Chapter

Analytical Tools for Lipid Assessment in Biological Assays

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Abstract

Lipids are heterogeneous biological molecules with many important roles. In human body, lipids can be energy substrates, steroid hormones, inflammatory lipid mediators, transporters, and feature as structural cell and organelle membrane elements. At the cell membrane, lipids influence the distribution of surface proteins and, in part, protein signaling and, consequently, the activation of transcriptional factors. One of the best explored relationships in chemistry and science is the structure/activity one. Therefore, if the composition of a mixture is discovered and the structure of its components is known, a task of proposing relationship among all components and their activity would be closer to understanding. There are many powerful and advantageous analytical and bioanalytical tools available for the study of lipids, but all show at least some limitations. Knowing the advantages/disadvantages of each technique is essential for choosing the most appropriate one for the analysis as to answer a scientific question about lipid composition and role within a biological model. Often, inexperience and little familiarity with the cited analytical resources may limit the validity of the obtained results. Our chapter aims to present and discuss different tools available for the study of lipids and their main applications in biological assays.

Keywords: lipids, bioanalytical tools, gas chromatography, mass spectrometry, nuclear magnetic resonance

1. Introduction

Lipids are a very heterogeneous group of biological molecules. Some of the most studied lipids are built from the fatty acids (FAs) or isoprenyl groups. FAs are carboxylic acids composed by an even number of carbon atoms connected by single or double bonds with a methyl group end. FAs can be classified into very long (>20 carbons), long (14–20 carbons), medium (6–12 carbons), and short (up to 6 carbons)-chain FAs, as well as saturated (no double chains), monounsaturated (1 double bond), and polyunsaturated (PUFAs, >1 double bond) FAs. Furthermore, unsaturated fatty acids can receive its omega (n) assignment according to the first double-bond position from the end methyl group. Biosynthetically, endogenous FAs have been made from acetyl-CoA/malonyl-CoA [1–3].

FAs represent a class of lipids on their own and do not make part of all lipids [4]. Some lipids, which are not formed from FAs but are biosynthetically related to them, are the polyketides, formed from the acetyl units. Other unsaponifiable lipids

are built from isoprene units, molecules with five carbons with a branch structure and alternated double bonds. Isoprenes have their biosynthesis in mevalonate (vegetables) or deoxyxylulose phosphate (animals) pathways. They can form sterols and prenols [2]; some sterols can also have FAs in their structure [3].

Actually, lipids comprise eight main classes within different chemical characteristics: fatty acids (1), glycerolipids (2), glycerophospholipids (3), sphingolipids (4), sterols (5), prenols (6), saccharolipids (7), and polyketides (8) (**Figure 1**) [3]. These classes show a high diversity of molecules and are grouped into several subclasses. Lipid classification based on their chemical information, described by the head-group and the type of a linkage between the head group and aliphatic chains [5, 6] is the most used among biochemists. Investigators have estimated the presence of ~180,000 lipid species in nature and ~40 common fatty acids as building blocks [7]. At the moment, 43,109 structurally distinct lipids are already registered at the Lipid MAPS consortium.

The high diversity of lipids reflects their multiple biological functions and can be attributed to the wide variety of their building blocks and numerous possible permutations [6, 8]. In the human body, lipids serve as: substrates for the synthesis of energy (9.3 kcal/g), steroid hormones, inflammatory lipid mediators, vitamins or liposoluble vitamins transportation, and structural elements of cell and organelle membranes [9–11]. As a part of the cell membrane, lipids can influence the distribution of surface proteins, protein signaling (as part of lipid rafts or as second messengers), and consequently, the activation of transcriptional factors [12, 13]. This means that besides their recognized biological functions, lipids can influence protein signaling and synthesis.

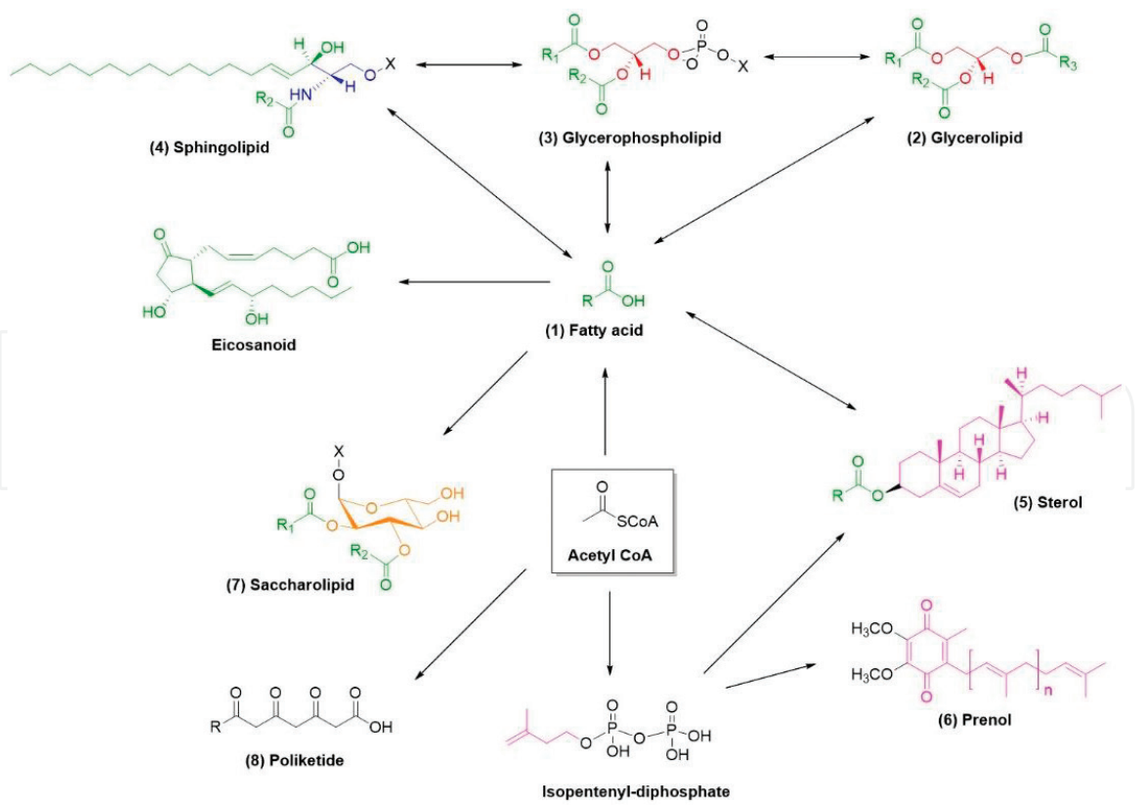

Figure 1.
Biosynthetic lipid network. Acetyl-CoA: fatty acids-FAs (class 1) are synthetized, enabling the production of other lipid classes: 2 (glycerolipids-GLs), 3 (glycerophospholipids-GPs), 4 (sphingolipids-SPs), and 7 (saccharolipids-SLs), as well the class of eicosanoids. Acetyl-CoA can also generate the class 8 (polyketides-PKs) and isopentenyl diphosphate molecule, through mevalonate. On the other side, isoprenyl is used as starting substrate for producing lipid classes 6 (prenols-PRs) and 5 (sterols-STs). Figure was inspired on Quhenberger et al. [4].

In a cell, lipids show different compositions, tens of thousands to hundreds of thousands of compounds, and concentrations from a mol/mg to nmol/mg of protein [5]. Facing the biological relevance of lipids, it is not surprising that the human organism has sophisticated machineries for the FA synthesis when its dietary supply flaws. Saturated and monounsaturated FAs can be endogenously generated from glucose and amino acids through enzymatic elongation (by adding units of two carbons) and desaturation (by forming new double bonds) reactions. However, a pitiful lack of the desaturating enzymes Δ-12 and Δ-15 desaturases preclude humans to add double bonds before the ninth carbon at the end of the methyl extremity for the synthesis of the polyunsaturated fatty acids (PUFAs) n-linoleic acid (C18:2 n-6, LA) and alpha-linolenic acid (C18:3 n-3, ALA). Consequently, LA and ALA are obtained exclusively from diet and, then, called as essentials. After ingestion, LA and ALA compete for sequential enzymatic processes of elongation and desaturation until their conversion into longer chain PUFAs: arachidonic acid (C20:4 n-6, ARA) from LA and eicosapentaenoic acid (C20:5 n-3, EPA) or docosahexaenoic acid (C22:6 n-3, DHA) from ALA [14].

ARA, EPA, and DHA have a high clinical interest once they influence the composition and steady-state of cell membranes. Also, they are precursors of the lipid mediators named eicosanoids involved in the activation of the inflammatory

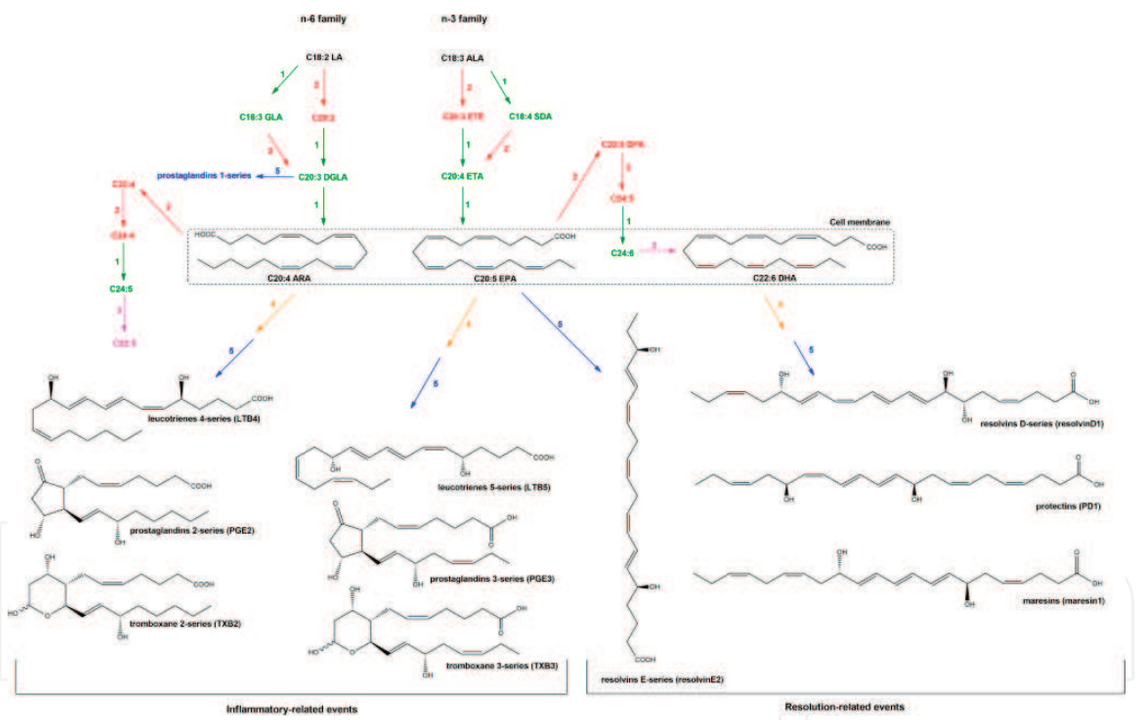

Figure 2.
Synthesis of lipid mediators from eicosapentaenoic (C20:2 n-6, EPA), docosahexaenoic (C22:6 n-3, DHA), and arachidonic (C20:4 n-6, ARA) acids. EPA, DHA, and ARA are previously synthetized from n-3 and n-6 fatty acid families in reactions mediated by enzymes: 1—desaturase, 2—elongase, 3—peroxisomal fatty acyl-CoA oxidase, 4—lipoxygenase (LOX), and 5—cyclooxygenase (COX). The cellular bioavailability of EPA decreases the production of ARA-produced eicosanoids, which include prostaglandins (PG)E2, thromboxane (TX) A2, and leukotriene (LT)B4. These eicosanoids have a higher pro-inflammatory potential than those contra parts produced from EPA (PGE5, TXA3, and LTB5) in promoting vasodilation and leukocyte chemotaxis and adhesion, events that stimulate the migration of neutrophils into the damaged tissue. As part of the neutrophil-monocyte sequence of inflammation, eicosanoids are no longer produced to initiate the synthesis of resolvins, protectins and maresins, lipid mediators from EPA and DHA. Other fatty acids shown are: linoleic acid (C18:2 n-6, LA), gamma-linolenic acid (C18:3 n-6, GLA), dihomo-gamma-linolenic acid (C20:3 n-6, DGLA), adrenic acid (C22:4 n-6), tetracosatetraenoic acid (C24:4 n-6), tetracosapentaenoic acid (C24:5 n-6), docosapentaenoic acid (C22:5 n-6), oleic acid (C18:1 n-9), octadecadienoic acid (C18:2 n-9), alpha-linolenic acid (C18:3 n-3, ALA), stearidonic acid (C18:4 n-3, SDA), eicosatrienoic acid (C20:3 n-3, ETE), eicosatetraenoic acid (C20:4 n-3, ETA), docosapentaenoic acid (C22:5 n-3, DPA), tetracosapentaenoic acid (C24:5 n-3), and tetracosahexaenoic acid (C24:6 n-3).

response. While ARA is a precursor of pro-inflammatory, immunosuppressive, and pro-thrombotic eicosanoids, EPA competes with ARA for lipoxygenase (LOX) and cyclooxygenase (COX) enzymes to generate functionally less intense and anti-thrombotic mediators [10]. Furthermore, EPA and DHA are precursors of resolvins and DHA is a precursor of protectins and maresins. These lipid mediators are collectively called as specialized pro-resolving mediators and have a relevant role in the inflammation resolution and homeostasis restoring [15]. In conjunction, these observations traduce an anti-inflammatory and pro-resolving potential of EPA and DHA (**Figure 2**).

Moreover, EPA, DHA, and their metabolites can exert anti-inflammatory and metabolic effects by modulating the activity of transcriptional factors, such as nuclear kappa B factor (NFκB), nuclear factor E2-related factor 2 (Nfr2), peroxisome proliferator-activated receptor (PPAR), and sterol regulatory element-binding proteins (SREBP). Due to their abilities, EPA and DHA can influence the transcription of genes enrolled in inflammation, cell survival, oxidative stress, and in carbohydrate and lipid metabolism [16]. Some of the EPA and DHA functions arise from the capacity of these n-3 PUFAs (mainly DHA) to interfere in protein receptors signaling by disrupting lipid rafts, membrane microdomains rich in saturated FAs (mainly cholesterol) who confer a rigidity needed for some protein dimerization through the fluid cell membrane [17, 18].

Due to biological properties, the importance of EPA and DHA for human health has been highly discussed and investigated by basic, translational, and epidemiologic scientists. However, studies on lipids and their biological relevance are not limited to n-3 PUFAs or other individual lipids, but also include the analysis of all

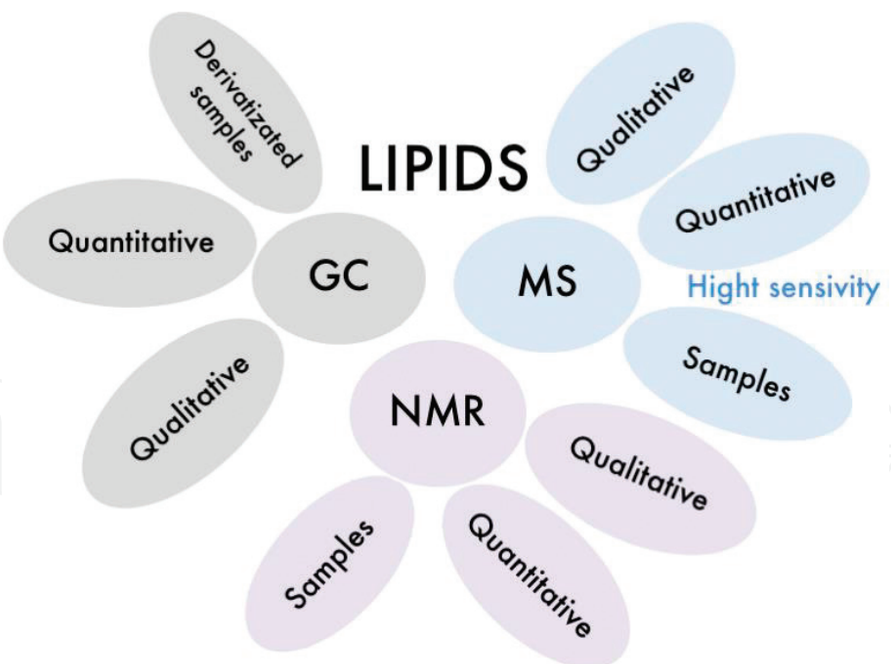

Figure 3.
The most common analytical techniques used in analyses of lipids and lipidomes are gas chromatography (GC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) spectroscopy. These techniques show some vantages and weaknesses and could be used in combination with other techniques in so-called hyphenated bioanalytical methods. All enable qualitative and quantitative analyses of lipids, but GC needs additional step in sample preparation as to increase the volatility of the compounds; thus, not all lipids could be analyzed by GC. Also, GC requires greater sample quantities when compared to MS, which is most sensitive. MS analyses require the use of ionization techniques, such as electron and chemical ones for gas samples, while electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are usually applied for liquid and solid samples. NMR is the only nondestructive technique and allows the noninvasive lipid analysis in intact cells and tissues, and enables to investigate changes in lipid and dynamic structures in biochemical cell functionalization, but it is not sufficiently sensitive and universal when compared to MS.

lipid species from a biological sample—the lipidome. Because lipids are intermediates and even signaling molecules of metabolic pathways, the lipidomic response (change of the lipidome pattern of a biological sample) to nutritional, pharmacological, or any intervention (i.e., surgery, exercise) treatments can reflect their biological effects [5]. Studies on lipidome can also add to the knowledge on the lipid content of a nutritional source (i.e., fish) aiming to found ones with the high n-3 PUFAs, for instance. These are examples of many applications of lipid analysis in biological systems.

There are several tools available for the study of individual lipids and lipidome (the total lipid content in a cell or an organism), all with their advantages and limitations (**Figure 3**). Understanding these points is essential for the application of that most appropriate techniques to answer a scientific question on lipids within a biological model. Often little familiarity with these analytical resources may limit the validity of the results. This chapter aims to present and discuss different tools available for different applications in the study of lipids aiming to assess biological hypothesis, with focus on nutrition and metabolism aspects.

2. Gas chromatography: principles, strengths, and weaknesses

According to the principles of chromatographic techniques, the gas chromatography (GC) is applied when aimed to separate organic compounds from a mixture in the gas form. For this purpose, the GC uses interaction among the sample components and the stationary phase and the mobile gas phase. After lipid extraction, the samples (lipid mixture) are usually liquids and must be exposed to a high temperature at the gas chromatograph entrance (injector). Vaporized, the samples are carried by a gas, which is usually a nonheavy and inert gas (i.e., hydrogen, helium), through a long capillary column containing a high or low polarity material (stationary phase) [19].

The gaseous compounds generated from the vaporized sample interact with the stationary phase what allows each compound to elute/separate at a different time (retention time). Because GC considers both chemical and physical properties of the vaporized compounds, those with more chemical affinity to the stationary phase will take longer time to be removed from the column and the temperature will influence the overall process. This explains why the column stays in an oven, which is programmed to work at different temperature ranges (i.e., temperature programming) in which the compounds are carried out by the gas according to their boiling point until they get to an electronic detector [20].

At the end of GC analysis, the electronic detector generates a chromatogram based on retention time by intensity. This allows a qualitative identification of the lipid compounds by comparing their retention times with certified standard using the flame ionization detector (FID) or by deduction of spectra information using a mass spectrometer as detector. Lipid quantification can also be performed using analytical procedures of external or internal certified standard in GC analysis [21].

Main points to be considered when assessing FAs by GC analysis are the carrier gas flow rate, column length, and the temperature because these can influence the order or retention time of the lipid compounds and then must be precisely standardized [22]. The column length of the stationary phase influences the resolution of the analytes, once the number of theoretical plates (hypothetical zone in which two phases establish an equilibrium with each other) is respectively high in longer column. As fat and oils have high boiling points not supported by the stationary phase, a previous derivatization reaction step is required after lipid extraction from the biological sample, in which triacylglycerol and free fatty acids are transformed

into their respective free fatty esters with lower boiling points (transesterification/esterification reaction) [23]. Several methods are available for FAs derivatization [24], and the most applied ones are described in the 969.33 AOAC's method [25].

Particularly for cholesterol analysis, the samples preparation must consider a derivatization reaction. This allows to block protic sites of steroids obtained after an unsaponifiable lipid extraction [26] had been performed, and also, to diminish dipole-dipole interactions, to increase the volatility of the compounds, and to generate products with reduced polarity. Cholesterol derivatization is usually achieved by using trimethylsilyl compounds as reagents (silylation reaction). A common method for this purpose is described by Bowden and collaborators [27], in which *N*,*O*-bis(trimethylsilyl-trifluoroacetamide/trimethylchlorosilane)—BSTFA/TMCS is used.

Nowadays, other more modern analytical tools than GC (next-generation techniques) do not require sample derivatization for lipid analysis. Needed lipid derivatization can be then consider a quite limitation step of the technique. In comparison with next-generation techniques, GC also implies in using greater sample quantities. This may be the main limitation in biological assays, which usually lead with restricted sample amounts. Nevertheless, by using certified standard and a powerful detector as FID, GC has the advantage to allow a precise and complete (by burning every compound, no one is lost in the detection) quantification of lipid compounds from biological samples, not always achieved by the other analytical techniques. In this context, GC continues to be accepted as an efficient and simple technique for FA and sterol analyses, mainly when combined with mass spectrometry (MS, detailed later in this chapter).

2.1 Gas chromatography: application in biological assays

In biological issues, GC is largely applied to assess the FA and cholesterol contents in animal models or human fluids and tissues, as biological markers of FA ingestion and cell incorporation. The technique is a powerful tool in studies assessing the effect of FA supplementation on a specific biologic response. For instance, the endogenous synthesis of EPA and DHA from ALA is low in humans, who have in the ingestion, oily fishes as the most relevant source. Therefore, studies on n-3 PUFAs have been focused on the effect of fish ingestion or fish oil/EPA/DHA supplementation in several clinical conditions, and cell and disease models. In such studies, the treatment compliance or effectiveness can be reflected by the cell or circulate contends of n-3 PUFAs [28]. Furthermore, GC can be applied to validate data generated by other lipidomic techniques.

A practical example in using GC for treatment compliance is the study of Nogueira et al. [29] assessing the effect of n-3 PUFAs supplementation in patients with nonalcoholic steatohepatitis against placebo (mineral oil). In this study, GC highlighted a similar increase in n-3 PUFAs plasma in both n-3 PUFAs- and placebo-treated patients. Because the authors have controlled compliance of n-3 PUFAs, they were able to discover off-protocol intake of PUFAs by some patients from the placebo group. When studying biochemical markers of lipid intake and cell incorporation, the biological sample nature matters. For instance, plasma and red blood samples can reflect periods of weeks and months of FAs ingestion and their effects, respectively, while the adipose tissue is the reference method, once it reflects such variables for years [28].

The study of Ravacci et al. [30] can illustrate the use of GC to assess treatment compliance. Applying this technique, the authors were able to demonstrate that the treatment of a lineage of breast cancer cell overexpressing HER-2 with DHA increased its availability in the cell membrane and was associated with the

disruption of surface lipid rafts that sustained cell signal for survival. Regarding the use of GC for data validation, a practical example is the study of Ouldamer et al. [31], which applied the technique to validate the fatty acid information generated by the ^{1}H NMR analysis on the PUFAs n-3 DHA and EPA content in the adipose tissue of mammary tumor model in rats exposed to controlled dietary intake of lipids.

3. Mass spectrometry: principles, strengths, and weaknesses

Modernization of MS used for lipid analysis raised the concept of lipidomics. Lipidomics is an emerging science that aims to analyze the total lipid content found in a cell or tissue (lipidome) through the application of analytical chemistry principles and techniques. As a part of the omics sciences, the processes applied in the lipidomic analysis are analogous to those applied in other life-building macromolecules, such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins, called as genomics, transcriptomics, and proteomics, respectively [32].

The basic principle of the MS technique is founded on the detection of the abundance of ions by their mass/charge ratio (m/z). To allow the analysis, such ions of compounds are generated by suitable methods and ions are separated according to their m/z. Ionization techniques can break some sample's molecules into charged fragments and are chosen according to the physical state of the sample. Also, the efficiency of various ionization mechanisms for the unknown species might help when picking the most appropriate ionization technique. The most common ones for gaseous samples are the electron and chemical ionizations, while for liquid and solid samples, the electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are usually applied [33].

Advances in ESI-MS and MALDI-MS have greatly facilitated lipidomic analysis [34] and enabled a great progress in lipid metabolic discoveries. This is because ESI is one of the softest ionization techniques, in which some complex dimers and solvent adducts can also be detected at the end. The efficiency of lipid ionization in ESI is incomparably higher than achieved by other traditional MS ion sources. MALDI-MS counts on a good solubility of analytes (lipids) and a matrix (for example, 2,5-dihydrobenzoic acid) in organic solvents, and provides excellent signal-to-noise ratio and reproducibility [32].

Mass spectrometers are made from three components: the ion source (1), which converts a sample into ions that are targeted through the mass analyzer (2) and run into the detector (3). The mass analyzer acts as ions organizer (classifier) using ion m/z ratios. This component accelerates ions as they face a strong electromagnetic field. The detector measures charged particles, such as an electron multiplier [35], and the abundances of each ion present in a sample are reported.

An advantage of MS is its high sensibility. A detection limit, expressed in concentration units, goes from a mol L^{-1} to as low as fmol L^{-1} and surely shall improve as the instruments modernize. For example, the instrument response factor for any individual molecular species detected is essentially identical within experimental error after ^{13}C deisotoping if the analysis is performed properly [34]. Also, MS ion source can act as a separation device if set to selectively ionize just a certain lipid class. Thus, it is feasible to analyze different classes of lipids and individual molecular species with high efficiency without prior chromatographic separation. Nevertheless, depending on the analysis aims, MS can also be combined with a chromatography system, as GC (early mentioned) and liquid chromatography (LC) [35].

Data obtained by MS are displayed as spectra of the relative abundance of detected ions as a function of the corresponding m/z. By correlating the known masses (e.g., an entire molecule) to the identified masses, or through the

compounds deposited characteristic fragmentation pattern, MS are used to identify compounds. The MS are also used to determine the elemental or isotopic signature of a sample, the masses of particles and molecules, and to elucidate the chemical structures of molecules [36]. Database platforms, such as LIPIDMAPS, LIPID Bank, LIPIDAT, Cyberlipids, and Lipidomics expertise platform, can help to identify the lipid molecules. Then, interpretation of MS-obtained lipid data must be conducted in accordance with the literature [7].

When assessing the entire lipidome profile, i.e., lipidomics by MS or nuclear magnetic resonance (NMR, detailed later in this chapter), big-data information is generated. Therefore, lipidomics require multistatistic tools for data interpretation. The additional information to MS lipidomics is mapping of the lipid pathway. For example, diacylglycerol is an essential precursor for glycerophospholipid and glycerolipid synthesis in eukaryotes [5].

Manual data interpretation using publicly available databases (i.e., KEGG pathways and the LipidMAPS databases) may add in to lipidomic results and provide meaningful biological context to data understanding from biological point of view. Indeed, using bioinformatics software platform, one can understand the changes in lipid composition and content, and understand adaptive or pathological changes in lipid metabolism. Lipids form networks, which are used to build their inter-relationships and connect them based on known metabolic pathways. Also, these relationships and the determined quantities of lipids are used to calculate the possible contributions to the production of a particular lipid class in the network, and the masses calculated are compared with the masses determined from the lipidomic MS data.

Several parameters involving the metabolic pathways can then be derived from computational simulation, such as those associated with enzymatic activities, as those analyzed by a lipid expertise, i.e., known principles of lipid biochemistry to calculate indexes of fatty acid unsaturation, fatty acyl chain length, or fatty acid precursor/product ratios to gain insight into the function of fatty acid remodeling or other relevant lipid metabolic pathways [5, 37]. Some useful tools that can be used for this purpose are the public platforms MetaboAnalyst (available from http://www.metaboanalyst.ca), VANTED, and MAVEN [37].

Once lipids have a high discrepancy of m/z within their categories and are susceptible to ion cleavage, the main disadvantage of MS in lipid analysis is that some compounds from a mixture may be determined as the same ion and incorrectly identified. Furthermore, lipid quantification by MS may be weakened by the loss of ion information due to the random collision of lipid molecules that may preclude that all of these get to the detector, the differing abilities of lipid species to form ions and hence varying signal intensity, and the ion-quenching phenomena. The last can occur when the signal from poor ionizing lipids is quenched by more easily ionized species (therefore suppressing the former signal), which is quite avoided by prior separation of lipid species for accurate quantitation or the use of specialized MS [38]. Altogether, these factors result in a loss of sensitivity for some nonpolar lipid metabolites.

It is worth to note that the limitations in identification and quantification of lipid species by MS described above have been minimized with advances of the technique (i.e., target MS). Currently, this analytic tool is considered accurate for characterization of lipids and the most efficient one to assess lipidomes.

3.1 Mass spectrometry: application in biological assays

In biological assays, lipidomics-MS analysis is highly applied to generate information related to metabolism and biological responses, once several known

pathways from metabolic networks in eukaryotes involve lipids as metabolic intermediates (mainly sphingolipids, glycerophospholipids, glycerolipids, and nonesterified fatty acids [NEFAs]) or signaling molecules (mainly oxysterols) [5]. For instance, changes of a lipidome profile can be identified by MS, allowing the interpretation of biological responses to external interferences (i.e., by comparing the lipidome before and after a medication) or enrolled in the pathophysiology of diseases (in comparison with healthy status) [5, 32, 39].

The ionization technique applied is a relevant point to be considered when designing studies for lipid assessment in biological samples using MS. For instance, MALDI can be used to analyze changes of lipid and their metabolites in single genetically identical cells from the RAW264.7 lineage after lipopolysaccharide (LPS) stimulation, using a Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS). MALDI analysis was chosen because single cells on a plate using a histology-directed workflow can increase the number of cells analyzed. Furthermore, the speed of MALDI-IMS enables high spatial resolution and high-throughput single-cell analysis. Combined with the high sensitivity of FTICR MS, hundreds of lipids can be measured from a large population of single cells (>100) in a few hours.

Tandem MS measurements (i.e., through precursor ion scanning and neutral loss scanning experiments) are useful for biological assays requiring the identification of all lipid molecular species. These methodologies are usually better than full scan MS because they apply sequential analyzers and are often associated with a target analysis (i.e., aiming to study a molecule species). This allows high sensitivity and enhanced signal/noise ratio, facilitating the characterization of minor but biologically relevant lipid species [40].

One example of the tandem analysis application is the work of Slatter et al. [41]. By using LC-MS/MS (tandem MS), they were able to characterize the lipidomic network of human platelets, where nearly 200 oxidized species were identified. These minacious data provided by the methodology allowed to display a direct link between innate immunity and mitochondrial bioenergetics in human platelets. Procedures enabling to achieve this conclusion from generated data included the selection of lipids upregulated under thrombin activation and the analysis on temporal dynamics of their generation, monitoring precursor-to-product ion transitions in multiple reaction monitoring (MRM) modes.

Also, through tandem MS, Morgan et al. [42] have proposed a novel role for 12/15-lipoxygenase in regulating autophagy. They have used LC/ESI/MS/MS in a target approach to determine the levels of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphate (DMPA), and 1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DMPG), using comparison technique with internal standards. In addition, the 1,2-dimyristoyl-sn-glycero-3-phosphoserine (DMPS) was determined by product ions and the analysis of cholesteryl esters was performed.

4. Nuclear magnetic resonance spectroscopy: principles, strengths, and weaknesses

Along with other analytical tools available for lipidome investigations, NMR spectroscopy allows identification of characteristic signals from the different classes of lipids and provides their successful quantification [43, 44]. The technique facilitates the analysis of hundreds of metabolites in a single sample with great advantage because there is no need for a previous sample treatment [8].

The principle of NMR spectroscopy is based on the physical resonance phenomenon in which spin-active nuclei in a strong static magnetic field respond to a perturbation (radiofrequency waves) by producing an electromagnetic signal with a characteristic frequency, which matches magnetic field observed by a given nucleus. This process of resonance happens when the oscillation frequency matches the intrinsic frequency of the nuclei, which depends on the strength of the static magnetic field, the chemical environment, and the magnetic properties of the isotope involved [45].

In a practical way, NMR spectroscopy provides information of the number (integrals) of magnetically distinct atoms (chemical shift of the resonance frequencies and peak splitting due to the coupling constants J or dipolar couplings between nuclear spins in the sample) of the studied isotope and provides all necessary information for determination of the structure of unknown molecules. Several nuclei can be studied by NMR techniques, but the most commonly available ones are hydrogen-1 and carbon-13. The most common experiments for lipid analysis by NMR are ^{1}H, ^{13}C, ^{31}P, and the bidimensional experiments involving ^{1}H-^{1}H and ^{1}H-^{13}C [45].

Usually, an NMR experiment starts with insertion of a liquid sample into the magnet, then, short radio-frequency pulses (from an electronic device named probe) are applied, and all emitted frequencies from the same type of nuclei are registered and reported as signals with a given chemical shift, multiplicity, and intensity. Also, multidimensional NMR as well as solid-state NMR has emerged to provide additional and relevant information on sample composition [45].

Also, the exact ratio of specific fatty acids in the lipid samples and their iodine values could be calculated considering integral values corresponding to characteristic peaks with the help of the corresponding spectral information and the existing references [46]. This type of experiment works as a relative quantification. Absolute and relative quantification experiments by NMR are possible; however, it is necessary to take care of some precautions. Direct quantitative information by NMR is due to the fact that the signal intensity of each resonance in the NMR spectrum is directly proportional to the number of spins associated with the particular resonance [38]. Thus, no standard with chemical similarity to the studied compounds is required as in other analytical methods; however, one certified standard must be used. This can be performed through relative quantification using ERETIC. For absolute quantification also, a certified standard is required now as an internal standard in a known concentration. For both methods, the pulse sequence needs to be calibrated to 90° to be sure that the spectral response is completely real, and it means that the longitudinal relaxation time (T_1) of spins is entirely returned [38]. Typically, this is achieved by waiting five times the longest T_1 (at five times T_1 approximately 99.3% of the equilibrium value is re-established) between two scans.

Proton magnetic resonance spectroscopic imaging (^{1}H-MRSI) has a major role in lipid assays, mainly used in the medical area with extreme importance for *in vivo* sampling. Both profiling and ratio quantifications are possible by the obtained spatial resolved spectra. The presence of so many compounds in living biological samples may require water or other signal suppression experiments to be performed in order to obtain better resolution on the target metabolites. The same approach is used in NMR samples but with greater implications due to lack of sample pretreatment [47].

Compared to the MS method, NMR technique is less sensitive and limited by the overlapping of signals in either, ^{1}H NMR or ^{31}P NMR, and also by the low natural abundance of ^{13}C for ^{13}C NMR. On the other hand, NMR is a nondestructive sample technique that allows a high analytical reproducibility, an easy identification of molecular moieties, and with relatively easy to get information on molecular dynamics [8, 38]. Furthermore, NMR does not require a standard curve or molecule species for quantitative measuring. Therefore, this technique has been emerging as

a promising approach for more accurate and faster quantitative analysis of lipids than other analytical methods [38]. Also, the sensitivity improvement of cryogenic probe in an equipment of 800 MHz LC-NMR is very promising in analysis of a trace amount of lipids in a faster experiment, once it is able to acquire ^{1}H NMR spectrum of approximately 1 μg sample within 30 min, whereas the current 500 MHz NMR needs 20 h or longer [38].

4.1 Nuclear magnetic resonance spectroscopy: application in biological assays

A wide variety of NMR experiments (e.g., HSQC, HMBC, TOCSY, etc.) besides the classics ^{1}H, ^{13}C, and ^{31}P NMR are being used to solve a variety of biological issues where biofluid samples such as serum, plasma, urine, cerebrospinal fluid (CSF), etc., are being investigated. More commonly used are ^{1}H, ^{13}C, and ^{31}P NMR experiments, which bring rich information on lipid profiling, for example, molecular identification of fatty acid chains and phospholipid structures. Furthermore, heteronuclear and multidimensional experiments can be used to elucidate lipid profiling information by signal interpretation and also using comparisons with databases. The ^{13}C NMR is also a complementary tool that can be used for fatty acyl residue identification [38].

Once NMR allows the noninvasive lipid analysis in intact cells and tissues, the technique prevents losses of chemical information in the analyte environment. This fact, together with the high sensibility of NMR to molecular dynamics (in timescales from picoseconds to seconds), enables to investigate changes in lipid and dynamic structures in biochemical cell functionalization. The experiment used for this application is the diffusion ordered spectroscopy (DOSY), which enables to separate signals according to their diffusion coefficients and then add chromatography-like capabilities to NMR [38, 48].

Lipoproteins consist mainly from cholesterol esters and triacylglycerols surrounded by a hydrophilic layer, which comprehend phospholipids, cholesterol, and proteins [8]. Lipoproteins perform the lipid transportation in blood circulation through the exogenous (dietary lipids) and the endogenous (liver-synthetized lipids) channels. The endogenous transportation begins in the liver through the production of a very low-density lipoprotein (VLDL). After being secreted into the bloodstream, VLDL interacts with other lipoproteins, through collisions, in which the contact with the high-density lipoprotein (HDL) is highlighted.

Kostara et al. [49] have found how blood lipoproteins influence the progression of coronary heart disease (CHD) by comparing the lipid profiles of atherogenic (non-HDL) and atheroprotective (HDL) lipoproteins from patients with CHD with those from patients with normal coronary arteries (NCA). They analyzed the lipid extracts of these lipoproteins using ^{1}H NMR experiments and statistical analysis and identified the potential target-lipid biomarkers for the early evaluation of the CHD onset. Furthermore, Lopes et al. [50] were able to find that circulating HDL increases, and LDL and VLDL decrease in obese patients after bariatric surgery by using DOSY experiments to monitor these lipoproteins. Notably, lipoprotein investigations and quantitative analysis of lipids can be performed using NMR of the same sample [51].

Also, selective recoupling of dipolar and chemical-shift interactions removed by magic-angle spinning NMR in the solid state allows the characterization of regulatory interactions, dynamics, and ion channels within biological membranes [52].

In this scenario, the NMR application has contributed to obtaining of important data on the structure and turnover of lipid species and the composition of lipids in cells, and to characterize pathways enrolled in lipid synthesis/transport and degradation [53, 54]. Also, the high-resolution magic-angle spinning NMR (HR-MAS NMR) has been applied to global lipidomic studies [52].

Besides the identification of lipid species and dynamics, NMR can be used for reliable quantification of lipid mixtures obtained from tissues, body fluids, and cell cultures [40, 55]. It can be allied to the bioinformatic tools available to a better quantitative analysis of lipid profiles [56]. For instance, using ^{1}H NMR and ^{31}P NMR, Fernando et al. [57] were able to identify an over-accumulation of lipids associated with the pathophysiology of ethanol-induced liver steatosis accompanied by mild inflammation.

Also, quantification can be used in magnetic resonance imaging (MRI) experiments as Vafaeyan et al. [47] have shown. They have used a time-domain quantification method namely as subtract-QUEST-MRSI algorithm to quantify alterations of the biomarkers, i.e., lipids and other metabolism molecules species such as choline, creatine, *N*-acetyl aspartate, lactate, myo-inositol, and glutamine in multiple sclerosis subjects in comparison with control group. This research aimed to know how lesion biomarker ratios in multiple sclerosis have affected human brains, through the imaging of different brain areas, which could present lesions.

Other MRI works have found that on brain imaging, lipids tend to be an almost undesired artifact, and consequently, scientists may use the approach of selective signal suppression pulses such as adiabatic frequency selective, spatial-spectral lipid suppression, or broadband outer volume suppression bands [58]. Trauner et al. [59] have used a dynamic saturation transfer technique in MRI experiments to assess dynamic Pi-to-ATP exchange parameters in nonalcoholic fatty liver disease (NAFLD) and steatohepatitis (NASH) aiming to report alterations of hepatic lipid, cell membrane, and energy.

5. Final considerations

Lipids *per se* exert several relevant biological functions making the single knowledge of the lipidome profile from a biological sample highly informative by itself. For instance, sphingolipids and glycerophospholipids are important components of the cell membrane and then can affect several cellular functions. Disorders of sphingolipid metabolism are associated with lysosomal storage diseases and of lysoglycerophospholipid by phospholipase A2 activation are associated with lipotoxicity and inflammation. Accumulation of triacylglycerol (a glycerophospholipid) is associated with lipotoxicity and insulin resistance, and the NEFA profile is a useful indicator of lipid metabolism and can add to understanding on molecular mechanisms underlying the metabolic syndrome [5].

Therefore, lipidomic tools are particularly useful to identify and understand changes in metabolic pathways and the underlying mechanisms enrolled in the pathophysiology of human health, such as metabolic diseases. One practical example is data from Meikle et al. [60] study that measured 259 lipid species in plasma samples from prediabetic, diabetic and normal glucose tolerant patients, including sphingolipids, phospholipids, glycerolipids, and cholesterol ester. The authors used electrospray ionization-tandem mass spectrometry in previous precursor ion and neutral loss scans on control plasma extracts, MRM experiments for the major species of each lipid class identified in plasma, and quantification using internal standards. These approaches highlighted that metabolic pathways altered in type 2 diabetes include a deregulation of lipid homeostasis, characterized by abnormal plasma-free fatty acids accumulation.

In lipidomic studies, beyond the care of equipment calibration and accuracy of the experiments, special cares of analytical procedures must be planned to have accurate information of data. The statistical recourses are necessary to process the data, but, also lipid knowledge is required for correct interpretation in all cases. The

choice of the most suitable lipidomic tool to be used for a specific biological assay is closely linked to the study aim. Next-generation techniques (MS and NMR) can provide detailed lipid information to assess more elaborated scientific questions. However, thousands of individual lipid molecular species are present in cells implying that no single technique can effectively study all the lipid species [38]. When possible, combining techniques can be the best choice, because one can compensate for the limitation of the other, and bring complementary information and/or can validate the previous analysis data.

Usually, combined lipidomic techniques are applied for data validation. For instance, data obtained by shotgun lipidomics (direct infusion MS) can be validated using LC-MS-based analyses and *vice versa*. Other methods, including NMR, or chromatography-based analysis might be used to validate the total lipid content of a lipid class [5]. However, the combined use of lipidomic techniques can also be useful to improve the data information on the lipids from biological samples. For instance, to assess lipid changes during the response of hypoxia stress to a treatment in cervical cancer-derived cells (HeLa cells), Yu et al. [40] applied NMR technique for the phospholipid profile analysis and MS for phospholipids characterization. Also, Whiley et al. [61] investigated the plasma phosphatidylcholine metabolism using NMR and MS to obtain a fingerprint of three phosphatidylcholines (PC) molecules that significantly decrease in individuals with Alzheimer's disease compared to healthy controls. Then, LC-MS and NMR were used for phosphatidylcholine and fatty acyl side chain validation and for total plasma choline validation, respectively. The study of Whiley et al. [61] illustrates the scientific value in combining different lipidomic tools to obtain complementary information and reinforce validation of the obtained data.

In conclusion, all available tools for lipidomic studies in biological samples have several advantages and limitations that can be overcome when combining more than one technique. Because this practice involves the availability of complex technologies and skilled labor, it is not always possible. In this scenario, the use of mass spectrometry alone can be the best alternative currently available when technique combination is impossible. However, NMR has a high potential and, in the future, may be expected to answer issues that MS is quite limited to do.

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Conflict of interest

Authors declare no conflict of interests.

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