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Tutorial on Lipidomics

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Abstract

The main stream of lipidomics involves mass spectrometry-based, systematic, and large-scale studies of the structure, composition, and quantity of lipids in biological systems such as organs, cells, and body fluids. As increasingly more researchers in broad fields are beginning to pay attention to and actively learn about the lipidomic technology, some introduction on the topic is needed to help the newcomers to better understand the field. This tutorial seeks to introduce the basic knowledge about lipidomics and to provide readers with some core ideas and the most important approaches for studying the field.

Keywords

Lipidomics; lipids; mass spectrometry; metabolic syndrome; neurodegenerative diseases; shotgun lipidomics

1. Introduction

The term “lipids” commonly refers to a large family of compounds that are generally soluble in organic solvents and are chemically and structurally non-homogeneous. They mainly include fatty acids and their naturally occurring derivatives (e.g., esters or amines) and other related complex substances [1]. The LIPID Metabolites and Pathway Strategy (LIPID MAPS) project, funded by the National Institutes of Health in 2003, divides lipids into eight major categories: a) fatty acyls, b) glycerolipids, c) glycerophospholipids, d) sphingolipids, e) sterol lipids, f) prenol lipids, g) saccharolipids, and h) polyketides [2, 3]. As of January 1, 2019, the LIPID MAPS Structure Database [4] contains more than 43300 unique lipid structures, not counting regioisomers, oxidized lipids, and other modified forms.

Lipids are the major constituents of biological membranes, which provide not only a barrier to the cell body but also a dynamic motif for appropriate membrane protein functions and signal transduction. The diversified cellular lipids serve numerous important biological functions. Aside from their participation in the regulation of a variety of life activities,

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including energy conversion, substance transportation, signal recognition and transduction, and cell growth, differentiation, and apoptosis, lipids are also associated with the abnormal metabolism in many diseases such as atherosclerosis, diabetes, obesity, Alzheimer's disease, and tumorigenesis [5–8].

A lipidome is a complete set of lipid species existing in a cell, an organ, or a biological system. The lipidome varies with time and with the different perturbations experienced by the organism [9]. The analysis of the lipidome was largely achieved with the development of biological mass spectrometry (MS), as first demonstrated by Han and Gross [10] and followed by many others, such as Kerwin et al. [11] and Brugger et al. [12]. In 2003, Han and Gross [13] first defined the concept of "lipidomics" in a review article published in a peer-reviewed journal. Their article described the evolution of electrospray ionization-mass spectrometry (ESI-MS) technology for the study of lipids from biological samples on a large scale and at the system level. The research in lipidomics involves characterization of the structures of cellular lipid species [14–16]; quantification of individual lipid species levels in biological samples [17–20]; and determination of the interactions of individual lipid species with other lipids, proteins, and metabolites *in vivo* [21–23].

At present, lipidomics has become one of the most important branches of omics and is a very active research field [24]. The importance of lipidomics in (pre)translational research has attracted widespread attention in the scientific community, and lipidomic tools have been applied to studies on obesity, atherosclerosis, the metabolic syndrome, and cancer, among others [25–27]. The analytical platform of lipidomics relies on advances in and applications of MS [28]. However, owing to the complexity of the structure and diversity of lipid molecules, there is still room for evolving and/or improving the lipidomic approaches—from sample preparation to MS analysis and data processing [29–31].

A typical lipidomic workflow consists of the following processes (Figure 1): the introduction of lipid molecules from lipid extraction or a tissue section into the mass spectrometer (i.e., direct infusion, liquid chromatography (LC), and MS imaging); data acquisition, identification, and quantification; and data mining and bioinformatic analysis.

In this tutorial, we present an introductory primer to the technical concepts and implementation approaches of lipidomics. We have noticed that increasingly more researchers in broad fields are beginning to pay attention to and actively learn about the lipidomic technology. This tutorial seeks to provide introductory knowledge about lipidomics and introduces readers to the core ideas and key approaches of lipidomics in an easy-to-understand way. We have also noticed that the topic of quantitative methods is difficult for beginners who are not specialized in MS-based methodological development to understand. Thus, this is particularly emphasized in the context of the current topic. Considering the broad backgrounds of the readers, we have striven to reduce the complexity of the technical discussion and focused instead on the understanding of the idea. Owing to spatial constraints, this article provides only brief descriptions of some other equally important analytical methods, such as separation-based analysis and MS imaging.

2. Mass Spectrometry-based Lipidomic Approaches

The development of MS technology has made comprehensive lipidomics possible. The classic triple quadrupole (QqQ) instruments still occupy a central position in lipidomic analysis, especially quantitative analysis. At the same time, high-resolution MS has greatly expanded the capabilities for analyzing lipid species. Table 1 provides a comparison of the different types of mass spectrometers used commonly in lipidomics. The development of ionization methods and their diverse variants fundamentally determined the range of species detectable. Currently, the frequently used ionization methods in MS for lipidomics include ESI, desorption electrospray ionization (DESI), matrix-assisted laser desorption/ionization (MALDI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and secondary ion mass spectrometry (SIMS). DESI is a variant of ESI, where its separation of the ionized solvent droplets from the analyte allows it to be suitable for *in situ* analysis. APCI uses a corona discharge method to generate high-energy metastable ions that react with the analyte to achieve ionization. APPI uses energy from a vacuum-ultraviolet lamp to directly or indirectly (chemical reaction of metastable ions) ionize the analyte. APCI and APPI are important additions to the ESI source. They can be coupled to LC or gas chromatography (GC) systems, and work well with solvents of low polarity. APCI is a soft ionization source that is more suitable for ionizing low-polarity molecules. Compared with APCI or ESI, APPI is not a soft ionization source, but it can improve the ionization efficiency of substances that are not easily ionized under both APCI and ESI. SIMS bombards the analyte with a focused high-energy ion (primary ion), causing ionization of the analyte (secondary ion).

In this tutorial, we present lipidomic approaches that have been developed mainly on the basis of the two most prevalent and representative ionization techniques (i.e., ESI and MALDI) that are critical to the analysis of biomedical samples.

2.1 Shotgun Lipidomics

2.1.1 Introduction—Shotgun lipidomics is also referred to as direct infusion-based lipidomics. The lipids are generally not separated by chromatography, and the continuously injected lipid extract mixture is electrospray ionized directly by MS under a constant lipid concentration [32]. Although there is no chromatographic separation, lipid extraction in the lipid sample preparation stage can be carried out by multiplexed extraction strategies according to the differences in hydrophobicity, stability, and reactivity among the lipid classes and subclasses [33].

The ionization of lipids in shotgun lipidomics employs a strategy of selective ionization. Different lipid classes have different charged characters in solution due to their distinctive structures (e.g., different polar head groups in polar lipids). These distinct lipids can be selectively ionized in the ion source under a given solution condition, referred to as intrasource separation [12, 34]. The idea of pseudo-separation in the ion source is similar to the electrophoresis of different compounds with different pI values.

This approach maximizes the original information of the sample. A long-term MS analysis time is available after continuous direct injection of the sample, allowing multiple

acquisitions in different MS scan modes for the analysis of the same component in a sample [35]. Since there is no change of lipid concentrations along the analysis time, it is possible to obtain a high signal-to-noise (S/N) ratio to facilitate accurate quantitative analysis using internal standards [35].

Currently, there are three intensively developed and widely used approaches in shotgun lipidomics; namely, tandem MS (MS/MS)-based shotgun lipidomics, high mass accuracy-based shotgun lipidomics, and multi-dimensional MS-based shotgun lipidomics (MDMS-SL). It should be noted that given the nature of shotgun lipidomics, MALDI, DESI, and SIMS could be included within its broad scope. Considering that the importance and application of these methods in lipidomics are more reflected in MS imaging, we will discuss them separately in Section 2.3.

2.1.2 Tandem Mass Spectrometry-based Shotgun Lipidomics—Lipid molecules contain structures that reflect their class characteristics. In MS/MS of lipids, especially of polar lipids, there are typical characteristic fragments associated with their head groups. On the basis of the characteristics of each lipid class, researchers have developed a shotgun-based lipidomic approach that “isolates” a class of individual lipid species of interest from a lipid mixture by a specific neutral loss scan (NLS) or precursor-ion scan (PIS) [12]. After collision-induced dissociation (CID), fragments with classification features, such as those detected by PIS or NLS, are used to identify lipid classes of interest, and the PIS or NLS of fatty acyl chains is used to identify specific molecular species present in a class [36]. After the MS/MS double-filtering process, the mass spectral S/N ratio can be increased by orders of magnitude. In general, researchers would like to use QqQ mass spectrometers (usually because of their multiple acquisition modes, high dynamic range, and low cost) to detect all species in a specific class directly from total lipid extracts in one MS/MS acquisition.

The advantages of the MS/MS-based approach include its simplicity, high sensitivity, ease of operation, and low instrumentation cost. However, there are some problems with this approach. For example, the length and unsaturation of the fatty acyl chain of lipid species are usually not determined. The specificities of characteristic fragments used in MS/MS scans may not be sufficient, which may introduce some false-positive results. Since the signal responses of the fragment to different species of a lipid class are not necessarily consistent, accurate quantification of the identified lipid species may be difficult. It requires at least two internal standards to achieve relatively accurate measurements for each lipid class [37], which increases the difficulty of internal standard selection.

2.1.3 High Mass Accuracy-based Lipidomics—High mass resolution/accuracy is important for both qualitative and quantitative analyses. The three main types of mass analyzers for high-resolution MS are quadrupole time-of-flight (Q-TOF), Orbitrap, and Fourier-transform ion cyclotron resonance (FTICR). Q-TOF or quadrupole-Orbitrap mass spectrometers offer high mass resolution/accuracy, where they have an excellent duty cycle compared with other high-resolution mass spectrometers [16, 38, 39]. Therefore, Q-TOF or quadrupole-Orbitrap is good for not only coupling with online LC separation to perform data-dependent analysis, but also for shotgun lipidomic analysis. The high mass accuracy-based approach can quickly determine all product ions in a small bin width across the entire

mass range of interest [40–43]. The high mass accuracy allows these instruments to accurately record the mass-to-charge ratio (*m/z*) information of the fragment ions in the selective window, and the high mass resolution minimizes false positives. This full range of coverage of a product ion scan is referred to as “top-down lipidomics” or “bottom-up shotgun lipidomics” [44, 45].

A high-resolution mass spectrometer can also perform data-independent analysis owing to its high mass resolution and high mass accuracy [46–48]. MS/MS^{all} is a representative data-independent analytical method developed in recent years that has been applied in lipidomic analysis [47, 49, 50]. In MS/MS^{all}, parallel scanning through the quadrupole is done across the full mass range, with 1 Da as the mass window. The precursor ions selected (usually with a quadrupole analyzer) are subjected to CID, and then all fragment ions are detected with a high-resolution mass analyzer. This method could provide a wider coverage of identification than that provided by data-dependent analysis, with better reproducibility and sensitivity. It is worth mentioning that this method theoretically maximizes the fragmentation information of the sample, if fragmentation of the lipid species in the mass range does not depend too much on CID energy; thus, it provides a large pool of data for mining, including the analysis of isomers and the identification of unknown species.

The high mass resolution/accuracy-based shotgun lipidomic approach can provide highly efficient, versatile, and sensitive measurements of lipid species. Within the dynamic range allowed by the instrument and the software package, all of the potentially existing lipid species can be covered and the method can be performed in an untargeted manner for the analysis of any lipid species present in the cellular lipidome. Since the method is essentially based on MS/MS technology, each lipid class needs to contain multiple (at least two) internal standards to achieve accurate quantification [37].

2.1.4 Multi-Dimensional Mass Spectrometry-based Shotgun Lipidomics—

MDMS-SL integrates a full mass scan and all MS/MS scans for head groups and acyl chains to totally identify individual lipid species (including isomers) and to accurately quantify these identified species with a two-step quantification procedure [51]. Therefore, all the concerns inherent in the MS/MS approach are overcome by this approach.

Specifically, in MDMS-SL, the characteristic structural building blocks of the lipid molecules are fully applied to identify individual lipid species [6, 52]. In MDMS, a two-dimensional (2D) mass map can be constructed, with the *m/z* range as one dimension and all spectra (including a full scan and unit-by-unit NLS or PIS scans) as another dimension [35]. Thus, for identification of a specific lipid class, it is sufficient to selectively scan the structural specific building blocks by PIS or NLS (Figure 2). The MDMS-SL approach enables us to achieve large-scale lipid identification and absolute quantification. The quantification principles are introduced in Section 3.3.2. Currently, this approach is capable of analyzing thousands of lipids from a small amount of biological sample, covering approximately 50 lipid classes. The total coverage of determination has exceeded 95% of the mass content of the cellular lipidome [31, 51].

MDMS-based shotgun lipidomics overcomes most drawbacks of the other two shotgun lipidomic methods, except that its throughput is relatively low. Moreover, the direct acquisition of multi-dimensional spectra provides a lot of information that can be mined for the accurate identification and quantification of individual lipid species, including isomers with different fatty acyl chains and regioisomers. Compared with the LC method, the MDMS-SL method is not limited by the peak width factor, so there is sufficient time to obtain an average spectrum with a high S/N ratio. Accurate absolute quantification can be achieved with two-step quantification, which is a unique quantification method for MDMS-SL [51]. Usually, shotgun lipidomics is not good at analyzing low-abundance or less-ionizable lipids owing to ion suppression. However, the analysis of these types of lipids can be improved by derivatization or pre-separation methods [53–55].

2.2 Separation-based Lipidomics

2.2.1 Liquid Chromatography–Mass Spectrometry-based Lipidomics—

The advantage of the LC-MS method is that the highly efficient separation of mixed components by LC and the highly sensitive detection by MS can be integrated for the even separation of components in extremely low abundance [56–58]. The LC-MS approach is also useful for the discovery and identification of novel lipids, as assisted by additional information on the retention time with a variety of LC columns.

Many LC-MS-based lipidomic approaches have similarities to proteomic methods. The most common approaches are selected/multiple reaction monitoring (SRM/MRM) and data-dependent analysis. SRM/MRM is a very powerful quantitative method in LC-MS-based targeted analysis. The accuracy of quantification is ensured by the high specificity and broad linear range of the SRM/MRM method, which is a mature, stable, and widely used method in metabolomic and drug analyses. Moreover, this method plays a very important role in clinical lipidomic analysis [59]. Data-dependent analysis is a strategy in which a fixed number of precursor ions are selected according to predetermined rules from a survey scan and then subjected to MS/MS analysis [60]. This method is very suitable for untargeted lipid analysis. Data-dependent analysis is one of the ideal methods for identifying and quantifying individual lipid species by LC-MS. However, the short peak elution time in LC separation limits the chances of detecting low-abundance species. In particular, the structural similarity of lipid molecules from the same class is higher than that of polypeptide fragments from digested proteins. Therefore, the fine-resolution separation of lipid molecules in chromatography is more difficult than that of peptides. Since data-dependent analysis usually selects a limited set of abundant ions in a peak width, this method requires reduction of the complexity of the injected sample (e.g., pre-separation).

Two-dimensional liquid chromatography (2D-LC) technology can be used to obtain a capacity of tens of thousands of peaks, and is the first technique of choice for untargeted analysis [61–64]. Among various 2D-LC methods, the combination of normal phase (NP)-LC and reversed phase (RP)-LC is the most advantageous setting, because NP-LC can be used as the first dimension to classify lipid compounds as different parts based on the polarities (head groups) of the molecules. The second dimension of RP-LC separates lipid species within the same lipid polarity fractions from NP-LC according to the hydrophobicity

(fatty acid chain(s)) of the molecules. Since the mobile phase of NP-LC is usually an organic solvent, and RP-LC is the mobile phase containing polar solvent(s), the interface of this type of two dimensions is a developing research field in analytical chemistry. Currently, most 2D (NP/RP) LC-MS/MS systems use a laboratory-built interface. The levels of automation and analytical reproducibility need to be improved, and the analysis time is long. Therefore, the development of a more complete and effective 2D (NP/RP) LC workflow to realize high-throughput automated analysis is an important research topic.

2.2.2 Other Separation Methods—

In addition to LC-based methods, the application of other separation methods is also an important complement to lipidomic analysis [65], including GC, thin-layer chromatography (TLC), capillary electrophoresis, and supercritical fluid chromatography (SFC).

GC is a widely used and mature separation method. Its most prominent advantages are its very high separation resolution and high accuracy of quantification. GC is usually suitable for the analysis of lipids with good thermal stability and high volatility, and is highly complementary to LC separation [66]. For non-volatile lipids, GC analysis can also be carried out by derivatization as needed.

The TLC method is a classic lipid separation method [67] with suitability for handling non-volatile substances. Although its separation resolution is low, it is very easy to operate and can be used as an upstream pre-separation step for other separation methods. In addition, the improved high-performance TLC has been coupled to MALDI in lipidomic analysis [68]. One disadvantage of TLC that differs from other separation methods is that it is difficult to achieve real-time separation for MS detection [69].

With regard to the complementarity of the separation spectrum, capillary electrophoresis is more suitable for separating molecules with high polarity; thus, it is rarely used for lipid analysis. Since its diameter can be made to be very small and it uses an electroosmotic flow as a means of separation, capillary electrophoresis achieves a much higher resolution than ion chromatography does [65]. In terms of coupling to MS, if not used for the study of the interaction of lipid molecules with other biomolecules, then the biggest difficulty is the low ionization efficiency due to the high polarity and high salt concentration of the mobile phase.

SFC is a sophisticated, fast, and high-resolution separation technique compared with classic high-performance LC [70]. The selectivity of SFC for separating lipids is similar to that of normal-phase chromatography and can be modulated by adding an additive solvent when using carbon dioxide as the mobile phase. SFC can therefore be applied to isolate most lipid classes. In addition, owing to the high permeability and low viscosity of the mobile phase, SFC is very advantageous for achieving high-resolution separation [71, 72]. The ion source of MS is widely available for coupling with SFC, including APCI, ESI, and APPI, according to the polarity of the mobile phase.

2.3 Imaging Lipidomics

Imaging analysis is the visual analysis of the spatial distribution of lipids in biological samples and can obtain dynamically changing data. Traditionally, lipid imaging analysis can

use fluorescence imaging in a high-resolution and highly sensitivity manner [73]. However, the samples need to be derivatized, and one imaging session can only obtain the information of one or several lipid compounds, which cannot meet the needs of lipidomic analysis. Although the sensitivity of MS is limited compared with that of optical detection methods, MS imaging can directly obtain information about hundreds of lipid compounds without the need for their labeling [74, 75]. Table 2 shows a comparison of the characteristics of various MS imaging methods used in lipidomics.

2.3.1 Matrix-assisted Laser Desorption/Ionization-Mass Spectrometry Imaging—

MALDI-MS imaging is an effective label-free imaging method that simultaneously detects a wide range of biomolecules, identifies unknown molecules, and displays their spatial distribution in tissue sections. MALDI-MS imaging has the most advantages among all MS-based imaging approaches in current development [76] mainly because the method can achieve high-resolution imaging by using laser modulation to obtain highly focused spots. Currently, commercial instruments provide a stable and reliable 10-micron level of imaging and achieve imaging at less than one-micron resolution in the laboratory [77]. MALDI-MS imaging is the most high-resolution method for MS imaging among all soft ionization methods. Since MALDI can be modulated with the energy of a laser source, the sensitivity in high-resolution imaging is higher than that of other methods [78, 79].

The successful imaging of lipids by MALDI-MS depends first on the choice of the matrix. The lipid molecule itself has a very low absorption capacity for the ultraviolet laser and cannot produce a molecular ion signal without a matrix. One of the main challenges for the MALDI-MS imaging of lipids is that the matrix should avoid the generation of strong, cluttered background interference signals in the detection range and be also capable of producing abundant lipid peaks. Many efforts have been devoted to finding or optimizing suitable matrices and associated application strategies [80–84]. The complexity of the distribution of lipids in cells or tissues is extremely high. Therefore, MS imaging of lipids requires diversified matrices. Different matrices can be used to ionize the target lipid classes selectively [85]. Depending on the knowledge about the distribution of lipid classes in different tissue sections and organs, the reasonable selection of a suitable matrix can also maximize data acquisition [84, 86–88]. For example, N-(1-naphthyl)ethylenediamine dihydrochloride is a highly selective matrix for the ionization of glycerophospholipids [84], whereas 9-aminoacridine is highly selective for the analysis of sulfatides [86]. In order to improve the detection of the lipids of interests and reduce unnecessary interference from other lipid classes, researchers can select the appropriate matrix according to the selectivity or preference. For example, for lipid mapping of the kidney or the liver, 9-aminoacridine is not the best choice because the kidneys and liver have abundant glycerophospholipids but a low abundance of sulfatides.

In addition to organic matrices, research on inorganic material-based matrices, especially those made of nanomaterials, has been a focused research area in recent years in order to eliminate the effects of the matrix background on interference [89]. This is because many inorganic substances are more stable under laser irradiation and do not have the propensity to generate fragmentation and free radicals. Many nanostructured materials have good

energy absorption under ultraviolet light and can transfer photon energy to lipid molecules. Nanomaterial matrices include the following categories: nano-metal oxides (e.g., TiO_2, WO_3, and ZnO) [90], nano-metal particles (e.g., platinum, gold, and silver) [91–93], nano-carbon materials (carbon dots, graphene, and diamond) [94–96], and other 2D nanomaterials (e.g., hBN nanosheet, MoS_2 nanosheet, and tellurium nanosheet) [97–99]. It should be pointed out that inorganic nanomaterial-based matrices having the advantage of low or free background interference are not a substitute for organic matrices because the current organic matrices are comparatively significantly superior in sensitivity. These two categories of matrices are complementary to each other.

2.3.2 Desorption Electrospray Ionization Imaging—DESI imaging (including nano DESI imaging) is a widely used ambient-MS imaging technique [100]. In DESI (a variant of ESI), charged primary organic solution droplets impact a sample surface with certain kinetic energy, and the secondary droplets containing sample molecules are sputtered from the surface upon impact by the primary droplets. The nanometer-scale sputtered sample droplets that are charged then undergo a desolvation and charge transfer process (similar to ESI) under the action of an electric field to guide the generated ions into a mass spectrometer. It can be understood from the principle of DESI that although its spatial resolution and sensitivity are not as good as those of MALDI-MS, real-time or even native imaging can be achieved by maintaining the original state of the sample because no pretreatment is required [101]. Therefore, DESI imaging has some advantages that MALDI imaging cannot replace, such as rapid or real-time intraoperative assessment, assisted diagnosis, and good performance in the surface analysis of forensic samples [102–105].

2.3.3 Secondary Ion Mass Spectrometry Imaging—SIMS imaging is performed by focusing a high-energy ion beam onto the surface of a sample to bombard it point-by-point in order to produce secondary ions. Its working principle is similar to that of electron microscopy, except that ions are excited and monitored instead of electrons. The most important significance of SIMS imaging is that it can reach the resolution level of optical microscopes, which is currently not possible for other MS imaging methods. SIMS imaging has been used for single-cell lipid imaging despite its low S/N ratio [106]. Since it is not a soft ionization method, it is currently hard to obtain rich intact lipid peaks without severe interferences with molecular fragments. In addition to the advantage of enabling ultrahigh resolution, SIMS also has unique advantages in three-dimensional (3D) imaging compared with the other MS imaging methods. SIMS can use the ion beam to peel the sample layer by layer at a nanometer-scale thickness to obtain a 3D image of a sample (also called sputter-then-image) [107]. Other MS imaging methods can only perform 3D reconstruction of the 2D images using layer-by-layer scanning of tissue sections owing to the nature of the ionization source (soft ionization) or the thermal effect of the laser.

3. Technical Aspects of Quantitative Lipidomics

3.1 Sample Processing

Analysis of lipid compounds begins with extraction of lipids from complex matrices to ensure the success of subsequent analyses. The most commonly used extraction method for

full-lipid compounds is the chloroform-methanol extraction-based methods originally from Folch extraction or Bligh-Dyer extraction [108, 109]. In recent years, there have also been reports of improvements to this method [110, 111], such as the BUME method, which shortens the extraction time to approximately 50% because researchers only need to perform one round of extraction instead of two rounds. Although these methods improve the extraction efficiency of certain lipid compounds, the sample processing throughput is still unsatisfactory. Therefore, in recent years, scientists have also been studying the use of solid-phase extraction, microwave-assisted extraction, supercritical fluid extraction, and other technologies to extract lipids from samples in order to increase the throughput of sample processing through automation [112–114]. In conclusion, the current sample processing in lipid analysis is still using traditional liquid-liquid extraction methods especially for quantitative lipidomics, because these extraction methods have a better extraction efficiency for most lipid compounds. Although the extraction time can be greatly shortened by the automated extraction instruments, the universality of different lipid compounds remains to be improved, and further research is still needed in this regard.

For quantitative analysis, quality control samples and internal standard(s) must be added during the sample preparation stage. Quality control samples can maintain the reliability, stability, and reproducibility of the analytical data acquired within a large-scale lipidomic study [115]. They can be classified into two types: pooled quality control samples (collected aliquots of every sample to be analyzed) and reference standards (where the m/z of each molecule is known) that monitor/correct for any shift in retention time or mass accuracy (e.g., The National Institute of Standards and Technology makes available Standard Reference Material 1950 Metabolites in Human Plasma, containing approximately 100 metabolites with certified and reference values). Background signals and contamination can also be monitored by blank extracts (parallel extraction without samples) [43].

For internal standards, isotopically labeled or structural analogs are selected to achieve sample consistency and standard signal responses [37]. The amount and type of internal standard(s) need to be considered during the sample preparation stage. The internal standard should be free of or contain less than 1% of the analyte of interest. It should be added at the beginning of lipid extraction, where the amount added should be optimized to an appropriate analyte-to-internal standard ratio (e.g., from 0.1 to 10).

The following operational advice could help beginners to improve the quality of the experiment. On sample preparation, the following points should be considered: 1) The tissue sample should be collected from an organ in the same way and from the same location; any blood contamination should be washed out with buffer (e.g., 1× PBS); any buffer residue on the tissue should be removed with wipers; tissue samples should be immediately frozen in liquid nitrogen and stored at −80 °C prior to lipid extraction. 2) The lipid levels can be normalized to the wet weight, dry weight, protein amount, or DNA level for tissue samples, whereas it can be normalized to the volume for biofluid samples. Therefore, the measurement of reference units, such as weight or protein concentration, should be accurate. 3) The procedure of tissue homogenization should always be conducted at a temperature below 4 °C to minimize enzyme activation. 4) The amount of individual internal standard must be added accurately and within a dynamic range of analysis. 5) The lipid solutions

(generally stored in a freezer) should be warmed up to room temperature before MS analysis.

3.2 Experimental Considerations for Mass Spectrometry

3.2.1 Infusion Solution and Intrasource Separation—One physical property of an electrospray ion source is that its electric field distribution can be utilized to achieve the intrasource separation of lipid species of different charged states [52]. Although this separation is conceptually analogous to electrophoresis, it is different in form because it does not have separate media and circuit loops. This concept can be used in shotgun lipidomics to achieve selective ionization, as well as to ensure a stable ion output, without fluctuations in matrix effects, and is suitable for the large-scale quantitative analysis of lipidomes. Lipids can be classified into three categories on the basis of the charged nature of the lipid molecules: (1) anionic lipids, (2) weakly anionic lipids, and (3) charge-neutral polar lipids [13, 32]. Anionic lipids usually carry at least one negative charge under weakly acidic conditions (e.g., pH ~4). Lipid classes in this category are cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid, and sulfatide, and their lysolipids; acyl CoA; etc. Weakly anionic lipids are usually not charged in a weakly acidic environment, but are dissociated in an alkaline environment and thus carry a negative charge. This category of lipids includes phosphatidylethanolamine (PE), lysoPE, non-esterified fatty acids and their derivatives, bile acids, and ceramide. As the name implies, charge-neutral lipids do not carry a net charge under any pH conditions. This category comprises both polar and nonpolar lipids, such as phosphatidylcholine (PC), lysoPC, sphingomyelin, hexosylceramide, acylcarnitine, monoacylglycerol, diacylglycerol, triacylglycerol, and cholesterol and its esters.

These three categories of lipids can be selectively ionized by an intrasource separation strategy, using different solutions and ionization modes. For example, by selectively adding lithium chloride to the solution, the pH of the solution can be changed and additive ions can be provided to assist with selective ionization (Figure 3). For lipids that are not readily ionizable in any case, derivative methods can be used to alter their charged nature.

3.2.2 Ionization Conditions and Collision Energy—The ion-source temperature plays an important role in effective ionization. This point is considered identical to all the ESI-based methods. Special consideration is that the temperature should not be too high, in order to avoid degradation of the lipid molecules. For example, when the source temperature is too high, PS degrades very easily, resulting in the generation of PA and thus causing interference signals, which affects the accurate quantification of both anionic lipids.

The concentration of the lipid solution entering the ion source should be diluted to a balanced one in order to avoid any aggregation of the lipids and to ensure an adequate signal. In addition, the flow rate, spray voltage, and collision gas pressure all need to be optimized and are also critical factors that affect the ionization efficiency and accuracy of quantification [34].

In CID, the selected ions that are accelerated under the electric field acquire specific kinetic energy to collide with inert gas in the collision cell. The collision energy that is either

excessive or too low does not provide sufficient fragmentation information. For quantitative analysis, the collision energy should be suitably optimized to provide a relatively stable response factor [116].

3.3 Quantification Method

3.3.1 Introduction and Principles of Quantification—Relative quantification, which refers to the determination of the ratio of lipid components between two samples without the need to know the actual amount, is commonly used in biomarker discovery and comparative studies. The use of relative quantification methods is common in LC-MS-based studies. The relative quantification determined by adding references or labeling is more common in proteomic analysis [117]. In metabolomic or lipidomic analyses, this type of method is generally not always practical because of the vast differences in the chemical structures of the analytes. In the usual untargeted metabolite analysis (where label-free analysis is acquired), relative quantification using ion counts of MS/MS spectra is commonly conducted [118]. This method is highly dependent on the chromatographic stability and separation ability because the MS signal of an ion species is susceptible to subtle differences in chromatographic and ion source conditions, which in turn affects the correlation between the concentration and signal. Hence, repetitive datasets acquired by a highly stable LC-MS system are the prerequisite for this approach, and a suitable software tool (e.g., XCMS) is needed to compare and match the peaks [119, 120].

Absolute quantification measures the amount of each lipid component under a specific physical unit reference, such as nanomole per milligram (nmol/mg) of protein. The absolute quantification of lipids is critical for the analysis of lipid metabolism pathways/networks and the elucidation of biochemical and biophysical mechanisms. The most common absolute quantification method is the one that spikes the sample with internal standard(s) and thereby directly compares the relative signal relationship between the analytes and the internal standard(s) under identical experimental conditions. It is assumed that the concentration of the sample is proportional to the signal (i.e., within the linear dynamic range). This proportional relationship can be established by subtracting the baseline effect, without lipid aggregation and within the linear detection range of the MS system [37].

It has been validated that individual species of polar lipids can have nearly identical ionization efficiencies in the low concentration range after correction for different ^{13}C isotope distributions [52, 121]. This is because the mass spectral signal response of polar lipids is mainly determined by the polar groups, whereas the length and degree of unsaturation of the fatty acyl chains have little effect on the signal under the same conditions. It can be noted that high polarity and low concentration are the critical factors that ensure the simple relationship of signals. This makes it easy to quantify with internal standards. However, for nonpolar lipids, such a relationship between the signal response factors of the analytes and the internal standard of a class does not exist, even in low-concentration regions (if not converted to polar species by chemical derivatization). The response factors of these species, or the response factors correlated with the fatty acid chain length and saturation, must be pre-corrected to ensure accurate quantification [122, 123].

The quantitative methods of high mass accuracy and MS/MS are both based on this principle, and their specific details are not mentioned here. Ultrahigh-resolution MS increases the accuracy of quantification by reducing the overlapping effect of the isobaric peaks. However, we should note that the linear dynamic range of ultrahigh-resolution MS (Orbitrap or FTICR) is relatively narrow.

3.3.2 Two-Step Quantification by Multi-dimensional Mass Spectrometry-based Shotgun Lipidomics—For polar lipids, a consistent response factor for the mass spectrometric signal of the lipid molecule at low concentrations suggests that any of the lipid molecules can be used as a new internal standard for the quantitative analysis of this lipid class [34]. This idea is the source of two-step quantification. In MDMS-SL, the lipid molecules identified by intrasource separation and MDMS screening are typically quantified using a two-step quantification procedure [32, 51].

This method is particularly useful in situations where lipids present with low-abundance signals, and peaks of different lipid classes overlap with each other to cause quantitative interference. In the two-step quantification process, the first step is to use the spiked internal standard of a class to directly and ratiometrically compare full-scan MS peaks from other lipid species of the class that have both high abundance and no interference. The quantitative results are corrected by baseline subtraction and ^{13}C deisotoping. The second step of quantification is based on the results of the first quantification step, where the low-abundance peaks and the peaks that overlapped with other lipid species are quantified. The difference here is that the second step uses PIS and NLS spectra to improve the S/N ratio of the low-abundance peaks and to exclude interference from other lipid classes. Since the signal response factors of different species are different in the PIS or NLS mass spectrum, it is necessary to use both the species identified quantitatively in the first step plus the originally spiked internal standard as the standards for the second step. An algorithm is used to correct the response factor for each lipid species. By using the second step of quantification, which is used as a dynamic range relay, it is possible to achieve a linear dynamic range of more than 5000-fold for many lipid classes.

3.3.3 Selected/Multiple Reaction Monitoring Quantification for Targeted Lipidomics by LC-MS—Selected ion monitoring (SIM) works in the first stage of the MS mode, recording only one or more selected ions and not the remaining ones [124]. SIM quantification can be achieved by comparing the intensity of the selected ions with that of the structurally equivalent internal standard ions. SIM is more advantageous than full-scan MS for quantitative analysis, since SIM allows the mass spectrometer to utilize more time to detect the ion flux of selected ions, thereby increasing the sensitivity. However, owing to limitations in the chromatographic resolution, this method is not suitable for a large-scale lipidomic analysis that requires high specificity.

Selected or multiple reaction monitoring (SRM or MRM) is an improvement over SIM [125]. They are similar to the idea of SIM quantification, quantifying by detecting characteristic ion pairs and reference standard curves. In SRM/MRM, the instruments are set up to detect only the signals of the precursor ions and the corresponding one (SRM) or more (MRM) product ions. The simultaneous detection of these ion transitions (precursor/product

ion pair) increases the detection specificity. Thus, SRM/MRM has higher sensitivity than SIM. This is because SRM/MRM has two ion selections, whereas only a single ion is selected at a time in SIM. Therefore, more noise and interference are eliminated in SRM/MRM, and the S/N ratio is improved.

The LC-MS-based SRM/MRM method has been widely used for quantitative targeted lipidomics [126–128]. The popularity of this method is due to the fact that the method is universal and the requirements for the instrument are not high. However, this method also has certain limitations. For example, it is necessary to pre-confirm large-scale and specific transitions. A large number of standard curves must be established to achieve accurate quantification. In addition, changes in the chromatographic conditions in repetition can affect the quantification accuracy. Therefore, the addition of quality control samples to the test samples is inevitable, which undoubtedly further increases the time of the entire test process.

4. Bioinformatics for Lipidomics

Lipidomic analysis produces a large amount of data. Without proper calculation tools and efficient mining of the data information, it is difficult to understand the biological significance of the data set. Bioinformatics advances tools for databases, algorithms, statistics, and data visualization. Therefore, bioinformatics has become an important part of biomedical science research and development as well as the development of lipidomics [129].

4.1 Lipid Databases and Identification Tools

LipidBlast, maintained by the Fiehn Laboratory at the University of California-Davis [36], is an *in silico* tandem mass spectral database containing hundreds of thousands of possible lipid structures. This library is very helpful for spectral annotation and lipid identification. Building-block concept-based theoretical databases are mandatory for MDMS-SL, which searches for this type of pre-established virtual lipid database that covers every lipid class and that contains all possible structural building-block combinations [51].

The LIPID MAPS website covers a wide range of content, including large virtual libraries, as well as a variety of bioinformatics tools for lipid analysis [4]. Its main tools involve several aspects, including structural classification and nomenclature, statistical tools, and computer-aided spectral comparisons. It also provides a lot of known MS/MS fragment information for structural analysis. These tools can help build and predict possible lipid structures from mass spectra.

Similarly, both the METLIN Metabolomics Database [130] and the Human Metabolome Database [131] are large, comprehensive metabolite databases (including lipids) that provide rich functional information and cross-indexed links to facilitate metabolic pathway studies. These databases also provide mass spectra under a variety of conditions.

Many software programs can execute the large-scale analysis of lipids in an automated or semi-automated manner. In general, these tools use the fragment information generated by

CID to identify and quantify lipid species and chain lengths. Typical representatives of these tools are LipidFinder [132, 133], LipidQA (data-dependent tools) [134], LipidMiner (for LC-MS data) [135], and LipidSearch (a commercial software). LipidFinder is a recently developed online tool that facilitates the automated search for LIPID MAPS for lipid identification and statistics. For shotgun lipidomics, typical tools include LipidProfiler [41], LipidInspector [42], AMDMS-SL [51], and ALEX [136].

4.2 Biostatistic Tools

Interpretation of the data requires the use of statistical methods. Descriptive statistics and hypothesis testing are the most commonly used methods for data analysis [137]. There are many general-purpose packages in this area. Advanced statistical methods, such as multivariate statistical analysis and biomarker discovery, are also widely used in lipidomic research. Online tools provide convenient and easy-to-use options that can meet most analytical requirements. For example, MetaboAnalyst is a set of online tools for metabolomic data analysis and interpretation [138]. It provides a variety of analytical tools, including descriptive statistics, multivariate statistical analysis, biomarker discovery, and metabolic pathway analysis. We believe that MetaboAnalyst is a good entry option for beginners. It contains a lot of statistical tools that cover a wide range of applications, is easy to understand, and allows the customization of operations. However, MetaboAnalyst may not meet the requirements for more advanced and complex statistics or complex image outputs. For example, for supporting vector machine analysis, only two groups of comparisons are supported in MetaboAnalyst. Many large, commercial statistical software programs provide powerful analytical capabilities, including SIMCA-p, SPSS, and more. The R language package, which is free, is an even more powerful alternative, but is more difficult to use.

5. Summary and Outlook on Future Directions

Lipidomics plays an important role in revealing the mechanisms underlying the pathogenesis of diseases and facilitating their diagnosis. The changes in lipidomes are not only present in metabolic diseases such as diabetes, but are also associated with other diseases such as neurodegenerative disorders and cancer [139, 140]. For example, lipidomic analysis revealed the association of sphingolipid and glycerophospholipid changes in brain tissue or blood with Alzheimer’s disease [141–143]. Numerous studies have shown that altered glycerophospholipids can be considered biomarkers for cancer diagnosis [144–146]. However, owing to the special complexity of the lipidome, unlike the genome and proteome that can be predicted by nucleic acid sequences, we do not know how many lipids are present in a cellular lipidome. Therefore, a more comprehensive approach for discovery and quantification is crucial for mapping lipidomes and studying their metabolism [24].

A standardized method for extracting total lipids or specific lipid types from different biological samples has not yet been formulated. A guideline for the analysis of plasma samples has recently been proposed [147]. The results obtained by different research groups using different lipid extraction methods are very different [148] and can lead to differences in result interpretation. Homogenization is an unavoidable step in the isolation and

purification of subcellular organelles and specific lipid regions in cells. Therefore, the resultant aggregation of lipid molecules or the reorganization of disrupted membrane structures raises difficulties for the study of the localization or dynamic changes of lipid molecules in organelles or specific membrane regions.

Although LC-MS and shotgun lipidomic technologies have features such as high throughput, high specificity, and high accuracy, the qualitative and quantitative analytical software with low manual intervention is still limited in variety and function; in particular, the highly automated analytical software is still lacking.

Bioinformatic technology systems, such as those that allow data integration and the visualization of lipid metabolic pathways, are still in the immature stage and need further development and improvement.

Combining the analytical results of lipids in biological samples with clinical pathology and pharmacology will become increasingly widespread. The following areas are important aspects of lipidomics in biomedical research: a) the large-scale, high-precision discovery/quantification of lipids and related metabolism; b) the robust and reliable biomarker discovery approach; c) the relationship between dynamic changes of the cellular lipidome and cell function: d) the changes and effects of drugs on lipidomes; and e) the analysis of the fine structure of lipid molecules and the biological functions of these structures.

In conclusion, lipidomic analysis should serve as a foundation of biomedical science since a limited understanding of the composition of biological systems usually stagnates biomedical research into a state of low efficiency and even contingency. To solve this problem, a detailed chemical description of the biological system is required, which includes qualitative and quantitative analyses. The future development of lipidomics should significantly facilitate our understanding of biological systems and disease mechanisms.

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List of Acronyms:

APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
CID	collision-induced dissociation
CL	cardiolipin
DESI	desorption electrospray ionization
ESI	electrospray ionization
FTICR	Fourier-transform ion cyclotron resonance

GC	gas chromatography
IS	internal standard
LC	liquid chromatography
LIPID MAPS	LIPID Metabolites And Pathway Strategy
MALDI	matrix-assisted laser desorption/ionization
MDMS-SL	multi-dimensional MS-based shotgun lipidomics
MS	mass spectrometry
MS/MS	tandem MS
NLS	neutral loss scan
NP	normal phase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIS	precursor-ion scan
PS	phosphatidylserine
QqQ	triple quadrupole
Q-TOF	quadrupole time-of-flight
RP	reversed phase
SFC	supercritical fluid chromatography
SIM	selected ion monitoring
SIMS	secondary ion mass spectrometry
S/N	signal-to-noise
SRM/MRM	selected/multiple reaction monitoring
TLC	thin-layer chromatography

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Highlights

- Introductory knowledge of mass spectrometry-based lipidomics approaches
- Principles and the technical aspects of quantitative lipidomics
- Basic bioinformatics for lipidomics

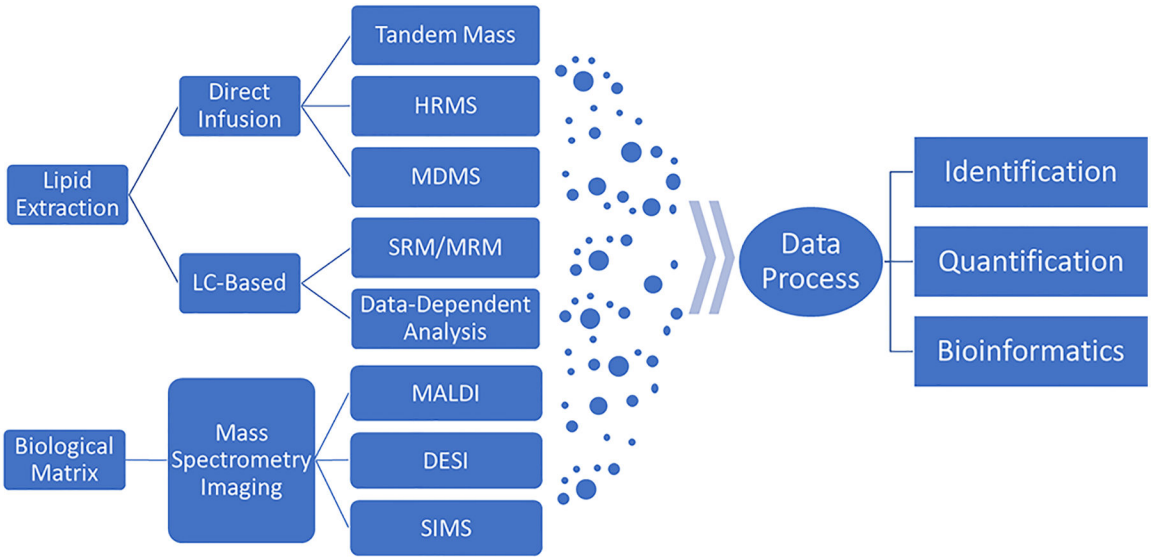

Figure 1.

A typical workflow of mass spectrometry-based lipidomics. HRMS, high-resolution mass spectrometry. MDMS, multi-dimensional mass spectrometry. SRM/MRM, selected/multiple reaction monitoring. MALDI, matrix-assisted laser desorption/ionization. DESI, desorption electrospray ionization. SIMS, secondary ion mass spectrometry.

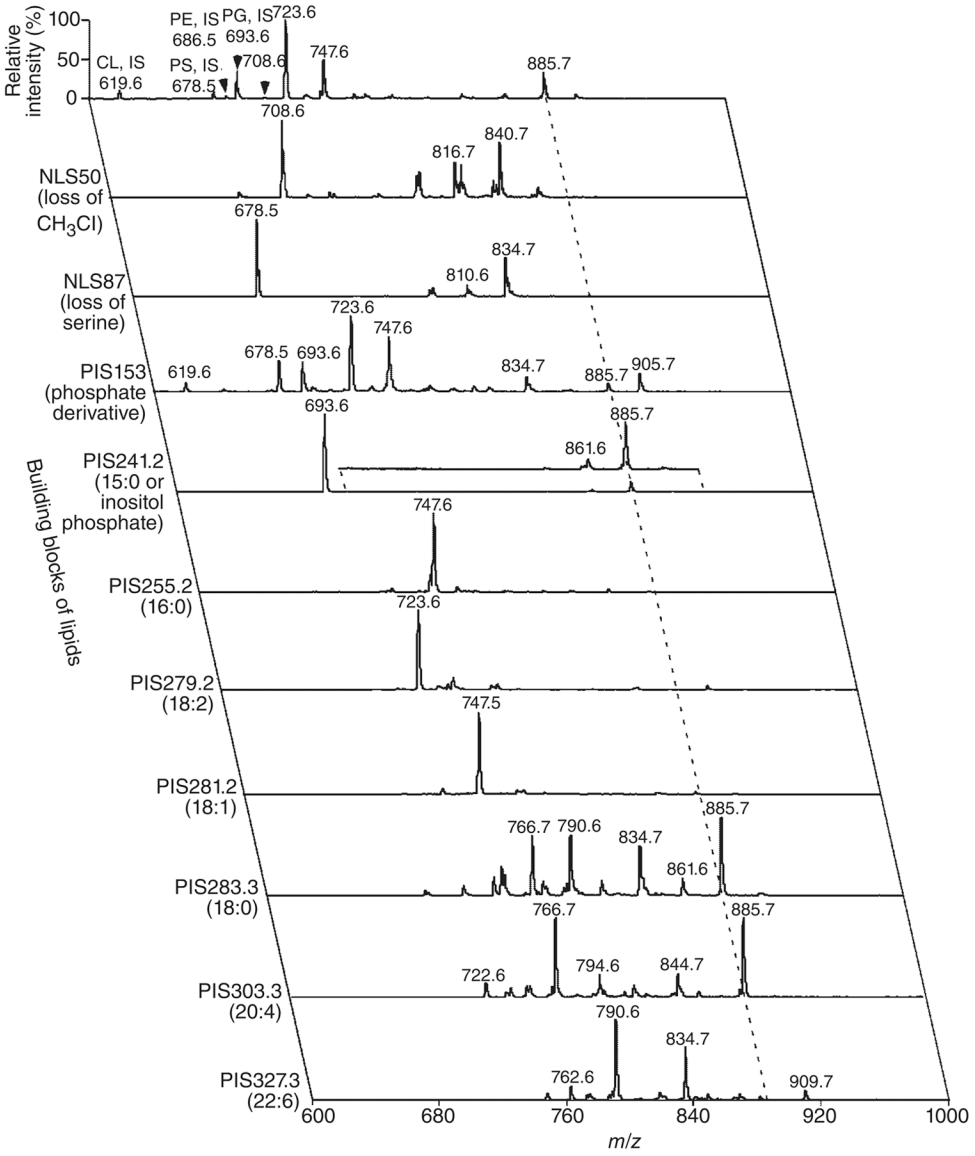

Figure 2.

A 2D mass spectrum of rat myocardial lipid extract in negative ion electrospray ionization for analysis of phospholipids. As shown, the first dimension represents the mass range from *m/z* 600 to 1000. The second dimension, composed of different PIS and NLS analyses, shows specific fragment (building block) information about these species. For example, the ion at *m/z* 885.7 was present in the negative ion full scan (the most top one), as well as PIS153, PIS241.2, PIS283.3, and PIS303.3 spectra. This indicates that this molecular ion is an anionic phospholipid and can yield fragments at *m/z* 153, 241.2, 283.3, and 303.3 corresponding to the building blocks of dehydrated glycerophosphate, inositol phosphate, and carboxylate ions of stearic acid (18:0) and arachidonic acid (20:4), respectively. These building blocks plus their intensities allow us to identify the ion as PI (18:0–20:4). Similarly, the 2D analysis identifies the ion peaks at *m/z* 619.6, 678.5, 693.6, and 708.6 as the internal standards (IS) of CL (14:0/14:0/14:0/14:0), PS (14:0/14:0), PG (15:0/15:0), and PC

(14:1/14:1), respectively, which were added prior to lipid extraction for quantification. The peaks at *m/z* 723.6 and 747.6 were also apparently identified as CL (18:2/18:2/18:2/18:2) and PG (16:0/18:1), respectively, which are the major species of lipids present in rat myocardium. The identified major species could be quantified in the full MS scan mode in comparison to the corresponding IS. By using these quantified species plus IS, the low abundance lipid species can be quantified by using PIS/NLS spectra.

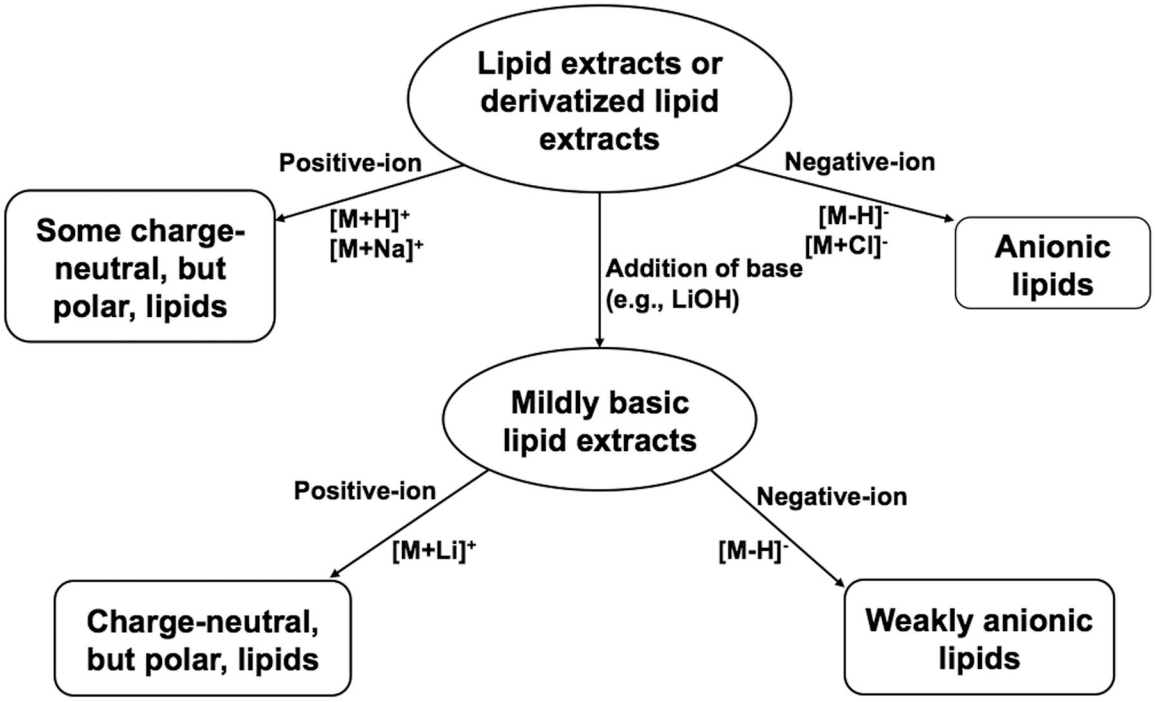

Figure 3.

Scheme of a typical experimental strategy based on the concept of intrasource separation for measurement of different categories of lipids from lipid extracts to achieve maximal lipidomics coverage.

Table 1.

Comparison of the features of some common mass analyzers.

Mass analyzer	Mass resolution[a]	Mass accuracy (ppm)	Sensitivity	Identification	Quantification
LTQ (LIT)	2000	100–500	Good	++	+
Q-q-Q	1000	100–1500	High	+	+++
TOF/TOF-TOF	10,000–40,000	5–50	High	++	++
Q-q-TOF	10,000–60,000	5–50	High	++	+++
Orbitrap	100,000–800,000	<5	Medium	+++	++
FTICR	>1,000,000	<1–2	Medium	+++	++

[a]Mass resolution at full width half maximum

Table 2.

Comparison of the features of major mass spectrometry imaging approaches.

	MALDI	DESI	SIMS
Typical spatial resolution	5–100 μm	100–200 μm	50 nm-2 μm
Mass range (*m/z*)	Full range	< 2000	< 1500
Mass analyzer	TOF/TOF, Q-TOF, FTICR, Orbitrap	Q-TOF, Orbitrap	TOF, magnetic section
Sample preparation	Tissue section; Dehydration; Matrix coating	No pretreatment	Tissue section; Dehydration; Fixation (optional)