Methods of Lipidomic Analysis: Extraction, Derivatization, Separation, and Identification of Lipids

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1 Sample Collection and Extraction

1.1 Sample Collection

The first step in lipidomics studies involves the collection of analytical samples from plants, animals, or microbes. These samples can be solid in nature (e.g., tissues [1], cells [2], solid fecal material [3], seeds [4], leaves [5], or root hairs [6]) or comprise highly complex fluids (e.g., plasma [7, 8], serum [9], urine [10], synovial fluid [11], milk [12], or oils [13]). A typical procedure begins when samples are frozen quickly in liquid nitrogen before they are stored at very low temperatures (e.g., −80 °C). This initial step helps to inhibit enzymatic activity and reduce the rate of oxidation, peroxidation, and hydrolytic degradation of lipids containing unsaturated bonds [14]. To ensure that the profile of the extracted lipids is a good representative of the entire sample, the next step of the protocol involves sample homogenization, and then appropriate extraction buffers are used to extract lipids from the homogenate. In contrast, with the focus solely on optimizing the extraction efficiencies of lipid classes of interest using different solvent systems, the sample preparation protocol for fluids tends to be more straightforward. Following lipid extraction, the stability of lipid species in the extraction solvent is also an important consideration, particularly if the samples are subjected to multiple freeze-thaw cycles. As described above, lipid molecules containing unsaturated double bonds may be subjected to oxidation and are also susceptible to hydrolysis in the presence of water. To minimize the breakdown of unsaturated bonds on these molecules, aliquots of lipid extracts into smaller volumes can be considered, which will reduce the number of freeze-thaw cycles.

1.2 Sample Extraction

In general, the application of lipidomics requires sample extraction methods that are highly efficient, reproducible, and able to cover a wide range of analytes with different polarities. The extraction protocol also needs to take into account that a limited amount of sample may be available for lipidomic analysis. With the goal of improving overall lipid coverage, liquid–liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction (SPME), and other emerging techniques have been applied to lipid extraction.

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1.2.1 Liquid–Liquid Extraction (LLE)

LLE is the most predominant extraction technique for lipids. In order to achieve exhaustive and comprehensive extraction of key lipid classes, LLE involves the use of two immiscible organic solvents – most commonly a mixture of chloroform and methanol with water, introduced more than 70 years ago by Folch et al. [15] (chloroform/methanol/water ratio 8:4:3 v/v/v) and subsequently modified by Bligh and Dyer [16] (chloroform/methanol/water ratio 1:2:0.8 v/v/v). Most lipidomics studies still rely on these general extraction procedures, often in modified versions. Due to its lower toxicity, dichloromethane has been used as a substitute for chloroform [17].

The high chance of contamination of the samples is a pitfall of two-phase extraction method, because of the need of retrieving lipids from the lower chloroform-rich layer. In 2008, Matyash et al. [18] demonstrated a new sample extraction procedure employing methyl tert-butyl ether (MTBE). The method involves addition of MeOH and MTBE (1.5:5, v/v) to the sample and phase separation is induced by adding water. Compared to conventional two-phase chloroform-containing solvent systems, this extraction method utilizes the low density of the lipid-containing organic phase to form the upper layer during phase separation. This greatly simplified sample collection and minimized dripping losses. Furthermore, compared to chloroform, MTBE is nontoxic and noncarcinogenic, which reduces potential health risks for exposed personnel. While the method has the advantage over conventional chloroform-containing solvents, unsatisfactory recovery for more polar lipid classes has been observed [19].

One-phase lipid extraction has recently been demonstrated, which is an "all-in-one-tube" approach eliminating the need for phase separation. This is achieved by using solvents such as butanol/methanol (3:1, v/v) [20] or MMC solvent mixture (MeOH/MTBE/$CHCl_3$, 1.33:1:1, v/v/v) [21] to denature proteins that are later removed by centrifugation. With an untargeted lipidomics approach, Andres et al. [21] explored the differences/similarities between the most commonly used two-phase extraction methods (Folch, Bligh and Dyer, and MTBE) and one-phase extraction method based on the MMC solvent mixture. The four extraction methods were evaluated and thoroughly compared against a pooled extract that qualitatively and quantitatively represents the average of the combined extracts. The results showed that the lipid profile obtained with the MMC system displayed the highest similarity to the pooled extract, indicating that it was most representative of the lipidome in the original sample. Furthermore, it had better extraction efficiencies for moderate and highly polar lipid species in comparison with the Folch, Bligh and Dyer, and MTBE extraction systems.

1.2.2 Solid-Phase Extraction (SPE)

While LLE represents a somewhat universal extraction method in lipidomic analysis, SPE could well enrich lipids of extremely low endogenous abundance via minimizing background matrix and ensure satisfactory detection upon mass spectrometric (MS) analysis. SPE is a well-established sample preparation method, which utilizes a solid (stationary) phase and a liquid (mobile) phase to capture selectively specific classes of molecules with similar properties [22]. Selective retention of specific molecules of interest on the solid phase can be achieved through the differential interaction of analytes between the two phases.

The most commonly used SPE-column chemistries for lipid extraction include normal-phase silica, reversed-phase (C8 and C18), and ion-exchange columns (packed with aminopropyl) [23]. Silica and aminopropyl columns are often used for separation and sub-fractionation of neutral and polar lipids, which can be achieved by changing the eluent solvents [24, 25], while C8 and C18 columns have been used to isolate PC, cerebrosides, gangliosides, and fatty acids from polar compounds in water based samples [26]. HybridSPE phospholipid (HybridSPE-PL, zirconia-coated silica stationary phase) has been successfully used to remove PL interferences of biological samples based on the Lewis acid-base interaction between zirconia and the phosphate moiety of PLs. Therefore, HybridSPE-PL is also an ideal choice for the isolation and enrichment of all kinds of PLs from complex biological sam-

ples. HybridSPE-PL instead of LLE was used to rapidly enrich and recover PL molecular species from human plasma by Wei et al [8].

1.2.3 Solid-Phase Microextraction (SPME)

SPME has also been introduced as a rapid equilibrium-based sample preparation technique, for which a small amount of extraction phase is coated, typically on a solid support, and then used to remove a small portion of analyte from the sample. This technique is commonly used in conjunction with gas chromatography (GC) or GC-MS analysis, as the headspace available in a SPME cartridge enables the enrichment of volatile analytes typically monitored by GC.

SPME has been used successfully together with GC-MS for the extraction of fatty acids and fatty acid esters from solid samples, such as lung tissue [27] and hair [28], and biofluids, such as sputum [29]. This approach can be useful when the sample amount is very limited (e.g., synovial fluid or cyst fluid) or when targeted compounds of interest are expressed at low concentrations. Due to the small sample sizes and extraction volumes required for SPME, sample cleanup tends to be highly efficient, resulting in fewer matrix effects, such as ion suppression or enhancement, in subsequent MS-based analysis [30].

1.2.4 Emerging Techniques for Lipid Extraction

In addition to these established lipid extraction techniques, there are alternative methods, which may have shorter extraction time and lower solvent requirements. These techniques include supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE), which are more commonly used for the extraction of metabolites from biological samples such as plant and food materials.

The principle of SFE is based on an increase in solvation power of a supercritical fluid when its pressure and temperature are raised above critical values. This increase in solvation power, coupled with the relatively low viscosity and high diffusivity of such fluids, allows to extract and separate effectively different compound classes (including oils, fats, and vitamins) in a sample [31]. SFE has been used for lipid extraction in plant, animal tissues [32], and, more interestingly, dried human-plasma spot samples [33]. Uchikata et al. [33] compared the extraction of PLs by SFE with a traditional LLE method (Bligh and Dyer) and concluded that SFE was more effective, as it resulted in higher levels of selected PL species, including PC, lysoPC, PE, and SM.

UAE) is an efficient and reproducible extraction technique. It helps improve the yield and quality of the lipid extract and does not raise the temperature of the system. This makes it attractive to extraction of heat-unstable lipids. In addition, UAE can be combined with conventional LLE methods to improve the extraction efficiency of lipid species present in biological samples. For example, Liu et al. successfully developed a combined UAE and LLE protocol for human serum samples, which led to a 5–60% increase in the levels of fatty acids compared to the conventional LLE method [34]. A similar approach was adopted by Pizarro et al. [35] for human blood plasma samples, of which the use of MTBE with UAE resulted in the detection of 30% more lipid species when compared to a conventional MTBE-based LLE method. The MTBE-UAE technique also showed high reproducibility, with relative standard deviation values of less than 6% and lipid-component recoveries of more than 70%.

2 Derivatization

2.1 Advantages of Derivatization

Many trace level compounds in complex matrices which have essential biological functions cannot be well detected by MS-based methods, especially if they are difficult to ionize or to fragment. Derivatization is a specific chemical reaction, which aims to modify the structure of the target compounds and, as a consequence, the chemical and physical properties. The advantages of combining derivatization with MS analysis include: (1) improvement of selectivity and sensitivity [36, 37], (2) enhancement of ionization efficiency [8], (3) improvement of struc-

tural elucidation [38], (4) increase accuracy for quantification [1], and (5) facilitation of isomer separation [39].

2.2 Lipid Analysis After Derivatization

Certain lipids, including fatty acyls, glyceride (GLs), glycerophospholipids (GPs), sphingolipids (SPs), sulfatides (STs), phenolic lipids, saccharolipids, and polyketides, contain functional groups, such as carbonyl, hydroxyl (alcohol or phenol), and amine group, and they are suitable for introducing a fragmentable moiety by chemical derivatization. There are challenges when performing derivatization reaction, including formation of by-products, nonquantitative reaction, requirement for harsh reaction conditions, long reaction time, and product degradation. To achieve an effective derivatization-based MS analysis, the derivatization reaction should be fast, efficient, and specific and form relatively stable products.

Fatty acids (FAs), a basic element of all lipids, contain at least one carboxyl group and a long aliphatic chain. Yang et al. developed a LC-MS method for the identification and quantification of FAs through derivatization with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide, forming 3-acyloxymethyl-1-methylpyridinium iodide (AMMP) [40]. This derivatization reaction attached a quaternary amine to analytes and enabled electrospray ionization (ESI)-MS analysis with the positive ionization mode. Detection sensitivity was generally 2500-fold higher than in the negative mode of ionization used for underivatized FAs. The main derivatives for FAs and modified FAs are quaternary amine derivatives [41, 42], tertiary amine derivatives [43], piperazine-pyrimidine derivatives [44], benzofuran derivatives [45], and other derivatives [46] for enhancing the assay sensitivity or accuracy of quantification. Kloos D et al. [47] reviewed the most recent trends in analysis of FAs by chromatography and MS employing derivatization techniques. Derivatization has also been applied to analysis of PLs [1, 8], glyceride [48, 49], and steroids [50, 51]. In addition, there are a number of derivatization strategies for determining the location of double bonds in lipids, such as derivatization of C=C bond with acetone [52], N-(4-aminomethylphenyl)-pyridinium [53], ozonolysis [54], ozone-induced dissociation, or olefin cross-metathesis [53].

3 Chromatographic Methods

3.1 Thin-Layer Chromatography (TLC)

TLC is not commonly applied for lipidomic analysis but shows great potential for the separation of lipids by class. The separated analyte species can be acquired from the spots on the TLC plate and extracted with chloroform and methanol. The lipids can then be analyzed by matrix-assisted laser desorption/ionization (MALDI)-MS, ESI-MS, or GC-MS. TLC-MALDI-MS has been demonstrated for analysis of PLs in bronchoalveolar lavage (BAL) fluids, and the study showed a prospective for direct MALDI-MS measurements on the TLC plates in the mass spectrometer [55].

3.2 GC Separation of Lipids

Because of their nonvolatile property, the use of GC for direct analysis of global lipids is impossible. Most early methods relied on hydrolysis of PLs by phospholipase C to diacylglycerols, which is followed by methyl transesterification, producing fatty acid methyl esters that are subsequently analyzed by GC [56].

The first application of GC for FAs can be traced back to the 1950s [57]. There are two methods available to quantify FAs. The first method is through simple peak integration. It may provide uncertain results if peaks are not fully recognized, which is often the case for molecules with similar structures, such as PLs that may only distinguish by a single double bond [58]. The second method is to use a flame ioniza-

tion detector (FID), which combusts the sample into fragments that are ionized by an electrode. The charged ions will flow to the electrode in the detector, yielding a current. FID is a very sensitive detector; however the disadvantage of using FID is that it destroys the sample [59].

3.3 LC Separation of Lipids

LC separates different classes of analytes according to their physicochemical properties. Reversed-phase LC (RPLC), normal-phase LC (NPLC), and hydrophilic interaction LC (HILIC) are commonly used for lipidomic analysis. The mechanism of action by RPLC for lipids lies in the basic of lipophilicity, which is regulated by the carbon chain length and the number of double bonds. Thus, lipid species containing longer acyl chains are eluted from the column later than shorter chain lipids, and saturated acyl structures are eluted later than polyunsaturated analogs. However, NPLC and HILIC typically separated lipid species based on their hydrophilicity. Therefore, lipids are separated according to their representative polar head group classes [60]. Other LC methods used for lipid separation include nonaqueous RPLC [61], silver-ion RPLC [62], chiral LC [63], and supercritical fluid chromatography (SFC) [64].

3.3.1 RPLC

RPLC utilizes a mobile phase that is more polar than the stationary phase, which permits complex lipidomes to be separated prior to MS analysis. The lipids are separated based on lipophilicity owing to the combined chain length and number of double bonds present in the fatty acid side chains [65].

Due to the hydrophobic property of lipids, the most common separation method for LC-MS-based lipidomics is RPLC with a C18 column. Lipids are adsorbed to the stationary phase and eluted based on the relative affinity, and the gradient allows for controlled elution of lipids over a wide range of polarities [66]. The mobile phase composition can be changed throughout the separation process, increasing the hydrophobicity of mobile phase and hence increasing the elution effect. By contrast, greater separation of lipid classes can be achieved if the mobile phase composition is held constant [67]. However, this amelioration in separation is only available for a narrow range of polarities and can result in long retention times.

3.3.2 NPLC and HILIC

Compared to RPLC, NPLC and HILIC utilize polar stationary phases, therefore more strongly retaining polar analytes. Retention of lipid classes on a RP column is influenced by acyl and alkyl chain length and desaturation; however, the retention on HILIC columns largely relies on the polarity of the lipid head groups [68, 69]. This allows efficient separation of lipid classes, which is not observed with RPLC.

In fact, HILIC and RPLC are highly orthogonal, leading to different elution profiling [70]. As neither method is capable of fully analyzing all lipid compounds in complex matrix, it is possible to couple the two modes of separation using two-dimensional LC (2D-LC). In this configuration, the co-eluting lipid species from the first-dimensional RPLC are loaded onto the second-dimensional HILIC for further separation [71]. However, challenges exist in the development of 2D-LC systems. First, the mobile phase composition for HILIC has a high elution strength in RP. Second, it is difficult to have the second dimension keep up with the sampling frequency of the first dimension. To overcome some of these challenges, trapping columns have been placed between the first and second dimensions to retain analytes while the second dimension is resolved. Alternatively, widely different column sizes and flow rates may be used between the first and second dimensions, generally requiring mobile phase splitters to reduce flow rate into the ion source [71].

3.3.3 SFC

Recently, SFC has emerged as a viable alternative to LC for lipidomic analysis [72]. The most common supercritical fluid used in SFC is CO_2 as it is cheap and easy to achieve and has a low polarity, similar to hexane. The requirement to

use mobile phase modifiers, such as methanol, to adjust polarity is important [73]. SFC has been demonstrated for fast separations of lipid classes [74]. Supercritical fluid has higher diffusivity and lower viscosity than a common liquid, thereby facilitating higher-throughput analysis as compared with LC [75]. In addition, supercritical CO_2 used as a mobile phase has almost the same polarity as hexane, and its polarity can be adjusted by adding a modifier such as methanol. These advantages make SFC eminently suitable for simultaneous analysis of lipids with a wide range of polarities. Studies have reported a higher detection sensitivity for carotenoids in SFC than LC, and structural isomers were successfully separated using SFC but not resolved by LC [64, 76].

4 MS Analysis of Lipids

4.1 Ionization Methods

The ionization mode used in MS detection plays an important role in lipidomic analysis. One ionization method may not work for all types of lipid classes, since some lipids are better ionized with one ionization mode while other lipids are ionized more evidently with another mode [77, 78]. Ionization efficiency can be enhanced by the additives present in the mobile phase leading to the formation of different type of adducts. ESI in positive mode (ESI+) is the most common mode in LC-MS because it can effectively ionize a wide range of lipids, while negative ionization mode (ESI−) provides superior results for certain lipid classes, such as PI, PS, and PA [79]. Atmospheric pressure chemical ionization (APCI) has been applied to lipidomic analysis and is preferred for more nonpolar lipids (e.g., triacylglycerols).

When using RPLC for lipid separation, 53% of the studies were performed with both ESI+ and ESI− modes and 38% with ESI+ mode only, while 45% of NPLC/HILIC studies were performed with both ionization modes and 41% with ESI− only [80]. The reason for these differences lies in that NPLC/HILIC methods are often used for analysis of PLs, in which ESI− allows effective ionization of these lipids, while RPLC well separates the lipids that can be effectively ionized with ESI (+), such as cholesteryl esters.

4.2 MS Detection

After lipid compounds are ionized and enter a mass spectrometer, the ions are then measured with a mass analyzer. Depending on the type of mass spectrometer, ions can be filtered by m/z using a quadrupole, accelerated along a flight path to measure their m/z, or orbited around an electrode to measure m/z ratios [81]. Quadrupole mass analyzers often act as mass filters by only allowing ions within a small m/z window to pass through and by contrast, time-of-flight, Orbitrap, and cyclotron-based mass analyzers acquire a mass spectrum in a single scan [82, 83]. Time-of-flight mass analyzers measure the time of flight of an ion in the flight tube, which is then converted to the m/z. Orbitrap, same as cyclotron mass analyzers, traps ions in an orbital motion. The image current from the trapped ions is detected and converted to a mass spectrum using the Fourier transform of the frequency signal [84]. These mass analyzers differ significantly in terms of mass resolution [81]. Compared to quadrupole and time-of-flight mass analyzers, Orbitrap and cyclotron mass analyzers in general have higher resolution and therefore superior resolving power [85]. However, due to the narrow peak widths generated with UHPLC and the long scan times for high-resolution mass spectra, operating these instruments at the highest mass resolution is only commonly used by direct infusion [86, 87].

In tandem MS (MS/MS) , two or more mass analyzers, e.g., triple quadrupole, quadrupole time-of-flight, and linear ion trap-Orbitrap, are coupled together using an additional reaction step to increase their abilities to analyze and identify biomolecules. In these configurations, quadrupoles can form mass filters or collision cells, whereby ions undergo collision-induced dissociation with an inert gas, causing fragmentation of the molecules [80]. Fragmentation can

also be accomplished within an ion trap which can supply complementary fragment ions to quadrupole-based fragmentation [88]. Ions entering the mass spectrometer from the ion source are referred to as precursor ions, while ions produced following fragmentation are referred to as product ions and fragmentation products can provide structural information about the precursor ions.

5 Quality Control

During large-scale lipidomics studies, drifts in LC peak shape and retention time may happen due to sample residue or column aging, and signal intensity attenuation may occur in direct infusion MS due to the contamination of ion source components of the mass analyzer. It appears difficult to maintain repeatability and stability during large dataset acquisition over the time. A potential solution is to utilize a standard quality control sample (QC sample) for real-time monitoring the stability of the MS system [89]. This is due to the following reasons: First, the analytical platform needs to be tested with QC sample to ensure that data are reproducible before vital samples are analyzed. Observed peak drift or signal attenuation prior to analyzing vital samples is not acceptable [90]. Second, QC samples analysis can be used as a quality assurance (QA) tool [91]. Third, data produced from QC samples can be used to correct signals between analytical runs and mine in-depth information within the data from different analytical batches [92]. Finally, standards are required for normalization if using community QC samples [93].

The matrix composition of QC samples should be theoretically similar to that of experimental samples. Four types of QC samples are commonly used: (i) internal standards (ISs) were often employed to evaluate the stability of the instrument in the past. However, the limited number of available ISs was not enough to estimate all the lipid features [94]; (ii) batch QC was formed from the small aliquots of each sample to be studied in a batch; (iii) pooled QC was prepared independent by pooling the related samples from laboratory's bank [95]; this type of QC, together with batch QC, is easily prepared and can monitor each lipid features to minimize the variations of intra- and inter-batch. Luo [94] compared the differences between the two QC samples; no significant differences were observed. However, pooled QC samples are more suitable to monitor the data of long-term lipidomics study to increase their comparability; (iv) Standard Reference Material (SRM) may be available as a QC sample in lipidomics studies. For instance, the "SRM 1950 Metabolites in Frozen Human Plasma" is intended primarily for validation of methods for determining metabolites such as fatty acids, hormones, and amino acids in human plasma and similar materials [96]. It may be used for data comparisons among different laboratories.

Regarding how to use QC samples in the analytical runs during lipidomic profiling analysis, the following setup may be considered: (i) before analysis of actual sequence of samples, five and ten QC samples are recommended to be run in order to equilibrate the shotgun-MS and LC-MS system, respectively [97]; (ii) the injection frequency of QC samples has been assessed in relation to the accuracy and robustness during signal correction process, 3–25 injections of samples between each QC injection were reported [98, 99]. Kamleh [95] evaluated the effect of the injection frequency of QC samples, and the signal drift correction procedure always represented better for the most frequency of injections. Considering the balance between the analysis time and the quality of the data acquisition, at least one QC sample should be injected for every ten sample injections; (iii) the NIST SRM 1950 should be injected during the analytical sequence if there is a need to compare the experimental data between laboratories [100]; (iv) in order to monitor possible sample contamination (e.g., contamination caused by extraction) and check the quality of mobile phases, every series of sample analysis are supposed to contain blank samples [95]; (v) randomizing the sample sequence is vital to ensure that there is no correlation among extraneous factors (e.g., preparation or analysis order) and no bias from the analyst [96, 101].

6 Data Processing and Analysis

6.1 Spectral Data Processing

Spectral data processing for MS-based lipidomic analysis may include spectral filtering, peak detection, alignment, and baseline correction. For peak picking from the features of the chromatographic data, a variety of algorithms [102–104] have been proposed. A widely recognized method is to cut the LC/MS data into slices a fraction of a mass unit (0.1 m/z) wide and then operate on those individual slices in the chromatographic time domain. The peak detection algorithm thus handles low-resolution, high-resolution, and centroided data in a flexible and robust manner.

According to Brown and colleagues [77], most of the methods [105–107] commonly used for retention time alignment are based, in some manner, on the correlation between spectra. Since the retention times in lipidomic analysis are well constrained within individual classes by the observed retention times of internal standards, species of interest are bracketed by the standards in time. This information is often used to effectively time-shift spectra within the time-m/z domain of each class without complex spectral computations. The required alignment shifts can then be chosen to maximize the correlation of time-lag-shifted spectrum against one arbitrarily chosen sample from that session of MS analysis.

The correction of the background contribution to the peak intensities of a mass spectrum is important for peak detection and accurate quantification of each analyte, particularly when the species is at low abundance. Accurate baseline correction could reduce the complications faced by uncertainty about the intercept of the standard calibration curve in LC-MS analysis and thus remove reliance on any latent subtraction of noise through the intercept term. Most of identification and noise reduction are typically based on filtering or smoothing functions of LC-MS data analysis tools. However, applying such kind of filtering tools, which implies a model of the noise and/or the peak shapes present in the original data, may lead to distortion of peak identification. According to the study by Smith et al. [105], group methods from LC/MS data, background subtraction may add more noise than it eliminates. Instead, the problem can be reduced to simply finding the appropriate retention time window boundaries for a given m/z.

6.2 Annotation of Lipid Species

Two metabolomic databases, Metlin [108] and HMDB (human metabolome database) [109], are commonly used for database search to identify metabolites including lipids. High-resolution LC-MS, combined with database searching, has been used for lipidomic profiling of human and animal model samples [110]. Derivatization and stable isotope labeling are usually used for the analysis of fatty acids of lipid species in LC-MS-based lipidomic analysis [111]. For instance, fatty acids can be derivatized with 2-bromo-1-methylpyridinium iodide and 3-carbinol-1-methylpyridinium iodide, forming 3-acyloxymethyl-1-methylpyridinium iodide (AMMP). AMMP derivatives have unique tandem mass spectra characterized by common ions at m/z 107.0, 124.0, and 178.0, and individual fatty acids also display unique fingerprint regions that allowed the identification of their carbon skeleton number, number of double bonds, and double bond position.

6.3 Bioinformatics Tools for Lipidomic Data Processing

To simplify the work of processing complex LC-MS data, software tools have been developed to perform multiple data processing steps, including spectral filtering, peak detection, alignment, normalization, and exploratory data analysis and visualization. Here we briefly discuss several software tools that are commonly used in lipidomic analysis. MZmine mainly focuses on LC-MS data analysis [106]. The functionality includes the identification of peaks using online databases, MS^n data support, improved isotope pattern support, scatter plot visualization, and a

new method for peak list alignment based on the random sample consensus algorithm. The current version of MZmine 2 is suitable for processing large batches of data and has been applied to both targeted and nontargeted lipidomic analyses. OpenMS 2.0 is an updated version of cross-platform software [112], which provides a set of 185 tools and ready-made workflows for common MS data processing tasks. It also provides implementations to address the most common tasks in quantitative proteomics and metabolomics, including quantification, identification, and visualization, as well as algorithms for isotopic deconvolution, chromatographic peak picking, and so on. The only challenge is to build your custom workflows which can be time-consuming. XCMS2 allows to automatically search MS/MS data against high-quality experimental MS/MS data from known metabolites contained in a reference library [113]. It features the same functions such as peak picking, peak alignment, and statistical analysis of features but with the added capability of automatic searching of MS/MS spectra against the METLIN database. MS-DIAL is a data processing pipeline for untargeted lipidomics applicable to either data-independent or data-dependent fragmentation methods [114]. Identification is achieved through analyses of retention time, mass accuracy, and isotope ratio along with MS/MS similarity matching to libraries from publicly available databases. Other software tools for shotgun lipidomics data analysis include LipidXplorer [115], LipidProfiler [116], AMDMS-SL [117], and so on. These software packages are very useful for identifying and quantifying individual lipid species from the data obtained with MS-based lipidomic analysis.

6.4 Biostatistical Analysis and Data Visualization

After qualitative and quantitative results are obtained from analysis of lipidomic data, the next step is to perform statistical analysis of the data to determine significant lipid species and reveal the biological interpretation. In a simplest setting, the descriptive data analysis can be carried out with statistical methods such as two-sample Student's t-test, Wilcoxon test, Wilcoxon signed-rank test, and Mann-Whitney U test. Analysis variance (ANOVA) may be used to compare the means of two or more groups assuming that sampled population are normally distributed. Correlational analysis can be performed to determine the degree of relationship between two variables, which is measured using a correlation coefficient. Statistical methods are also in place for analysis of multivariate data. Principal component analysis (PCA) is a useful statistical technique for multivariate analysis of correlated variables. It is mostly used as a tool in exploratory data analysis and for making predictive models. Hierarchical clustering analysis (HCA) is widely used for multivariate data analysis, displaying cluster analysis results with heat maps. Partial least square-based discriminant analysis (PLS-DA) is a widely used, supervised classification algorithm when dimensionality reduction is needed, and discrimination is sought in multivariate analysis. Multivariate analysis of variance (MANOVA) is a statistical test procedure for comparing multivariate (population) means of several groups, which uses the variance/covariance between variables in testing the statistical significance of the mean differences. Many of the abovementioned tools are available from commercial software (e.g., SAS, NCSS, IBM SPSS, SIMCA-P). Graphic display is often used for data visualization and presentation in lipidomic analysis. Many software tools such as MetaboAnalyst 2.0, Prism, Origin, and R package can be useful for graphic display of lipidomic data.

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