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ER Membrane Lipid Composition and Metabolism: Lipidomic Analysis

Laetitia Fouillen, Lilly Maneta-Peyret, and Patrick Moreau

Abstract

Plant ER membranes are the major site of biosynthesis of several lipid families (phospholipids, sphingolipids, neutral lipids such as sterols and triacylglycerols). The structural diversity of lipids presents considerable challenges to comprehensive lipid analysis. This chapter will briefly review the various biosynthetic pathways and will detail several aspects of the lipid analysis: lipid extraction, handling, separation, detection, identification, and data presentation. The different tools/approaches used for lipid analysis will also be discussed in relation to the studies to be carried out on lipid metabolism and function.

Key words Ceramides, GC-FID, GC-MS, Glucosylceramides, HPTLC, LC-MS, Long chain bases (LCB), Phospholipids, Phytosterols, Triacylglycerols

1 Introduction

ER membranes are known to be involved in many key cellular functions and understanding more precisely their role, being able to analyze their lipid composition and metabolism/homeostasis is as crucial as it is for ER proteins.

Of course to be able to perform such lipid analysis, we must handle highly purified ER membranes devoid of other membrane contaminants, something which is not so obvious as it will depend on the plant material considered. According to the starting material, different methodologies with various efficiencies in isolating highly purified ER membranes (differential ultracentrifugation, free-flow electrophoresis, two phase partition systems, affinity purification) alone or in combination have to be used ([1–3] and references therein).

Table 1 details the lipid composition of the ER membranes from different plant species and tissues, that have been combined from the literature to give a simplified view of the different lipid families/species and their relative abundance. The corresponding biosynthetic pathways of the different lipid classes of the ER

Table 1
Lipid composition of plant ER membranes in mol%^a

| PA ^o | IPC | PC | PE | PG | PI | PS | GlcCer | Free sterols | Conjugated sterols |
|-----------------|---------|-------|-------|-------|----------|-----|--------|--------------|--------------------|
| 0.5–7 | 0.8–1.7 | 36–65 | 11–23 | 3.5–5 | 2.5–17.5 | 1–7 | 2–8 | 3–14 | 1–4 |

^oGenerally, PA is considered and found to be low/very low in the ER membranes and in most plant cell membranes because it is an intermediary metabolite and a signaling phospholipid. So most of the time, high amounts of PA are probably due to Phospholipase D activation during material handling and/or lipid extraction

^aData combined from several plant species and tissues ([12–14] and references therein, and unpublished results). *PA* Phosphatidic Acid, *IPC* lysoPhosphatidylCholine, *PC* PhosphatidylCholine, *PE* PhosphatidylEthanolamine, *PG* PhosphatidylGlycerol, *PI* PhosphatidylInositol, *PS* PhosphatidylSerine, *GlcCer* Glucosylceramides. Conjugated sterols: sum of sterol glycosides + steryl esters

membranes are presented in Fig. 1 for phospholipids, in Fig. 2 for glucosylceramides, and in Fig. 3 for sterols.

The pretty large variations in lipid amounts which can be observed (Table 1) can be due of course to the differences in the plant species/tissues but also to some extent to the purity of the ER membranes according to the isolation procedures used. Anyway, Table 1 indicates that the major lipid families/species are represented by the phospholipids, followed by the sterols (free and conjugated) and the glucosylceramides (GluCer). Other lipid species such as phosphoinositides (PIP, PIP2), acyl-CoAs, and ceramides (precursors of GluCer) are also present but in lower amounts. The levels of diacylglycerol in ER membranes (precursors of phospholipids and also of triacylglycerols for lipid bodies) are not mentioned because their levels can even be more variable.

The purpose of this chapter is not to give an extensive overview of the various lipid methodologies because this has already been done by well-recognized lipid experts in exhaustive set of publications such as in the AOCS Lipid Library (<http://lipidlibrary.aocs.org>) or in the arabidopsis book [4], but to select some lipid technologies that were, are and will be relevant for studying several aspects of plant ER membranes lipid metabolism.

2 Materials

2.1 Technologies/ Equipment

1. Lipid extraction-preparation/Bench centrifuge, 110 °C dry bath incubator, 110 °C resistant screwed glass tubes, bench vortex.
2. Densitometric analysis/TLC sampler Linomat 5 (Camag, Muttenz, Switzerland), TLC scanner 3 (CAMAG, Muttenz, Switzerland).
3. HPTLC (High Performance Thin Layer Chromatography)/ADC2 automatic developing chamber (Camag, Muttenz, Switzerland), HPTLC plates F254 (Merck, Darmstadt, Germany).

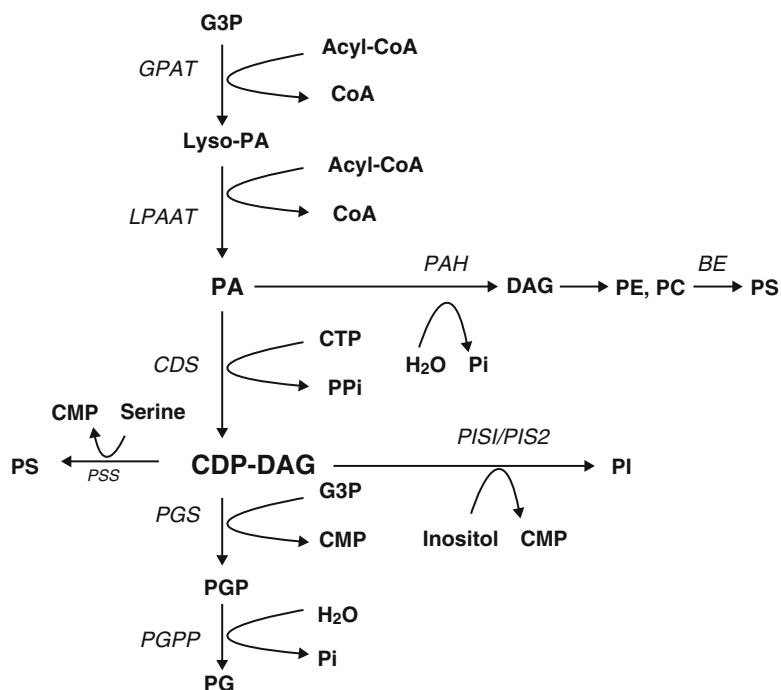


Fig. 1 Simplified phospholipid biosynthetic pathways in the ER. *CDP-DAG* cytidine diphosphate diacylglycerol, *CoA* coenzyme A, *CMP* cytidine monophosphate, *CTP* cytidine triphosphate, *DAG* diacylglycerol, *G3P* glycerol-3-phosphate, *Lyso-PA* lysophosphatidic acid, *PA* phosphatidic acid, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerol, *PGP* phosphatidylglycerol phosphate, *PI* phosphatidylinositol, *PS* phosphatidylserine. *Enzymes*: *CDS* cytidine-diphosphate diacylglycerol synthase; *GPAT* glycerol-3-phosphate acyltransferase, *LPAAT* lysophosphatidic acid acyltransferase; *PAH* phosphatidic acid phosphohydrolase; *PGPP* phosphatidylglycerol phosphate phosphatase; *PGS* phosphatidylglycerol phosphate synthase; *PIS* phosphatidylinositol synthase; *PSS* phosphatidylserine synthase

4. GC-FID (Gas Chromatography-Flame Ionization Detector)/Agilent 7890 gas chromatograph equipped with flame ionization detection (Santa Clara, CA, USA), Agilent GC Column DB-Wax 15 m × 0.53 mm × 1.0 μm.
5. GC-MS (Gas Chromatography-Mass Spectrometry)/Agilent 6850 gas chromatograph coupled with a MS detector MSD 5975-EI (Santa Clara, CA, USA), HP-5MS capillary column (5% phenyl-methyl-siloxane), 30-m, 250-μm, and 0.25-μm film thickness (Agilent, Santa Clara, CA, USA).
6. LC-MS (Liquid Chromatography-Mass Spectrometry)/QTRAP 5500 (ABSciex) mass spectrometer coupled to a liquid chromatography system (Ultimate 3000, Dionex). Luna C8 150 × 1 mm column with 100-Å pore size and 5-μm particles (Phenomenex).
7. Radiolabeling analysis/PhosphorImager (such as STORM or TYPHOON from GE Healthcare).

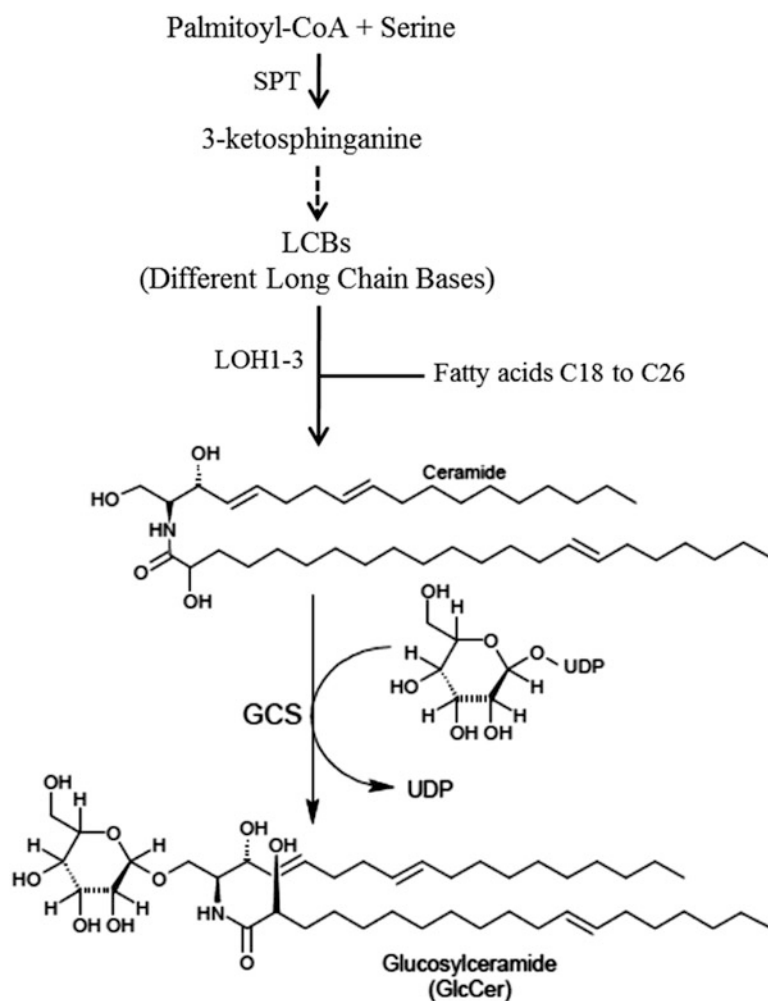


Fig. 2 Simplified GlcCer biosynthetic pathway in the ER. *GCS* glucosylceramide synthase, *LOH1–3* ceramide synthases 1, 2, and 3, *SPT* serine palmitoyl transferase. The *dotted arrow* indicates several enzymatic steps. All the enzymes involved in the biosynthesis of glucosylceramides and its precursors are located in the ER

2.2 Lipid Standards

1. Lipid standards are purchased from Avanti Polar Lipids (Alabaster, AL, USA), Nu-Chek-Prep (Elysian, MN, USA), Matreya, (State College, PA, USA), and/or Sigma (St. Louis, MO, USA). *See Note 1.*
2. For HPTLC analysis, all the phospholipid standards (PC, PE, PI, PG and PS) are from soybean extracts excepted PA which can be from any other source, DAG (for example di-18:1), TAG (for example tri-18:1), ceramides (bovine extract), glucosylceramides (soybean extract), sterol (plant extract). *See also Note 2.*

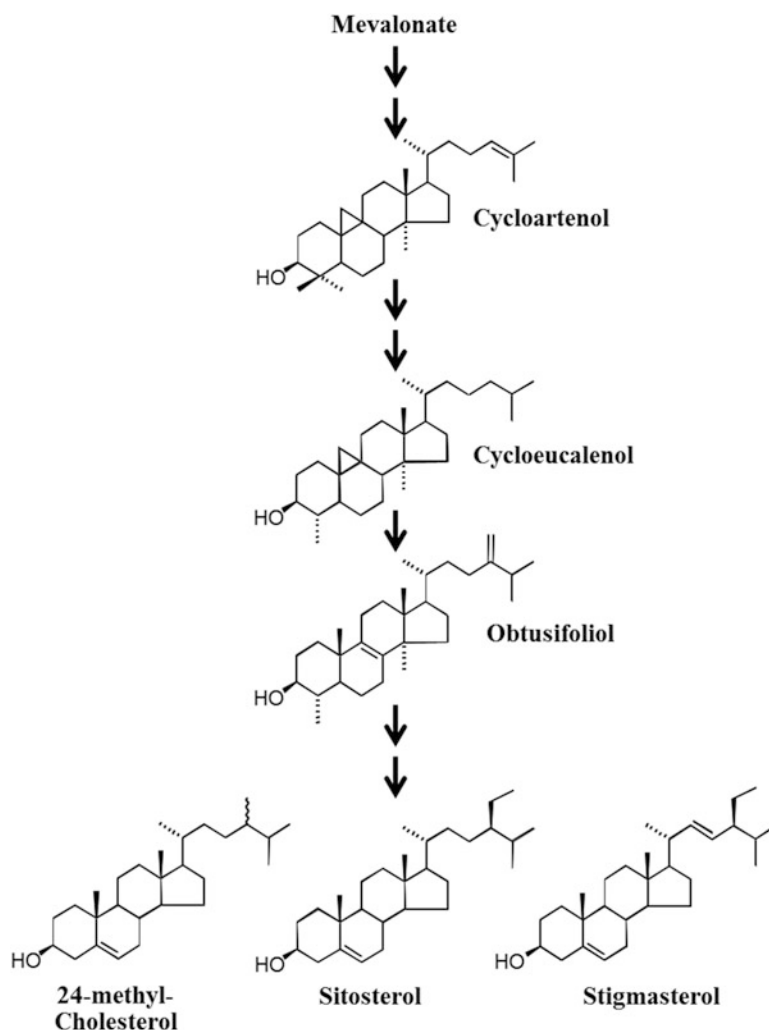


Fig. 3 Simplified sterol biosynthetic pathway in the ER. The *two successive arrows* indicate two or more enzymatic steps. The biosynthesis of the precursor mevalonate by the HMG-CoA reductase is the first step of the sterol biosynthetic pathway which is located in the ER

- For GC-FID or GC-MS analysis, C17:0, h14:0, d20:0, t17:0, d14:1^{4(E)}, and cholestanol.
For LC-MS analysis, PC, 17:0/17:0; PI, 17:0/14:1; PE, 17:0/17:0; PG, 17:0/17:0; PS, 17:0/17:0, TAG 17:0/17:0/17:0, DAG 17:0/17:0.

2.3 Solutions

- Eluent A: isopropanol/methanol/H₂O 5/1/4, v/v containing 0.2% formic acid and 0.028% NH₃.
- Eluent B: isopropanol containing 0.2% formic acid and 0.028% NH₃.

3. Eluent C: acetonitrile/methanol/H₂O (19/19/2, v/v) containing 0.2% formic acid and 0.028% NH₃.
4. Eluent D: isopropanol containing 0.2% formic acid and 0.028% NH₃.

3 Methods

3.1 Lipid Extraction

Whatever the isolation procedure of the purified ER membranes (differential ultracentrifugation, free-flow electrophoresis, two phase partition systems, affinity purification), the ER fraction will be generally obtained as a pellet after centrifugation in order to concentrate the membranes.

Analyzing lipids requires extraction using organic solvents. Extraction should be rapid in order to avoid lipid modifications during treatment. In the case of ER membranes, extraction should be carried out immediately after recovering the ER membranes; otherwise the membranes can be stored at $-80\text{ }^{\circ}\text{C}$ (but there is a risk of changes to the lipids).

1. The pellet should be suspended in a small volume (100–300 μl) of distilled H₂O (if protein activity is not necessary to be measured) or in buffer, by gentle grinding rather than vortexing, and transferred into glass tubes (with Teflon protected screw caps) for lipid extraction (plastic apparatus and containers (other than Teflon) should be avoided). One part (5%) of the aliquot should be conserved for protein measurement.
2. Then extract the lipids with chloroform/methanol 2/1, v/v [5] (*see Note 3* for acyl-CoAs), using a ratio of chloroform, methanol, and aqueous phase in the final mixture close to 8/4/3, v/v to be efficient.
3. The solution is vortexed or shaken for 5 min, at room temperature. Centrifuge the solution (10 min 3000 rpm) and discard the upper phase (as well as interphase).
4. This organic phase is washed with $\frac{1}{4}$ of volume of H₂O or 0.9% NaCl; after vortexing for 1 min, the solution is centrifuged (10 min, 3000 rpm ($3000 \times g$)) and the lower phase recovered in a new glass tube.
5. The organic solvent is evaporated under air (or nitrogen if particular protection of unsaturated fatty acids is needed). Resuspended the lipids into appropriate solvents required for the subsequent analysis.

The principal methods for lipid class analysis are HPTLC and LC-MS/MS, and for their fatty acid content are GC-FID or GC-MS. Long chain bases of sphingolipids and sterols can be analyzed by GC-MS (a general scheme of lipid analysis is given in Fig. 4).

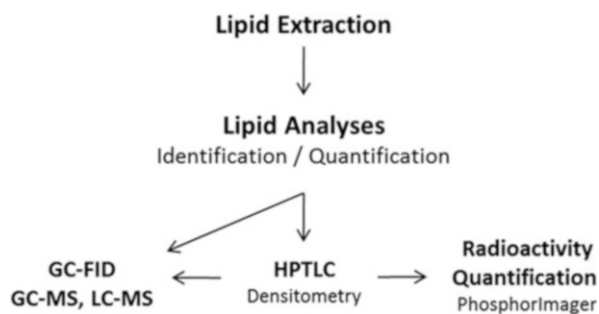


Fig. 4 Different steps of lipid analysis processes. The principal methods for lipid class analysis are HPTLC and LC-MS, and for their fatty acid content are GC-FID or GC-MS. Long chain bases of sphingolipids, and sterols, can be analyzed by GC-MS. *GC-FID* gas chromatography-flame ionization detector, *GC-MS* gas chromatography-mass spectrometry, *SPE* solid phase extraction, *HPTLC* high performance thin layer chromatography

3.2 HPTLC Analysis of Phospholipids and Acyl-CoAs

1. For analysis of phospholipids with HPTLC plates, the lipids should be resuspended into 50–200 μl of chloroform/methanol 2/1, v/v. Close the cap until use to prevent solvent loss and changes in lipid concentration.
2. Phospholipids may be analyzed by loading the extract, using a CAMAG Linomat IV, onto 10 \times 10 or 10 \times 20 cm HPTLC plates, which are developed in methyl acetate/n-propanol/chloroform/methanol/0.25% aqueous KCl (25/25/25/10/9, v/v) according to Heape et al. [6].
3. External phospholipid standards (10 μg per standard; *see Note 2*) are loaded on the same plates as the samples to be analyzed.
4. The migration may be carried out into a simple developing chamber (20 min saturation/45 min migration/45 min drying; developing solvent volume 20 ml) or into an ADC2 automatic developing chamber (developing solvent volume 10 ml; program: migration distance 85 mm; drying time 5 min; saturation time 0 min).
5. Lipids can be detected by dipping the plates into a 3% copper acetate (w/v) in 8% aqueous phosphoric acid solution [7, 8], followed by heating at 110 $^{\circ}\text{C}$ during 30 min.
6. Densitometric analysis of phospholipids must then be performed in the following hour (stability of coloration), using for example a TLC scanner 3 at 366 nm. Lipids are quantified (μg of lipids) by comparing lipids and standard lipids peak areas (*see Note 2*).
7. If analysis of the fatty acid content is needed, the lipids from the HPTLC plates are only labeled by iodine vapors. Place the chromatogram 5–15 min into a strong iodine atmosphere (into a covered beaker in which some iodine resublimed

crystals have been placed); double bonds containing lipids will be stained in yellow over a few minutes. The desired lipid bands are scraped off and transferred into glass tubes (with Teflon covered screw caps) for further analysis by GC-FID or GC-MS.

8. For HPTLC analysis of acyl-CoAs, the lipids should be resuspended into 50–200 μl of chloroform/methanol/ H_2O , 3/3/1, v/v.

3.3 GC-FID Analysis of Fatty Acids from Phospholipids

1. Fatty acid methyl esters (FAMES) are prepared by resuspending the scraped lipid bands in 1 ml of 2.5% H_2SO_4 (v/v) in methanol using heptadecanoic acid 2 $\mu\text{g}/\text{ml}$ as internal standard.
2. Tubes are heated at 80 $^\circ\text{C}$ for 1 h and cooled to room temperature.
3. Then 400 μl hexane and 1.5 ml H_2O are added to extract fatty acid methyl esters.
4. The tubes are vortexed vigorously for at least 1 min and centrifuged 10 min at 2000 rpm ($2000 \times g$) with a bench centrifuge and the organic (upper) phase is transferred (preferentially with a syringe) to injection GC vials.
5. GC-FID can be performed using an Agilent 7890 gas chromatograph equipped with an Agilent GC Column DB-Wax 15 m \times 0.53 mm \times 1.0 μm and flame ionization detection, with Helium as carrier gas at a flow rate of 3 ml/min (4 psi).
6. The temperature gradient conditions can be: 160 $^\circ\text{C}$ for 1 min, increase to 190 $^\circ\text{C}$ at 20 $^\circ\text{C}/\text{min}$, increase to 210 $^\circ\text{C}$ at 5 $^\circ\text{C}/\text{min}$ and then remaining at 210 $^\circ\text{C}$ for 5 min.
7. FAMES are identified by comparing their retention times with commercial fatty acid standards and quantified using ChemStation to calculate the peak areas, and then comparing them with the response of an internal standard (C17:0).

3.4 Phospholipidic Analysis by LC-MS

1. For the analysis of phospholipids by LC-MS, phospholipid extracts were dissolved in 100 μl eluent A.
2. Synthetic internal lipid standards (PE, 17:0/17:0; PS, 17:0/17:0; PC, 17:0/17:0; PI, 17:0/14:1; and PG, 17:0/17:0) were added at different amounts adapted for the detection.
3. LC-MS/MS (multiple reaction monitoring mode) analysis can be performed with a model QTRAP 5500 mass spectrometer coupled to a liquid chromatography system Ultimate 3000. Analysis was performed in the negative (PE, PS, PI, and PG) and positive (PC) modes with fast polarity switching (50 ms); nitrogen was used for the curtain gas (set to 15), gas 1 (set to 20), and gas 2 (set to 0). Needle voltage was at -4500 or $+5500$ V without needle heating; the collision gas was also nitrogen. The multiple reaction monitoring mode is based on

the loss of the fatty acid part for the negative analysis (PE, PS, PI, and PG), and on the loss of the polar head in positive mode for PC analysis. The declustering potential was adjusted at -160 V for PE, -130 V for PG, -90 V for PS, -85 V for PI, and $+40$ V for PC; collision energy is set to -48 eV for PE and PG, -60 eV for PS, -62 eV for PI, and $+47$ eV for PC. The dwell time was set up to 3 ms. Reverse-phase separations are performed at 50 °C on a Luna C8 150×1 mm column with 100 -Å pore size and 5-mm particles. During the run, the proportions of eluent A and eluent B vary: 0 min, 30% B; 5 min, 50% B; 30 min, 80% B; 31–41 min, 95% B; 42–52 min, 30% B. The flow rate is set up at 40 μ l/min, and 3 μ l sample volumes are injected. The areas of LC peaks are determined using the MultiQuant software for phospholipid quantification.

3.5 Long Chain Bases (LCB) Analysis by GC-MS

The long chain bases (LCB) are extracted from sphingolipids by basic hydrolysis. The free LCB are chemically oxidized in their aldehyde form to be analyzed in GC-MS [9].

Add 1 ml of 10% (w/v) of barium hydroxide (dissolved in H_2O) to the ER membrane lipid extract. Be sure that the aqueous solution was well shaken before pipetting and place the samples in tubes of about 8 ml (*see Note 4*). Add 1 ml of dioxane with standards (d20:0, t17:0, and d14:1^{4(E)}, *see Note 5*). Incubate at 110 °C overnight, then, after cooling add 6 ml of distilled H_2O , and transfer the content in a new bigger tube (approximate volume of 12 ml). Add 4 ml of ether 100%. Shake vigorously and centrifuge at $700 \times g$ for 5 min. Pick up the upper phase and place it in a new tube of about 8 ml. Add another 4 ml of ether 100% in the previous 12 ml tube, and shake vigorously and centrifuge at $700 \times g$ for 5 min. Pick up the upper phase and place it in the previous 8 ml tube. Evaporate with N_2 gas flow. Do not warm in a H_2O bath, as LCBs are quite labile. After complete evaporation, add 1 ml of methanol. Add 100 μ l of a 0.2 M metaperiodate ($NaIO_4$) solution in H_2O (*see Note 6*). Incubate at room temperature *in the dark* for 1 h sharp and shake slightly. Add 1 ml of hexane 99% and 1 ml of distilled H_2O and shake vigorously. Centrifuge at $700 \times g$ for 5 min. Pick up the upper phase. Transfer to a new tube (8 ml) and evaporate with N_2 gas flow at room temperature (do not warm in a H_2O bath for evaporation as fatty aldehydes might be unstable in this condition). Suspend in 100 μ l of hexane 99% and vortex. Transfer to GC vials and run GC-MS.

GC-MS can be performed using an Agilent 6850 gas chromatograph coupled to a MS detector MSD 5975-EI. An HP-5MS capillary column (5% phenyl-methyl-siloxane, 30-m, 250- μ m, and 0.25- μ m film thickness) can be used with Helium carrier gas at 2 ml/min; injection is done in splitless mode; injector and mass spectrometry detector temperatures are set to 250 °C; the

oven temperature is held at 50 °C for 1 min, then programmed with a 25 °C/min ramp to 150 °C (2-min hold) and a 10 °C/min ramp to 320 °C (6-min hold).

3.6 Ceramide and Glucosylceramide Analysis by HPTLC and GC-MS Analysis of Their Fatty Acids

Under the conditions of polar lipid (phospholipids) separation described above, glucosylceramides and sterylglucosides do not separate. These lipids are separated on HPTLC plates using the solvent system chloroform/methanol (85/15, v/v) [10]. In this elution system, ceramides can also be isolated on HPTLC plates. Quantification by densitometry using ceramide and glucosylceramide standards (10 µg per standard) can be performed as explained for phospholipids in 3.2.

For acyl-chain characterization of ceramides and glucosylceramides by GC-MS, scraped lipid bands (after iodine vapor revelation, *see* Subheading 3.2) are directly incubated with 1 ml of 5% sulfuric acid solution in methanol (implemented with standards: 5 µg/ml of C17:0 and 5 µg/ml of h14:0) for transesterification which is made overnight at 85 °C to produce FAMES. Those are then washed by adding 1 ml of NaCl 2.5% and 1 ml of hexane 99%. After vigorous shaking and centrifugation at $700 \times g$ for 5 min at room temperature, the higher phase is harvested, placed in a new tube and buffered with 1 ml of 100 mM Tris, 0.09% NaCl, pH 8 with HCl. After vigorous shaking and centrifugation at $700 \times g$ for 5 min at room temperature, the higher phase is harvested, placed in a new tube and evaporated with needles evaporating pan. Then, 200 µl of N,O-Bis(trimethylsilyl)trifluoroacetamide + 1% trimethylsilyl (BSTFA + 1% TMCS) are added and incubated at 110 °C for 20 min. After evaporation, FAMES are resuspended in 100 µl of 99% hexane and run on GC-MS with the same program described in Subheading 3.3.

3.7 Neutral Lipid Analysis by HPTLC and GC-FID

To isolate and quantify neutral lipids (including phytosterols, DAG, FFA, TAG, and steryl esters), the lipids, resuspended into 50–200 µl of chloroform/methanol 2/1, v/v, are loaded onto 10 × 10 or 10 × 20 cm HPTLC plates developed with hexane/ethylether/acetic acid (90/15/2, v/v) as described [8, 11]. External neutral lipid standards (10 µg per standard; *see* Note 2) are loaded on the same plates than the samples to be analyzed. The migration may be carried out into a simple developing chamber (10 min saturation/20 min migration/30 min drying; developing solvent volume 20 ml) or into an ADC2 automatic developing chamber (developing solvent volume 10 ml; program: migration distance 85 mm; drying time 5 min; saturation time 0 min). Quantification by densitometry can be performed as explained for phospholipids in Subheading 3.2.

For GC-FID analysis of fatty acids from fatty acid-containing neutral lipids, FAMES are analyzed and quantified as described in Subheading 3.2.

3.8 Phytosterol Analysis by GC-MS

Phytosterols are prepared by resuspending the corresponding scraped lipid bands in 1 ml of ethanol containing the internal standard α -cholestanol (25 mg/ml) in screwed glass tubes. For saponification, add 100 μ l of 11 N KOH (56.11 g/mol, 12.3 g/20 ml H₂O), close the tube(s) under nitrogen, incubate in a dry bath incubator at 80 °C for 1 h, add 1 ml of hexane, vortex for 15 s, add 2 ml of distilled H₂O, vortex for 15 s, centrifuge 5 min at 2000 rpm in a bench centrifuge, and recover the phytosterol-containing upper phase in a new screwed glass tube and evaporate the solvent under an N₂ gas stream. For silylation of phytosterols, add 200 μ l of BSTFA/TMCS, incubate 15 min at 110 °C in a dry bath incubator, evaporate the solution to dryness under nitrogen.

GC-MS analysis: add 200 μ l of hexane before injection on GC-MS and use the same program as described in Subheading 3.3.

3.9 LC-MS Analysis of DAG and TAG

For the analysis of DAG and TAG by LC-MS, phospholipid extracts were dissolved in 100 μ l of eluent C; synthetic internal lipid standards (DAG 17:0/17:0; TAG, 17:0/17:0/17:0) were added at different amounts adapted for the detection.

LC-MS/MS (multiple reaction monitoring mode) analysis is performed with a model QTRAP 5500 mass spectrometer coupled to a liquid chromatography system Ultimate 3000. The multiple reaction monitoring mode is based on the loss of the fatty acid part during the fragmentation.

Analysis was achieved in positive mode and nitrogen was used as curtain gas (set to 30), gas1 (set to 25) and gas2 (set to 0). Needle voltage was at +5500 V without needle heating. The collision gas was also nitrogen. The multiple reaction monitoring mode is based on the loss of the fatty acid part of the TAG or DAG molecules. The declustering potential was set at +40 V for TAG and +86 V for DAG; collision energy was adjusted at +34 V for TAG and DAG. The dwell time was set to 3 ms. Reversed phase chromatography was carried out at 30 °C using a Luna 3u C8 150 \times 1 mm column, with 100-Å pore size, 3- μ m particles. Eluent C and eluent D were then used as follows. The gradient elution program was 0–5 min, 15% D; 5–35 min, 15–40% D; 35–50 min, 55% D; 51–58 min, 80% D; 59–70 min, 15% D. The flow rate was 40 μ l/min and injection volume was 3 μ l. The relative levels of DAG and TAG species were determined using MultiQuant and normalizing to the area of the DAG or TAG internal standards (Yuan et al., submitted).

3.10 Radiolabeling In Vivo

Radiolabeled lipid precursors can be used for incubation with plant cells/tissues to investigate ER membrane lipid metabolism in vivo. According to the starting material, ER membranes can be isolated through different methodologies (*see* Subheading 1). Subsequently, ER membrane lipids are then extracted and washed as detailed in Subheading 3.1. Polar and neutral lipids can then be separated onto HPTLC plates as described in the corresponding

paragraphs. Their radiolabeling can then be determined using a PhosphorImager equipment. HPTLC plates are generally exposed to the adapted screens for 24–48 h and then the screens are scanned with the PhosphorImager. The ImageQuant software translates the radioactivity on HPTLC plates into peaks and gives a surface area for each. Then, from a calibrating curve (made with any radioactive standard) defining the correspondence between peak surface area and labeling amount, we can calculate for each lipid the level of labeling, and from the specific radioactivity of the lipid precursor used, we can determine the amount of each lipid in moles.

3.11 Radiolabeling In Vitro

Radiolabeled lipid precursors can also be used for incubation with ER membranes to investigate in vitro the enzymatic activity of ER membrane enzymes involved in lipid metabolism. According to the starting material, ER membranes can be isolated through different methodologies (*see* Subheading 1). Subsequently, ER membrane lipids are then extracted and washed as detailed in Subheading 3.1. Polar and neutral lipids can then be separated onto HPTLC plates as described in Subheading 3.2. Their radiolabeling can then be determined using a PhosphorImager equipment. The procedure determining the formed products amounts in moles is described above.

4 Notes

1. All chemicals and solvents were either of analytical or mass spectrometric grades. Prepare all solutions using deionized H₂O (18.2 MΩ cm at 25 °C), for example produced using a Synergy UV Millipore System. Follow all disposal regulations when disposing waste material.
2. For HPTLC analysis, each of the lipid classes should be calibrated with appropriate standards, i.e., with a similar fatty acid degree of unsaturation to the samples because the revelation with both cupric acetate/phosphoric acid or iodine vapor solutions is sensitive to the degree of fatty acid unsaturation.
3. If acyl-CoAs are required, lipids should be extracted with six times the sample's volume of chloroform/methanol 1/1 in order to obtain a homogeneous phase permitting to conserve the more polar molecules such as the acyl-CoAs.
4. Be extremely careful that all tube caps contain a Teflon disk.
5. Standards (for example, d20:0, t17:0, and d14:1^{4(E)}) should be added in the dioxane. Each standard initial concentration (1 g/L in methanol) should be added at 5 µg/ml final, i.e., 10 µl in the 2 ml of barium hydroxide + dioxane.
6. The NaIO₄ stock concentration should be prepared only freshly.
Equivalent instrumentation can be used.

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