-H	1.21E+06	753.456
16:0_14:1	M-H	1.56E+05	779.4716
18:0_12:0	M-H	8.84E+05	781.4873
16:1_16:1	M-H	6.34E+05	805.4873
16:0_16:1	M-H	5.16E+06	807.5029
16:1_18:1	M-H	3.71E+05	833.5186
16:0_18:1	M-H	9.96E+06	835.5342
18:1_18:1	M-H	2.64E+05	861.5499
18:0_18:1	M-H	4.96E+06	863.5655
26:0_16:1	M-H	2.19E+05	947.6594
26:0_18:1	M-H	1.98E+05	975.6907

value	Lipid Class	Hydrophobic tail composition
	PS	17:0_17:0
	PS	8:0_16:0
	PS	16:0_16:1
	PS	16:1_18:1
	PS	16:0_18:1

Addudct-	MS Peak Area	Calculated m/z value
M-H	1.98E+06	762.5291
M-H	2.94E+04	622.3725
M-H	8.00E+05	732.4821
M-H	2.72E+05	758.4978
M-H	2.09E+06	760.5134

Lipid Class	Hydrophobic tail composition	Adduct+	MS Pea
TAG	28:1_10:1_10:1	M+NH4	1.27
TAG	10:0_10:0_22:1	M+NH4	1.32
TAG	10:0_10:0_22:0	M+NH4	1.67
TAG	18:0_10:1_16:1	M+NH4	2.62
TAG	18:0_10:1_16:0	M+NH4	7.01
TAG	28:0_10:1_10:1	M+NH4	4.13
TAG	16:0_10:1_22:0	M+NH4	1.46
TAG	16:0_16:0_16:0	M+NH4	1.07
TAG	30:1_10:1_10:1	M+NH4	2.06
TAG	30:0_10:1_10:1	M+NH4	2.26
TAG	18:1_10:1_24:1	M+NH4	1.21
TAG	18:0_10:1_24:0	M+NH4	2.15

ak Area	Calculated m/z value
E+08	818.7232
E+07	738.6606
E+07	740.6763
E+07	764.6763
E+07	766.6919
E+08	820.7389
E+08	822.7545
E+07	824.7702
E+08	846.7545
E+08	848.7702
E+08	874.7858
E+08	878.8171