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Chapter 10

Lipidomic Analysis of Biological Samples by Liquid Chromatography Coupled to Mass Spectrometry

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Summary

Lipidomics studies the large-scale changes in nonwater-soluble metabolites (lipids) accompanying perturbations of biological systems. Because lipids are involved in crucial biological mechanisms, there is a growing scientific interest in using lipidomic approaches to understand the regulation of the lipid metabolism in all eukaryotic and prokaryotic organisms. Lipidomics is a powerful tool in system biology that can be used together with genomics, transcriptomics, and proteomics to answer biological questions arising from various scientific areas such as environmental sciences, pharmacology, nutrition, biophysics, cell biology, physiology, pathology, and disease diagnostics. One of the main challenges for lipidomic analysis is the range of concentrations and chemical complexity of different lipid species. In this chapter, we present a lipidomic approach that combines sample preparation, chromatographic, and intrasource ionization separation coupled to mass spectrometry for analyzing a broad-range of lipid molecules in biological samples.

Key words: Lipidomics, Lipids, Liquid chromatography mass spectrometry, Fatty acids, Phospholipids, Cholesterol, Lipid profile, Lipid biomarkers, Large-scale analysis

1. Introduction

Lipids are natural molecules that are insoluble or partially soluble in water. These hydrophobic or amphipathic molecules can be either biosynthesized or absorbed from the environment and are vital for the life of all eukaryotic and prokaryotic organisms. Lipids play crucial biological roles through three general mechanisms (1) they affect the cellular membrane structures and protein–membrane interactions, (2) they provide a source of energy through processes of oxidation, and (3) they serve as signaling molecules, binding to
plasma membrane or nuclear receptors mediating transmembrane signaling and cell-to-cell communication (1).

The development of mass spectrometry (MS) techniques marked the beginning of a new era for the study of lipids, opening a series of unprecedented experimental opportunities. Indeed, the implementation of atmospheric-pressure ionization techniques such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), capable of coupling liquid chromatography (LC) with MS, made it possible to separate and analyze even the most hydrophobic lipids with much greater accuracy than ever before possible.

Such technological advances have contributed to the advancement of lipidomics, the discipline that studies the large-scale changes in lipid composition accompanying perturbations of biological systems (see Note 1). The ultimate goal of lipidomics is to understand the role of lipids in the biology of living organisms. It represents a rapidly evolving tool in system biology, which integrates multidisciplinary sets of data derived from molecular-profiling techniques such as genomics, transcriptomics, and proteomics. Therefore, there is a growing scientific interest in using lipidomics to answer various biological questions, arising from living organisms with all degree of biological complexity, such as animals, plants, fungi, protists, bacteria, archaea, and viruses. Lipidomic approaches can be used to investigate the following main research areas:

1.1. Ecophysiology
Lipidomics can be applied to study the impact of both abiotic environmental factors (e.g., climate, radiation, toxins, gravity, CO₂ and oxygen levels, insolation, light-dark cycle, habitat) and biotic environmental factors (e.g., plants, animals, pathogens, and micro-organisms) on lipid metabolism (2–5).

1.2. Nutrition
Lipidomics is applied to study the effect of nutrients (e.g., carbohydrates, fats, proteins, vitamins, minerals, water, and beverages), nutraceuticals (e.g., antioxidants, fibers, omega-3 fats), food additives and fertilizers on lipid metabolism (6, 7).

1.3. Pharmacology and Toxicology
Lipidomics can be utilized to study the effects of pharmacological treatments (e.g., medications, vaccinations) and other synthetic products (e.g., cosmetics, contaminants, drugs of abuse, chemicals) on lipid metabolism (9, 10).

1.4. Genetics, Transcriptomics, and Proteomics
Lipidomic approaches can be used to study the effects of genetic diversity (e.g., genotypes, epigenetic regulation, mutations, and polymorphism), messenger RNA expression profiles and protein diversity (e.g., isoforms, post-translational modifications, cofactors) on lipid metabolism. Furthermore, lipidomics can be applied to investigate the biological functions of genes and proteins by...
studying the lipid profiles associated with genetic manipulation (i.e., gene overexpression or knock down) in biological systems. Indeed, fluctuations in lipid composition can be used to uncover alterations in the transcriptome and proteome (11, 12).

1.5. Biophysics

Lipidomic approaches can be used to investigate the effects of lipid composition on biophysical parameters (e.g., fluidity, compressibility), the biological functions of membrane structures (e.g., lipid rafts), as well as lipid–protein and lipid–nucleic acid interactions (13).

1.6. Cell Biology

Lipidomics can study the role of lipid metabolism in critical cellular processes (e.g., cell cycles, survival/death, morphology, organelles) together with the circadian regulation of biological processes (e.g., development, aging, hormone production, hunger, thirsty, sleep) (14–17).

1.7. Biochemistry and Molecular Biology

Lipidomic approaches can be used to understand the biochemical mechanisms for the biosynthesis and the metabolism of lipids in living organisms. In this area of research, lipidomics may lead to the discovery of novel lipid molecular species and lipid-related biochemical pathways (e.g., enzymes, proteins, receptors, and genes) (18, 19).

1.8. Physiology and Psychology

Lipidomics can be applied to understand the physiological roles played by endogenous lipids in crucial biological processes (e.g., learning and memory, immune response, pain, and inflammation) (20, 21). Also, lipidomic approaches can unveil the role of lipids in the sensory perception (chemoreception, photoreception, mechanoreception, and thermoreception), in mental processes and behavior (e.g., cognition, emotion, personality, social, and sexual behaviors), and physical activity (e.g., exercise) (22).

1.9. Pathology and Disease Diagnostics

Lipidomic strategies can be used to investigate the role of lipid metabolism in the pathology of plant and animal diseases. Indeed, epidemiological studies revealed that many human diseases are characterized by specific alterations in lipid metabolism (e.g., cancer, obesity, diabetes, insomnia, depression, stress, trauma, dementia, as well as infectious, cardiovascular, and neurodegenerative diseases) (23, 24). Therefore, lipidomics can be used to profile lipid composition of biological samples for disease diagnosis and drug discovery. In fact, lipid composition can provide a “snapshot” of the biological state of an organism and, consequently, be considerate as an index (biomarker) of healthy or diseased state (25, 26). Furthermore, such lipid biomarkers can serve also as indicators of pharmacologic responses to a therapeutic intervention (24).
One of the main challenges of lipidomic analysis is the range of concentrations and chemical complexity of lipid compounds in biological samples (27) (Scheme 1). In fact, a comprehensive lipidomic analysis is expected to take into consideration “structural lipids” (e.g., phospholipids, which serve both as building blocks of the cell membranes and as precursors for signaling lipids), “storage lipids” (e.g., triacylglycerols, which are hydrolyzed to produce either energy or signaling lipids) and the less abundant, but equally important, “signaling lipids” (e.g., fatty acids and their derivatives) (Scheme 1). Therefore, there is a need to develop analytical approaches that allow for the comprehensive analysis of structural, storage, and signaling lipids.

In this chapter, we present current methodologies utilized in our laboratory for lipidomic analysis of biological samples. We describe in some detail an analytical approach that combines sample preparation, chromatographic, and intrasource ionization separation coupled to mass spectrometry for analyzing the lipid composition of cells, biological fluids and tissues (see Note 2).

2. Materials

2.1. Equipment

1. Analytical balance.
2. Chemical fume-hood.
3. Homogenizer.
4. Vortex.
5. Centrifuge.
6. Pierce Reacti-Therm III Heating/Stirring Module Thermo Fisher Scientific (Somerset, NJ, USA).
7. Spectrophotometer for protein measurement.
8. Agilent 1200-LC system (with autosampler) coupled to Ion-Trap XCT or single quadrupole 1946D MS detectors and interfaced with ESI or APCI (Agilent Technologies).
9. Gas: ultra-high purity compressed helium (for MS fragmentation) and high-purity N₂ (for drying samples and for atmospheric pressure ionization functioning).

2.2. Reagents

A representative list of internal standards may include the following lipids.

1. Fatty acyls
   Fatty acid: heptadecanoic acid from Nu-Chek Prep (Elysian, MN, USA); d₈-arachidonic acid from Cayman Chemicals (Ann Arbor, MI, USA);
Scheme 1. Lipid classes. Chemical classification of lipids. (a) Fatty acyls are fatty acids and their derivatives (oxygenated, amides, esters). (b) Glycerolipids are fatty acid esters of glycerol and comprise mono-, di-, and tri-acylglycerols. (c) Glycerophospholipids contain phosphoric acid in ester form with a glycerolipid. (d) Sphingolipids contain a common sphingoid base moiety. (e) Sterol lipids contain a fused four-ring core. 

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Prostaglandin: d₃-prostaglandin E₂ from Cayman Chemicals (Ann Arbor, MI, USA).

Fatty-acid ethanolamide: heptadecenoylethanolamide (synthesized as previously reported, see (28)).

2. **Glycerolipids**
   - Triacylglycerol: Trinonadecenoin from Nu-Chek Prep;
   - Diacylglycerol: dinonadienoyl-sn-glycerol from Nu-Chek Prep;
   - Monoacylglycerol: monoheptadecanoyl-sn-glycerol from Nu-Chek Prep; d₈-2-arachidonoyl-sn-glycerol from Cayman Chemicals.

3. **Glycerophospholipids**
   - Phosphatidylethanolamine: 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine from Avanti polar Lipids;
   - Phosphatidyglycerol: 1,2-diheptadecanoyl-sn-glycero-3-phosphoglycerol from Avanti Polar Lipids;
   - Phosphatidylcholine: 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine from Avanti Polar Lipids;
   - Phosphatidylserine: 1,2-diheptadecanoyl-sn-glycero-3-phosphoserine from Avanti Polar Lipids;
   - Phosphatidylinositol: 1,2-dipalmitoyl-sn-glycero-3-phosphoinositol from Avanti Polar Lipids.

4. **Sphingolipids**
   - Ceramide: N-Lauroyl-ceramide from Avanti Polar Lipids;
   - Sphingomyelin: N-Lauroyl-sphingomyelin from Avanti Polar Lipids.

5. **Sterol lipids**
   - Cholesterol: d₇-cholesterol from Avanti Polar Lipids.

6. **Solvents and chemicals**
   - Water, methanol, chloroform (HPLC grade) are purchased from Thermo Fisher Scientific (Somerset, NJ, USA). Acetic acid and ammonium acetate are from Sigma (Saint Louis, Missouri, USA).

### 2.3. Supplies

1. LC columns.
2. Glass Vials (8 ml, 40 ml, 1.5 ml for autosampler and LC analysis).
3. Glass pipettes (5 ml, 10 ml).
4. Glass Pasteur pipettes.
5. Caps with Teflon-liner.
6. Conical insert for reducing the volume of the autosampler vials.
7. Vial racks.
8. Vial trays.
9. Dry ice.
3. Methods

3.1. Sample Preparation for Lipidomics Analysis

Sample preparation includes the extraction of lipids from the biological matrix and the removal of any nonlipid contaminants from the extract (see Note 3). We use a modified Folch procedure for lipid preparation of biological samples from cells, biological fluids, and tissues (see Notes 4 and 5) (Scheme 2). Lipid molecular species are quantified by normalizing the individual molecular ion peak intensity with an internal standard for each lipid class. Therefore, a mixture of nonendogenous lipid species used as internal standards for each lipid class before the extraction process (see Subheading 2.2). These internal standards allow the lipid levels to be normalized for both extraction efficiency and instrument response.

3.1.1. Cells

1. Label 8-ml glass vials based on the number of tissue samples to analyze.
2. Wash cells with phosphate-buffered saline (PBS, 1×), remove all PBS.
3. Add 1 ml of methanol containing the internal standards to each well keeping the plate on ice.
4. Scrape and collect the cells in 8-ml glass vials (see Note 6).
5. Sonicate in ice for 10 s (5 pulses (×2) at 200 V).
6. Save 20 µl aliquot for protein measurements. Protein concentration is measured using the Bradford protein concentration assay (Bio-Rad Laboratories Inc., Hercules, CA) or the BCA protein assay (Pierce, Rockford, IL).
7. Add 2-ml chloroform and vortex for 10 s.
8. Wash with 0.75 ml of water (or better 0.7% KCl solution) and vortex for 10 s.
9. Centrifuge 1,000 × g for 15 min at 4°C to separate the mixture into two phases with a protein disk at their interface. The lower phase is mainly chloroform and contains most of the lipids; the upper phase is methanol and water containing more polar metabolites.
10. Take the organic (bottom) layer using a glass Pasteur pipette and transfer into another 8-ml glass vial. Discard the protein disk and the upper (aqueous) phase.
11. Dry down using a gentle N₂ steam.
12. Resuspend in 50–100 µl chloroform/methanol (1:3, vol:vol).
13. Transfer into 1.5-ml glass vials with the 0.2-ml conical inserts and proceed to LC/MS analysis.
14. Normalize lipids for mg protein (mol/mg protein).
Tissues are rapidly collected and snap-frozen in liquid N\textsubscript{2}.

1. Label 8-ml glass vials according to the number of tissue samples to analyze.

2. Add 1 ml of methanol containing the internal standards in each vial, while keeping the vials in ice.

3. Weigh the frozen tissues (10–100 mg) \textit{(see Note 6)} and transfer them into the previously prepared vials containing methanol with internal standards.

4. Homogenize the tissues keeping the vials in an ice bath and collect 20 μl aliquots for protein measurements \textit{(see Note 7)}.

5. Add 2 ml of chloroform and vortex for 10 s.

6. Wash with 0.75 ml of water (or better 0.7% KCl solution) and vortex for 10 s \textit{(see Note 8)}.

7. Centrifuge at 1,000 × g for 10 min at 4°C to the mixture into two phases with a protein disk at their interface. The lower phase is mainly chloroform and contains most of the lipids; the upper phase is methanol and water containing more polar metabolites \textit{(see Note 9)}. 

\begin{figure}
\centering
\includegraphics[width=\textwidth]{lipidomicApproach.png}
\caption{Lipidomic approach. Flow chart of the strategy used for a broad-range analysis of lipids from biological sample. MAG monoacylglycerol, FAE fatty acid ethanolamide, LPC lysophosphatidylcholine, FA fatty acid, oxFA oxygenated fatty acids, DAG diacylglycerol, TAG triacylglycerol, SP sphingolipid, PC phosphatidylycholine, PE phosphatidylethanolamine PS phosphatidylerine, PI phosphatidylinositol; PG phosphatidylglycerol; PA phosphatidic acid.}
\end{figure}
8. Prepare another set of 8-ml glass vials using the same labeling system as described before.
9. After centrifugation, collect the lower (organic) phase using a glass Pasteur pipette.
10. Re-extract the protein disk and the upper (aqueous/methanol) phase with 2-ml of chloroform.
11. Centrifuge and add together the two organic phases.
12. Evaporate the solvent to dryness in the vials using a gentle N₂ stream.
13. Resuspend in 50–100 µl chloroform/methanol (1:3, vol:vol).
14. Transfer the resuspended lipid solution to the 1.5-ml vials with the 0.2-ml conical inserts and proceed to LC/MS analysis.
15. Normalize lipid amount per grams of tissue (mol/g) or per mg protein (mol/mg protein).

3.1.3. Biological Fluids

Plasma and Serum

For plasma preparation, blood is centrifuged in EDTA-containing tubes at 1,000 × g for 10 min at 4°C, and the top layer (plasma) is recovered using a glass Pasteur pipette. For serum preparation, blood is immediately centrifuged in glass tubes at 1,000 × g for 10 min at room temperature and the top layer (serum) is recovered using a glass Pasteur pipette.

Cerebrospinal Fluid (CSF)

CSF samples are checked for blood contamination by measuring the total cell count, total protein, CSF/serum albumin and IgG quotients, and determination of oligoclonal bands by isoelectric focusing and silver staining. Normal cell counts, normal CSF/serum albumin ratios, and no oligoclonal bands indicate healthy blood–brain barrier function and lack of intrathecal immunoglobulin G synthesis.

1. Label 8-ml glass vials according to the number of tissue samples to analyze.
2. Transfer 0.2 ml of plasma/serum/CSF samples into the 8-ml vial in ice.
3. Add three volumes of ice-cold acetone containing internal standards.
4. Vortex for 10 s.
5. Shake and refrigerate sample for 30 min.
6. Centrifuge at 1,000 × g for 10 min at 4°C to pellet out the precipitated proteins.
7. Take the supernatant and evaporate the excess acetone under N₂ stream.
8. Add 1-ml of methanol and vortex for 10 s.
9. Add 2-ml of chloroform and vortex for 10 s.
10. Wash with 0.8 ml of water (or better 0.7% KCl) and vortex for 10 s.
11. Centrifuge at 1,000 × g for 10 min at 4°C.
12. Collect the lower phase with a glass Pasteur pipette and transfer to a clean 8-ml glass vial.
13. Evaporate the eluates to dryness under N₂ stream.
14. Resuspend in 50–80 µl of a solution chloroform/methanol (1:3, vol:vol).
15. Prepare 1.5-ml vials with conical glass inserts.
16. Transfer the resuspended lipid solution to the 1.5-ml vials with 0.2-ml conical insert and proceed to LC/MS analysis.
17. Normalize lipid amount per ml of biological fluid (mol/ml).

Blood

Blood can be fractionated in plasma and blood cells, which is made of white blood cells (WBCs) and red blood cells (RBCs). Blood cells are normally discarded when collecting the plasma. However, the same procedure used for plasma collection, also allows the recovery of the buffy coat (mainly WBCs) and the RBCs, which can be used to measure biomarkers for dietary fat (29) and diseases (30).

1. Fractionate whole blood samples by centrifuging in EDTA at 1,000 × g for 10 min at 4°C. This will separate the blood into an upper plasma layer, a lower RBCs layer, and a thin interface (buffy coat) containing the WBCs.
2. Recover the plasma.
3. Recover the WBCs, wash with PBS (1×) three times and centrifuge discarding the supernatant; freeze in distilled water (1:1, vol:vol).
4. Recover the RBCs, wash with PBS (1×) three times and centrifuge discarding the supernatant; freeze in distilled water (1:1, vol:vol).

3.2. LC/MS Analysis of Lipids

Mobile phase A is methanol containing 0.25% acetic acid and 5 mM ammonium acetate; mobile phase B is water containing 0.25% acetic acid and 5 mM ammonium acetate. Lipids are identified based on their retention times and MS² properties.

3.2.1. Small Lipids Analysis

Small lipid molecules are separated using a reversed-phase Zorbax XDB Eclipse C-18 column (50 × 4.6 mm i.d., 1.8 µm particle size, 80 Å of porous diameter, Agilent Technologies). Detection and analysis is controlled by Agilent Chemstation and Bruker Daltonics software.

1. Fatty acyls

Lipids are eluted using a linear gradient from 90% A to 100% B in 2.5 min at a flow rate of 1.5 ml/min with column
temperature at 40°C. ESI is in the negative mode, capillary voltage is set at −4 kV and fragmentor voltage is 100 V. N₂ is used as drying gas at a flow rate of 13 l/min and a temperature of 350°C. Nebulizer pressure is set at 60 psi. We use commercially available fatty acyls as reference standards. They are analyzed monitoring the mass-to-charge ratio (m/z) of the deprotonated molecular ions [M – H]⁻ in the selected-ion monitoring mode (Fig. 1).

3.2.2. Large Lipids Analysis

Large lipid molecules are separated using a reversed-phase Poroshell 300SB C-18 column (2.1 × 75 mm i.d., coating layer of 0.25 μm on total particle diameter of 5 μm, 300 Å of porous diameter, Agilent Technologies). Lipids are identified based on their retention times and MSⁿ properties. Detection and analysis is controlled by Agilent Chemstation and Bruker Daltonics software.

1. Glycerolipids, glycerophospholipids, sphingolipids

A linear gradient is applied from 85% A to 100% B in 5 min at a flow rate of 1.0 ml/min with column temperature set at 50°C.
The capillary voltage is set at 4.0 kV and skimmer voltage at 40 V. N₂ is used as drying gas at a flow rate of 10 l/min, temperature at 350°C and nebulizer pressure at 60 psi. Helium is used as collision gas, and fragmentation amplitude is set at 1.2 V. MS detection is both in the positive and in the negative ionization modes. Ion charge control is on, smart target set at 50,000 and max accumulation time at 50 ms, scan range of 100–1,500 amu, 26,000 $m/z$ per second.

3.2.3. Sterol Lipids Analysis

Lipids are separated using a linear gradient from 75% A to 100% B in 4-min period at a flow rate of 1.0 ml/min with column temperature at 50°C. APCI is set in positive mode. Drying gas is set at 350°C and a flow of 8 l/min. Nebulizer gas pressure is set at 30 psi and vaporizer temperature at 475°C. Capillary voltage is set at 300 V with the corona current set at 5 μA.

3.3. Results: Chromatographic and Intrasource Ionization Separation of Lipid Molecules

Lipids exist in nature in a wide variety of chemical complexities and dynamic range of concentrations (Scheme 1). In order to simplify the analysis in biological tissues, lipids are schematically divided into three main classes (a) small lipids, defined here as molecules containing one aliphatic group such as fatty acids and their derivatives (amides, esters, oxygenated compounds); (b) large lipids, molecules containing two or more aliphatic groups, such as phospholipids, diacylglycerols, triacylglycerols, sphingolipids; and (c) sterol lipids, molecules containing a rigid four-ring backbone such as cholesterol and its derivatives. Therefore, in order to analyze the different classes of lipids by LC/MS, two separate chromatographic approaches are applied, using different reversed-phase C-18 stationary phases (Scheme 2). Furthermore, because lipid classes with different functional groups have characteristic ionization efficiencies, a combination of ESI set in either positive or negative mode, and APCI set in positive mode is used (Scheme 2).

3.3.1. Small Lipids Analysis

To separate lipids containing one fatty acyl group, a reversed-phase C-18 column packed with conventional porous silica particles of small spherical diameter (sub-2 μm) is used. Fatty acyl species are separated both by chain length and by degree of unsaturation of their fatty acid chains. For example, fatty acids containing shorter or more unsaturated acyl chains elute earlier than those with longer and more saturated chains (Fig. 1). Generally, in positive ESI mode small lipids are detected as protonated molecular ions or sodium and ammonium adducts. In contrast, in negative mode small lipids are detected as deprotonated molecular ions (Fig. 1).

3.3.2. Large Lipids and Sterol Lipids Analysis

To separate large and sterol lipids, a reversed-phase C-18 column packed with superficially porous particles (Poroshell,
Agilent-Technologies, coating layer of 0.25 μm on total particle diameter of 5 μm) is used (18, 31). This allows for fast flow rates and good peak shapes (Fig. 2). Usually, because of diffusion limits in totally porous silica, large lipid molecules give tailing peaks at high flow rates. However, superficially coated columns allow for faster diffusion at the surface, allowing high flow rates and good peak shape. Indeed, the thin shell allows the slowly diffusing hydrophobic macromolecules and the rigid structures of sterol lipids to rapidly penetrate the superficial packing material (since the solid core prevents further diffusion). To decrease the retention times, a high flow velocity is applied. To decrease mobile phase viscosity and avoid exceeding the column back-pressure limits, a relatively high column temperature is used. A combination of high temperature and high flow velocities improves the separation speed, resulting in better peak shape of the lipid analytes. Notably, lipids are stable at high temperatures using high flow rates (32). Although lipids are separated when differing in a single fatty acyl chain, their combinatorial nature makes only a partial separation of the isomeric species possible (Fig. 2). Therefore, to obtain more information on the lipid structure, LC separation is coupled with MS* fragmentation data (Fig. 3). Generally, large lipids are detected in the positive ESI mode as sodium or ammonium adducts or as deprotonated molecular ions in the negative mode. For sterol lipids, which are highly hydrophobic and hard to ionize, APCI is used in the positive mode and the protonated molecular ions are detected after loss of water.

Fig. 2. Analysis of large lipids by LC/MS. Representative LC/MS chromatograms of phosphatidylethanolamines (PE) extracted from biological samples.
Fig. 3. Analysis of large lipids by MS/MS. Representative MS/MS spectrum of a selected phosphatidylethanolamine (1-stearoyl,2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine) derived from biological samples.
3.4. Maintenance and System Suitability Test for LC/MS Analysis

3.4.1. LC/MS Maintenance

In the following sections we report a general procedure for the maintenance and system suitability testing used to validate the LC/MS lipidomic analysis.

To avoid contaminations, routinely preventive maintenance is performed.

1. Replacing the spray needle and electron multiplier; cleaning ionization spray chamber or other accessible MS components.
2. Replacing inline filters and frits, the injector needle and capillaries or other accessible LC components; flushing the system with a mixture of cyclohexane/acetonitrile/isopropanol (1/1/2, v/v/v).

3.4.2. Quality Assurance

1. To check for the LC column status, assure that the column has a constant backpressure, which usually is a guarantee of good column performance. Increased pressure indicates column contamination or fouling.
2. To check for contaminations, blank samples are run before and between biological samples.
3. To check for accuracy of quantification, quality control samples are run at the end of the run (three concentrations that are representative of the concentration range of the analyte of interest).
4. To check for linearity of the detection response, calibration curves are run before running the samples.
5. To avoid sample cross-contaminations, the injector needle is washed automatically between each sample injection.

3.4.3. Storage of Lipid Extracts

Lipid extracts are generally stored in a freezer at –80°C. They are solubilized in chloroform–methanol solutions using glass vials closed with Teflon-lined caps and secured with Parafilm. To prevent oxidation, air is removed by flushing the vials or tubes with N₂ before closing them. It was shown that after storage up to 4 years at –80°C, the blood lipid composition is practically unchanged (33). If storage is brief, lipids can be stored at –20°C.

3.4.4. Contaminations

Contaminants can be detected as extra-peaks or high background noise in LC/MS chromatograms. They strongly affect the specificity and sensitivity of our analysis. During sample preparation, common sources of contamination are mineral oils, grease, detergents, and plasticizers from plastics, including lipid molecules such as oleamide (34). Plastic pipettes, tips, beakers, and vials can leach contaminants into organic solutions. Therefore, all operations are generally carried out in glass and all vials or tubes are closed with screw caps including a Teflon-covered liner. Furthermore, all
operators must wear gloves during the procedures to prevent any contaminations by skin surface lipids. Change gloves frequently and keep vials closed or covered with aluminum foil.

4. Notes

1. Because lipids are a set of small-molecule metabolites, lipidomics is considered to be part of metabolomics, which is the large-scale study of all metabolites (both water-soluble and water-unsoluble) in biological organisms. The distinction originated as consequence of the metabolome (complete set of small-molecule metabolite) complexity, which required the development of analytical approaches specific for nonwater soluble metabolites (lipids) (35).

2. The described fast lipidomic approach is suitable for the determination of a broad-range of lipid alterations occurring in biological samples. The combination of the chromatographic resolving power in conjunction with the ionization source selection and the mass detection can be used to analyze even the lipids present at very low concentration. In contrast, the direct infusion of the lipid extract into the MS detector is subject to ionization suppression effects and loss of sensitivity and accuracy. Furthermore, because lipids may differ in mass by only two units, a partial chromatographic separation helps avoid the isotopic effects, which affect the actual mass abundance (36).

3. Particular attention should be given to sample preparation: It is worth remembering that there is no good LC/MS analysis without a good sample preparation.

4. Alternative extraction procedures that use less toxic organic solvents such as methyl-tert-butyl ether (37), hexane–isopropanol, and ethyl acetate/ethanol mixtures have been proposed for a wide range of tissues (38, 39). Surprisingly, it is not always made clear in the laboratory environment that methanol and chloroform are toxic and potentially carcinogenic (38, 40, 41). Furthermore, the methanol/chloroform mixture is extremely irritating to skin and eyes. Therefore, it is particularly important to train students and new laboratory personnel to handle organic solvents with gloves in a chemical fume-hood, avoiding health-hazard by accidental spills, skin contact and breathing of vapors.

5. For the recovery of acidic phospholipids such as gangliosides and phosphoinositides, alternative extraction methods have
been suggested which use strong HCl solutions instead of water during the washing step of the Folch procedure\textsuperscript{42}.

6. Lipid composition is altered during thawing at room temperature. Therefore, to avoid tissue degeneration (1) cells are kept on ice or (2) tissue samples are cut and weighted while still frozen.

7. Sometimes it is useful to normalize the lipid levels in tissue samples by protein amount. Indeed, very small amount of tissue are often difficult to weigh without thawing them and, consequently, altering the lipid composition. Therefore, the samples are directly added to methanol (without the weighting step) and prior to extraction, 20 μl aliquots from the homogenate solutions are taken for protein measurements, which can be conducted using the Bradford protein concentration assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) or the BCA protein assay (Pierce, Rockford, IL, USA).

8. The lipid extraction requires a ratio of chloroform, methanol, and water of 8:4:3. In these conditions, after centrifugation and phase separation, the approximate proportion of chloroform, methanol, and water in the upper phase is 3:48:47 by volume. In the lower phase, the respective proportion is 86:14:1.

9. To avoid contaminations from the upper aqueous phase into the pipette tip during the recovery of the bottom phase, insert the glass Pasteur pipette through the upper phase with gentle positive-pressure (i.e., gentle bubbling). Also, carefully withdraw the bottom phase through the pipette from the bottom of the vial. Furthermore, to avoid the interface or upper phase, it is better not to recover the entire bottom phase, but leaving the last drops (5–10% of the total organic phase) in the vials.

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