Biochimica et Biophysica Acta xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Reporting of lipidomics data should be standardized☆

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ARTICLE INFO

Article history:
Received 15 January 2017
Received in revised form 20 February 2017
Accepted 21 February 2017
Available online xxxx

ABSTRACT

This article highlights, to our opinion, some of the most pertinent issues related to producing high quality lipidomics data. These issues include pitfalls related to sample collection and storage, lipid extraction, the use of shotgun and LC-MS-based lipidomics approaches, and the identification, annotation and quantification of lipid species. We hope that highlighting these issues will help stimulate efforts to implement reporting standards for dissemination of lipidomics data.

1. Introduction

Mass spectrometry (MS)-based lipidomics has revolutionized the study of lipids by its power to provide detailed quantitative information about the extensive number of lipid molecules in cells, tissues and biofluids. This capability is currently drawing major attention as it offers new ways and opportunities to study lipid biology in health and disease. However, this positive trend brings the downside that an increasing number of studies are reporting poor quality lipidomics data with misidentification and inaccurate/inappropriate quantification of lipid molecules. This in turn can side-track the fields of investigation that utilize lipidomics technology, impede the progress of cumulative knowledge production and waste resources [1]. Notably, the problem with poor data quality primarily stems from studies using untargeted metabolomics approaches [2], an area which has received enormous amount of funding and interest for its expected power in biomarker and clinical discovery. The reasons for poor data quality are multifactorial covering both the analytical, bioinformatic and educational aspects. For instance, adequate controls of sample handling and processing are often missing as are the use of appropriate internal standards and lipid annotation. Importantly, when such technical details are not considered or not described in detail it becomes very challenging, if not impossible, to judge the scientific merit of lipidomics data and establish reasons behind potentially poor data quality.

In the sections below, we highlight some of the most pertinent issues related to producing high quality lipidomics data. Moreover, we believe that some of these issues could help stimulate efforts to implement reporting standards for lipidomics data that is being shared with the scientific community

2. Sample collection and storage

A well-known fact among analytical chemists is that the analysis can only be as good as the sample material, i.e., garbage in–garbage out. Thus, care must be taken at all the steps of the analysis starting from sampling and sample preservation through to actual processing and analysis of the sample. In terms of preanalytics, special attention is necessary when lipid mediators are analyzed, e.g., immediate production of lysophospholipids upon blood drawing [3]. Moreover, hydrolytic enzymes may degrade lipids and may be active even in organic solvents [4,5]. Another concern is that proper vials should be used for storage of lipid extracts as this can otherwise degrade lipids [6]. Many lipids, especially the polyenoic ones, are prone to oxidation induced by either atmospheric oxygen, metal ions or peroxides present in e.g., impure solvents (e.g., old chloroform). Furthermore, ester, (vinyl)ether and amide bonds present in lipids are sensitive to various acids and bases. Due to these issues appropriate sample collection must be carefully considered and controlled. Samples should always be snap frozen in liquid nitrogen and stored at −80 °C until further processing steps. Moreover, samples should be aliquoted to avoid freeze-thaw cycles that potentially stimulate hydrolysis of lipids. In this way the potential creation of artefacts can be minimized. Moreover, appropriate handling of tissues is of great importance. For example, liver should be perfused to remove blood components, including cells, lipoprotein particles and albumin.

☆ This article is part of a Special Issue entitled: BBALIP_Lipidomics Opinion Articles edited by Sepp Kohlwein.

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http://dx.doi.org/10.1016/j.bbalip.2017.02.013
1388-1981/

3. Lipid extraction

Most lipidomics workflows apply nonpolar organic solvents like chloroform, methyl tert-butyl ether (MTBE) and heptane to extract lipids from samples. Beside the common Bligh and Dyer, and Folch lipid extractions [7], more recently modified protocols using MTBE [8], butanol:methanol (BUME) [9] and different chloroform/methanol ratios have been developed to improve the extraction recovery and mass spectrometric detection of lipids [10,11]. These protocols are well-established for a number of sample materials and lipid classes but should, if the sample matrix is changed, be evaluated in terms of recovery, reproducibility and generation of artefacts (e.g., lysophosphatidic acid generation from lysophosphatidylcholine upon strong acidification of human plasma [3]). The removal of polar metabolites, proteins and salts during lipid extractions employing a partition step greatly reduces the complexity of the samples. In contrast, simple protein precipitation frequently used in metabolomics approaches bears a potential of adverse matrix effects leading to misidentifications and inaccurate quantification. A proper lipidomics analysis includes the addition of internal standards prior to extraction, to facilitate monitoring recoveries and absolute quantification. Moreover, biphasic lipid extractions should be done with as little sample amount as possible in order not to exceed the capacity of the organic phase (e.g., for parallel quantification of abundant glycerolipids, glycerophospholipids, sphingolipids and sterol lipids no more than ~10 μl of plasma in a 1000 μl Folch lipid extraction should be used [12]).

4. Shotgun vs. LC-MS-based lipidomics approaches

The two main approaches in lipidomics research are today based on direct infusion MS analysis (also known as shotgun lipidomics) and liquid chromatography (LC)-MS analysis. In the former approach, a crude lipid extract is infused into the MS instrument and, dependent on the resolving power, either direct MS scans and/or specific precursor ion scans, neutral loss scans, selected ion monitoring or data dependent MS^n scans are used to detect different lipid species [10,13–15]. The latter approach employs LC together with online MS detection [16,17].

Both approaches have their advantages and disadvantages. In general, the infusion-based shotgun lipidomics is simpler as it does not involved operating a LC system in parallel, and it also does not require implementation of additional quality controls that monitor drifts in LC-peak shapes and retention times. It provides simple means for quantitation since all analytes and internal standards are present in the same sample matrix, and thus, subject to the same ion suppression and matrix effects. However, this benefit is also its main downside – ion suppression from both abundant lipid analytes and impurities present in the sample limit its sensitivity for low abundant lipid molecules (this is however, flow rate-dependent). LC-MS, by separating both analytes and impurities can relieve ion suppression effects thus (typically) improving sensitivity, especially for low abundant lipids. Additional benefits include the characteristic retention times of lipid analytes that can be used for more confident lipid identification [17], resolution of isobaric and isomeric lipid species as well as reducing issues related to the so-called ^{13}C-isotope effect, if lipids are baseline resolved (see below). However, compared to shotgun lipidomics analysis, short LC peak windows may not permit identification and structural analysis by MS/MS of all precursor ions. The main downside for the LC-MS, especially when using reversed-phase liquid chromatography (RPLC) approaches is that lipid analytes and internal standards elute at different times, thus experience different matrix effects and different solvent compositions that influence their ionization efficiencies and result in inaccurate quantification. Thus, accurate absolute quantitation requires isotopically labelled standards for every lipid molecule that is measured [18,19], whereas in shotgun lipidomics a single non-endogenous synthetic standard is often sufficient to quantify all lipid molecules belonging to the same lipid class [10,20], which is similar in normal phase-LC (NPLC) and hydrophilic interaction chromatography (HILIC) (see below).

5. Lipid species identification

Correct identification and annotation of detected lipid molecules are important steps in a lipidomics workflow and frequently fails due to multiple reasons. MS/MS analysis of lipid molecules yields the so called "lipid class-selective fragments" that are common to lipids belonging to the same lipid class (e.g., all PC molecules release *m/z* 184.0733, all PE species undergo neutral loss of 141.0191) [15]. Detection of such lipid class-selective fragments supports annotating detected lipids by "sum compositions" and is incapable in differentiating bond types such as isobaric ester and ether species PC O-34:1 ($[M + H]^+$ *m/z* 746.6) and PC 33:1 ($[M + H]^+$ *m/z* 746.6) when using low resolution triple quadrupole and ion trap instruments. Today, with high resolution mass spectrometers, such as Orbitrap or FTICR instruments, the *m/z* of an ion corresponding to a lipid species can be determined solely based on mass accuracy as this allows assigning elemental composition. This way makes it easy to differentiate isobars like ether-bond PC O-34:1 ($[M + H]^+$ *m/z* 746.6058) from diacyl PC 33:1 ($[M + H]^+$ *m/z* 746.5694). However, isomers such as PC 31:1, DMPE 32:1, MMPE 33:1 and PE 34:1 can still not be differentiated without fragmentation. Moreover, fragmentation of the intact precursor lipid should still be performed to verify the presence of the lipid molecule and make sure it is not misidentified chemical noise. Lipid molecules detected based on fragment ions matching hydrocarbon chains can be annotated as "molecular lipid species" (e.g., PC 16:0_17:1 or alternatively PC 16:0–17:1) [20,21]. This annotation does not warrant assignment of *sn*-positions of hydrocarbon chains in glycerolipids and glycerophospholipids, although some investigators claim this. To infer this information (e.g., PC 17:1/16:0) requires validated assays based either on monitoring ratios between fragment ions of positional isomers (direct infusion [21], LC-MS [17]) or gas phase reaction (e.g., with ozone [22]) or ion mobility-MS [23].

Notably, there are many issues that need to be considered when identifying lipid species by MS/MS or multistage (MS^n) analysis. In complex samples the presence of isobaric and isomeric lipid species, different adduct ions as well as ^{13}C and other isotopes can lead to co-isolation of multiple distinct chemical species producing fragment ion spectra containing composites of multiple precursors. Hence, for MS/MS analysis it is mandatory to isolate lipid precursors using an ion isolation window of 1 Da (i.e., unit resolution) or lower. This helps make identification and quantitative assessment of easier, especially when acyl fragments from isomeric and isobaric lipid molecules are prominent. Moreover, in-source fragmentation may generate additional precursor ions, e.g., loss of water observed for ceramides [24].

A way to reduce sample complexity and to differentiate isobaric or isomeric species is to use LC-MS [16,17]. Since different lipid species are temporally resolved (when using relatively long gradients) this reduces overlap of isotopic patterns. Hence, co-isolation of multiple precursors is less frequent, which makes the identification of the precursor simpler. While separation of glycerophospholipid positional isomers is not possible by RPLC, NPLC and HILIC methods, lysoglycerophospholipid positional isomers can be resolved by RPLC [17], HILIC [25,26] or di- and monoacylglycerol positional isomers by NPLC [27]. Moreover, lipid class-specific separation by HILIC and NPLC may resolve lipid isomers [28,29].

In summary, identification of lipid species requires consideration of co-isolation of isobaric and isomeric lipid molecules, isotopic overlaps, interferences from potential adduct ions and in-source fragmentation (hence, ion isolation for MS/MS must be performed with unit resolution). These considerations are relevant also for LC-MS methods which should use in addition retention times for identification (often chemical noise is misidentified as a lipid molecule). Retention times of lipid species in RPLC should be evaluated in respect to their double bonds and

Please cite this article as: G. Liebisch, et al., Reporting of lipidomics data should be standardized, Biochim. Biophys. Acta (2017), http://dx.doi.org/10.1016/j.bbalip.2017.02.013