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# Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry – What, how and why?

**Miao Wang**[1], **Chunyan Wang**[1], and **Xianlin Han**[1,2,*]

[1]Center for Metabolic Origins of Disease, Sanford Burnham Prebys Medical Discovery Institute, Orlando, FL 32827 USA

[2]College of Basic Medical Sciences, Zhejiang Chinese Medical University, 548 Bingwen Road, Hangzhou, Zhejiang 310053, China

## Abstract

Lipidomics is rapidly expanding because of the great facilitation of recent advances in, and novel applications of, electrospray ionization mass spectrometry techniques. The greatest demands have been in successful quantification of individual lipid classes, subclasses, and individual molecular species in biological samples at acceptable accuracy. This review addresses the selection of internal standards in different methods for accurate quantification of individual lipid species. The principles of quantification with electrospray ionization mass spectrometry are first discussed to recognize the essentials for quantification. The basics of different lipidomics approaches are overviewed to understand the variables that need to be considered for accurate quantification. The factors that affect accurate quantification are extensively discussed, and the solutions to resolve these factors are proposed-largely through addition of internal standards. Finally, selection of internal standards for different methods is discussed in detail to address the issues of why, how, what, and how much related to internal standards. We believe that thorough discussion of the topics related to internal standards should aid in quantitative analysis of lipid classes, subclasses, and individual molecular species and should have big impacts on advances in lipidomics.

## I. INTRODUCTION

Cellular lipidomes are complex (Han & Jiang, 2009; Shevchenko & Simons, 2010). Hundreds of thousands of individual lipid molecular species which are divided into different classes and subclasses based on their backbone structure, head groups, or aliphatic chain linkage (Han & Gross, 2005a) are present in cellular lipidomes. The changes in the levels and/or composition of lipid species and/or classes occur after perturbation or during cell growth. Lipidomics has been developed to identify and quantify these changes, to investigate the functions and interactions of these involving lipids, and to delineate the biochemical mechanism underlying lipid changes under patho(physio)logical conditions (Han & Gross,

*To whom correspondence should be addressed: Xianlin Han, Ph.D., Center for Metabolic Origins of Disease, Sanford Burnham Prebys Medical Discovery Institute, 6400 Sanger Road, Orlando, Florida 32827, USA, Telephone: (407) 745-2139, Fax: (407) 745-2016, xhan@sbpdiscovery.org.

2003). Despite that research in lipidomics has already provided some insights into different disease states by extensive quantification of alternations in cellular lipidomes under the conditions (Bleijerveld et al., 2006; Cheng et al., 2013; Han et al., 2002; Miao et al., 2014; Postle & Hunt, 2009; Wang et al., 2015b), to accurately determine the levels of individual lipid species by global lipidomics still remains a major challenging in the field.

Numerous modern technologies, such as mass spectrometry (MS), fluorescence spectroscopy, nuclear magnetic resonance, UV-visible absorbance spectroscopy, and microfluidic devices have been developed and applied to determine the complexities inherent in cellular lipidomes (Feng & Prestwich, 2006). Most importantly, recent advances on electrospray ionization mass spectrometry (ESI-MS) have greatly revolutionized the progress on lipidomics (Blanksby & Mitchell, 2010; Han et al., 2012; Ivanova et al., 2009; Wenk, 2010). Currently, ESI-MS based approaches are widely used in the field and can be classified into two major platforms (i.e., direct infusion and liquid chromatography coupled MS (LC-MS)) that depend on whether analysis of lipids is conducted under a condition of constant lipid concentration. The major feature of direct infusion (generally termed shotgun lipidomics) is that analysis is performed at a constant concentration of lipid solution. The LC-MS platform features quantitative lipid analysis performed after separation of lipid classes and/or species with an LC column. In this latter case, the concentrations of lipid species in LC eluents vary with time.

In analytical chemistry, the goal of quantification is to determine the relative or absolute abundance of one, several, or all component(s) present in a sample of interest. For lipidomic analysis, relative quantification measures the pattern changes of lipid species in a lipidome. This approach is very useful and common for biomarker discovery and for readout after biological treatment or stimulation. Absolute quantification determines the mass levels of individual lipid species, and derives the total amounts of lipid subclasses and classes of a cellular lipidome of interest. Absolute quantification is critical to elucidate biochemical mechanism(s) responsible for the changes after a change of a biological system, to discover corresponding biomarkers, and to develop new drugs through quantitative pathway/network analyses.

In light of the importance of absolute quantification of lipids, this article focuses mainly on this topic. The strategies and principles of absolute quantification of lipids are first overviewed. Topics related to internal standards for absolute quantification, including what types of internal standards can be chosen, how many internal standards are needed, what amount is added, and why these internal standard types and levels are essential for accurate quantification of lipid species, subclasses, and classes, are extensively discussed. It should be recognized that the phrase “accurate quantification” to chemists and biochemists may have different expectations. To a chemist, accurate quantification means an accuracy of 99% or greater. To a biochemist, the expectation for accurate quantification is relatively loose (e.g., > 90%) since many uncertainties are present in the process from sampling, sample preparation, and analysis. It is our hope that the description presented herein can provide a foundation for absolute and accurate quantification by using an appropriate numbers and amounts of internal standards, and can serve as a reference for further expansion of one's knowledge in this important area.

## II. PRINCIPLE OF QUANTIFICATION OF COMPLEX LIPID SPECIES WITH MS

In MS analysis, the amount of an unknown compound can be, in principle, quantified with the relationship between the concentration and ion current (which is proportional to ion intensity as commonly measured) of the analyte within a specific linear dynamic range, as follows:

$$I = I' - B = A^* c \quad (1)$$

where $I'$ is the measured ion intensity of the analyte with MS; $B$ is the spectral baseline from electrical drifting and/or chemical noise, particularly in a triple quadrupole mass spectrometer (Papan et al., 2014); $I$ is the actual ion intensity of the analyte after baseline correction; $A$ is an overall response factor of MS analysis; and $c$ is the concentration of the analyte. It should be emphasized that the effects of response factors and baseline on general ESI-MS analysis has been extensively discussed previously (Cech & Enke, 2001; Gundlach-Graham & Enke, 2015).

Obviously, the actual ion intensity must be corrected for baseline noise $B$ in Equation 1 before quantification unless the ion intensity of analyte of interest is very abundant. The presence of baseline noise in a triple quadrupole mass spectrometer may be easily recognized and corrected (Yang et al., 2011a) in shotgun lipidomics is obvious. However, this type of noise might not be directly visible in LC-MS lipidomics, particularly if the separation is performed with gradient elution, where the noise level is difficult to evaluate due to the variable composition of matrix background. Although the extracted ion current at a neighboring elution time is applied to evaluate the baseline, it cannot represent the entirely level of noise at the time where the lipid species of interest elutes. Tandem MS analysis can significantly reduce baseline noise by nature of double filtering; however baseline correction is still required for accurate quantification of low-abundance species.

Instead of correcting for baseline noise, if $I'$ is much greater than $B$, such as signal to noise (S/N) ratio higher than 20, which results in an approximately 5% of systematic error (Han et al., 2008), then $B$ is negligible. In that case, Equation 1 is transferred into

$$I \approx I' = A^* c \quad (2)$$

If the response factor $A$ of an analyte of interest were constant, it could be determined with its standard compound in MS analysis and absolute quantification of its concentration would be readily calculated with Equation 2 within the linear dynamic range. In this case, MS analysis would be similar to the Lambert-Beer law in optical spectroscopic analysis, where the response factor (molar attenuation coefficient) could be determined and absolute quantification of an analyte could be performed with the determined molar attenuation coefficient. Unfortunately, the actual ion intensity of an analyte in MS measurement could easily be affected by even very minor alterations in sample preparation, analyte ionization condition, and/or instrumentation variation, such as tuning, calibration, and detector sensitivity. Therefore, the response factor $A$ with MS analysis might be varied or

irreproducible (e.g., > 20%) for an identical analyte with a fixed concentration by a same mass spectrometer, and would never be constant in MS analysis. Even more complicated is that most of the altered factors that affect the response factor $A$ in MS analysis are difficult to control and even impossible to be noticed. Hence, it would be hard to acquire a constant response factor for an analyte of interest, like in optical spectroscopic analysis. Consequently, direct quantification according to Equation 2 in MS analysis would be impractical.

Either an external standard or an internal standard is required to quantify an analyte of interest in MS analysis. When an external standard is used, usually it is the identical species to the endogenous one synthesized or purified to high purity, and a calibration curve of the standard, concentration *vs.* ion intensity, in its linear range is established with a series of concentrations under identical experimental conditions, including the sample matrix. Quantification of the analyte of interest is achieved by measuring the ion intensity of the analyte by MS and comparing it to the established standard curve. The advantage of quantification with an external standard is that there is no concern about overlap of the standard with endogenous molecular species because the standard solution is analyzed separately from the sample. Moreover, the analyte used for the external standard is also readily available. However, as described above, it is idea to maintain identical experimental conditions for analysis of the standard and sample. If we assume that stable MS instrument conditions are achieved, sample preparation, which involves multiple steps (extraction and separation), could lead to differential recovery and carryover from sample to sample, and the composition of the analyzed sample matrix could vary, either due to variations of solvent gradients or presence of differential co-eluents during any chromatographic separation. All of these factors can contribute to the variations of the analyzed solution from the solution used for analysis of external standard, and cause differential ionization responses between analyses. Even small variations in spray stability during ESI-MS analysis or other factors might lead to remarkably different ionization efficiency from time to time. Collectively, quantification with external standards alone is usually not the best choice for analysis of a complex system, especially when the analysis is associated with a complicated process such as comprehensive analyses of an entire cellular lipidome.

When an internal standard is used for quantification, the standard which is usually an analog of the analyte (e.g., its stable isotopologue) should be added at the earliest step possible during sample preparation and extraction. Both the internal standard and analyte are analyzed simultaneously to compensate for any possible variation during the entire process of sample preparation and analysis. The internal standard should be absent from the sample or present at extremely low abundance (e.g., << 1% of the analyte of interest). The added amount of the internal standard should be in an appropriate ratio to the analyte after consideration of the dynamic range (see Section IV for further discussion). The advantage of quantification with an internal standard is its simplicity and accuracy because of the simultaneous process and analysis of internal standard and analyte of interest. No calibration curve is required as long as the concentrations of internal standard and the analyte(s) fall in the linear dynamic range. Any adverse influence due to the factors mentioned above in sample preparation and MS analysis could be minimized because of the simultaneous process and analysis of the internal standard and the analyte of interest, which facilitates the

possibility of analysis accuracy. However, selection of an appropriate internal standard(s) to analyze a biological sample may be difficult as different systems possess varying endogenous species at differing levels, and therefore might require different internal standard species and/or amounts. Specifically, utilization of non-endogenous standards or isotopically labeled counterparts of the analyte might be necessary to avoid any potential overlap with endogenous species in the analyzed system. Therefore, selection of an appropriate internal standard is never straightforward, and requires some expertise and pre-determination.

When an internal standard is selected for quantification of an analyte of interest, Equation 3 can be derived from Equation 2 by dividing the parameters from the selected internal standard

$$I_u/I_s=(A_u/A_s)^*(c_u/c_s) \quad (3)$$

where $I_u$ and $I_s$ are the measured baseline-corrected ion intensities of the analyte and the internal standard from a MS spectrum (or an LC-MS run), respectively; $c_u$ and $c_s$ are the unknown concentration of the analyte and the known concentration of the added internal standard, respectively; and $A_u$ and $A_s$ are the response factors of the analyte and standard, respectively, under experimental conditions. If, under certain experimental conditions, the analyte and its selected standard have identical response factors (i.e., $A_u/A_s = 1$), then the concentration of the analyte is readily determined from the following simplified equation without knowing the response factors:

$$c_u=(I_u/I_s)^*c_s \quad (4)$$

This relationship has been proposed for quantification by MS a long time ago (Hammar et al., 1968) and is referred to as a “ratiometric comparison” in literature (Han et al., 2004a; Han et al., 2008; Yang et al., 2009a).

In order to satisfy the requirement of identical response factors, the stable isotopologue form of the analyte is the ideal internal standard because it possesses nearly the same structure and MS ionizability as the analyte. However, for a complex biological system such as a cellular lipidome, selection of internal standards to match every lipid species is impractical, if not impossible, since there are hundreds to thousands of species of interest (Ekroos et al., 2002). Fortunately, numerous experiments have showed that all the individual lipid species in the same polar lipid class possess nearly identical response factors after taking into consideration of isotope effects (de-isotoping) as long as the MS analysis is performed in the low concentration region (Christie & Han, 2010; Han & Gross, 1994; Han & Gross, 2001; Koivusalo et al., 2001). Even though each species in a polar lipid class contains aliphatic chains differing in length and unsaturation, the species exhibit essentially identical ionization efficiency because ionization of these species is predominantly dependent on their identically charged head groups. Aliphatic chains only minimally affect ionization under certain experimental conditions as discussed below and in Section IV. Because individual

species in non-polar lipid classes (e.g., cholesterol esters, diacylglycerols (DAG), triacylglycerols (TAG), etc.) do not have a dominant ionizable head group, species in these lipid classes do not exhibit identical response factors even in a very low concentration region. For accurate quantification, response factors of individual non-polar species must be pre-determined in relation to their acyl chain length and unsaturation (Han & Gross, 2001). Alternatively, the molecular species in these non-polar lipid classes can be derivatized with polar reagents to generate a “permanent” polar head group prior to quantitative analysis (Wang et al., 2012; Wang et al., 2013; Wang et al., 2014a).

A low concentration of lipids in the sample solution induced into MS is one of the critical conditions for their accurate analysis. This is because a unique physical property of lipids is that they tend to form aggregates as the concentration of a lipid solution increases. Formation of lipid aggregates is related to combined effects of the length and unsaturation of their aliphatic chains. This tendency leads to variation in the ionization efficiencies and differential response factors (i.e., different $A_u$) for individual lipid species with varied aliphatic chains (Koivusalo et al., 2001). Therefore, it is very critical to perform lipidomic analysis of polar lipid species at a low concentration where no lipid aggregation occurs in order to achieve a linear dynamic range comparable to an internal standard

## III. PLATFORMS FOR QUANTIFICATION OF COMPLEX LIPID SPECIES

Our central goal of this article is to discuss and understand what types of internal standards should be selected, how many internal standards are needed, what amount of internal standards should be added, and why these internal standard types and levels are essential for accurate quantification of lipid species, subclasses, and classes. To better answer these questions, it is necessary to understand all platforms present in LC-MS based strategies and direct infusion-based shotgun lipidomics, which are the two major approaches in lipidomics. In this section, we discuss these platforms, including their fundamentals, advantages, and possible limitations to a great degree based on the principles of quantification of lipid species by MS described in Section II.

### A. Quantification with LC-coupled ESI-MS

In order to accurately quantify lipid species with LC-coupled ESI-MS, the analysis must meet at least one of the following requirements. First, if an external standard is used in the analysis, then the calibration curve of the external standard must be established under identical experimental conditions to the sample analysis. This approach or its variants has been broadly used for quantitative analysis of eicosanoids, endocannabinoids, and their metabolites (Kingsley & Marnett, 2009; Mesaros et al., 2009). Second, a stable isotopologue of the analyte of interest, if commercially available, may be used an internal standard for quantitative analysis. This approach is only practical for quantification of one or a limited number of species (Deems et al., 2007), and is impractical for a complex biological system such as a whole cellular lipidome. Third, one or two lipid species in a polar lipid class may be used as standards for the quantification of species in the same class, if the ionization efficiencies of the internal standard and species to be determined are identical under identical experimental conditions after use of some appropriate correction factors, if

necessary. The fragmentation kinetics of the internal standard and the species determined should be identical as well if tandem MS analysis, such as selected reaction monitoring (SRM) or multiple reaction monitoring (MRM), is involved in the experiment. Although it is possible to use one internal standard or one external standard curve to quantify all individual lipid species in a polar class, usually this only provides a rough quantification (see below for further discussion) (Laaksonen et al., 2006; Shui et al., 2007; Sparagna et al., 2005).

As long as the LC elution conditions are effectively coupled with the mass spectrometer, in theory, many stationary phases, including normal-phase, reversed-phase, ion exchange, hydrophilic interaction, would be suitable for LC-MS quantitative analysis of lipids. Prior to LC-MS analysis of lipids, the linear dynamic range, limit of detection, calibration curves of the lipid species of interest, and reproducibility of analysis are generally pre-determined. The reconstituted ion intensity (or peak area) of each determined lipid species can be compared to the pre-determined standard curve obtained under "identical" experimental conditions to quantify these lipids.

There are two common techniques for quantitative analysis of lipid species with LC-MS, including selected ion monitoring (SIM), and SRM (or MRM). The former detects ion intensities in a survey MS mode in which only a single *m/z* or a limited number of *m/z* ratios is transmitted for its/their quantification if a quadrupole is used as an analyzer; however, unlimited *m/z* could be detected if a time-of-flight or an Orbitrap analyzer is used. The latter performs tandem MS scan(s) to monitor a particular pair(s) of precursor/product ions at a specific elution time in an LC chromatogram to quantify the particular precursor ion(s).

**1. LC-MS Quantification of Individual Complex Lipid Species—**SIM quantification with a quadrupole analyzer monitors a single *m/z* at its maximum sensitivity solely due to the high duty cycle in this technique during a chromatographic separation. The reconstituted ion peak of each *m/z* that corresponds to a species of interest in SIM chromatography can be compared to either an external standard curve of the molecular species and/or to the reconstituted ion peak of a control compound (or internal standard) under identical experimental conditions for quantification. The advantage of SIM is its simple instrumentation requirement because no tandem MS is necessary. However, the monitored *m/z* in SIM could represent a combination of the species of interest and its isobaric/isomeric counterparts with a quadrupole analyzer to lead to less-specific identification and inaccurate quantification. A high mass accuracy/resolution MS would be preferable in this approach as recently demonstrated (Fauland et al., 2011). In practical analysis, at least one control compound (more compounds are preferred) for each lipid class should be included in an analyzed sample in consideration of the differential ionization efficiencies among lipid classes with differential head groups (Bollinger et al., 2010a; Bollinger et al., 2010b; Zhao & Xu, 2010). Accordingly, each reconstituted ion peak intensity (or area) of the species can be normalized to the built-in control compound of the class prior to comparison with the appropriate standard curve for accurate quantification in order to avoid any variations of HPLC separation conditions and/or ESI-MS conditions. These variations could dramatically alter the detected absolute ion signals of a specific species (but much less affect the relative ion counts) when normalizing to the built-in control compound under the identical experimental conditions. For example, Masukawa and

colleagues comprehensively identified and quantified as many as 182 ceramides and related species in human stratum corneum with LC-MS (Masukawa et al., 2009). In that study, numerous ceramide analogs were used as external standards to establish linearity of calibration curves, and a synthesized ceramide species (d18:1-N17:0) was used as an internal standard normalized all analyzed ceramide species. Hermansson employed a diol-modified silica column to separate over 100 lipid species, and identified and quantified them with two-dimensional spectra of elution time and *m/z* of the detected ion (Hermansson et al., 2005). Although this approach is suitable to analyze a limited number of lipids in a particular lipid class, quantification of lipids on a global lipidomics scale is quite limited (Hermansson et al., 2005; Shui et al., 2007) because it is impractical to generate the necessary standard curves for all lipid species in a lipidome, and it is very inefficient to pre-isolate every individual lipid class of interest. Moreover, the possible presence of multiple isobaric molecular species might complicate the analysis if a high mass accuracy/resolution MS is not used for analysis, whereas the presence of isomeric species makes the quantitative analysis more complicated even a high mass accuracy/resolution MS is employed.

**2. LC-MS/MS Quantification of Individual Complex Lipid Species—**In order to improve specificity and retain high sensitivity, SRM/MRM is appropriate. SRM is a technique where a mass transition from a parent (precursor) ion to a fragment (product) ion is monitored by a tandem mass spectrometer (de Haffmann, 1996); MRM applies SRM to multiple product ions from one or more precursor ions (Kondrat et al., 1978). If the monitored precursor-product pair(s) in SRM/MRM is/are specific to the targeted precursor(s) eluted at a specified time and co-eluents do not possess such transitions, then SRM/MRM is specific to the monitored pair(s) of precursor-product transition(s). In comparison to SIM, SRM/MRM has higher specificity and better sensitivity of detection. The specific monitoring of a pair of ion transitions leads to higher specificity, whereas better sensitivity is due to the significant reduction of the noise level through tandem MS monitoring. However, preliminary experiments are required to discover an appropriate specific transition pair for a targeted precursor ion and to determine how many such pairs of transitions exist at an elution time. A mass spectrometer with a high duty cycle capability is also important to quantify multiple species, because the specific elution from an LC column that contain an analyte of interest are time-limited for monitoring.

In practice, many applications in lipid identification and quantification are performed with the LC-coupled ESI-MS/MS platform. Giera separated almost 70 different lipid components from distinct lipid classes with a reversed-phase capillary LC-MS/MS screening platform, and identification and quantification were achieved for the phosphatidylcholine (PC), lysoPC, and eicosanoid species in human synovial fluid (Giera et al., 2012). The laboratories of Sommer, Byrdwell, Merrill, etc. have used normal-phase coupled with reversed-phase LC-MS to analyze lipid species in different classes in complex lipid mixture samples (Byrdwell, 2008; Merrill et al., 2009; Sommer et al., 2006). Neutral lipids, such as cholesterol and related metabolites, in dried blood samples have also been separated and quantified with reversed-phase LC-MS-MRM in a few minutes (Becker et al., 2015). Bohlinger and coworkers derivatized a positive charge-reversal head group with eicosanoid species, and significantly increased their sensitivity in LC-ESI-MS/MS analysis (Bollinger

et al., 2010b). Lipid MAPS consortium has developed comprehensive protocols for quantification of complex lipid species by LC-coupled ESI-MS/MS and applied for global analysis of plasma lipids (Quehenberger et al., 2010).

**3. Advantages and Limitations of LC-MS based Lipidomics—**The major advantage of LC-coupled ESI-MS or MS/MS analysis is that the complex lipid extracts are simplified with chromatographic separation. A complex lipid extract could be separated into individual lipid class with normal-phase LC, whereas a reversed-phase LC might resolve individual lipid species based on their different hydrophobicities. Due to the separation, LC can also reduce interferences of low- or trace-abundance lipid species from high-abundance lipids, and simultaneously enrich the low- or trace-abundance species for their identification and quantification. Therefore, another major advantage of LC-MS quantification over direct infusion-based lipidomics is its capability to discover and quantify low- or trace-abundance lipid species. Additionally, isomers of lipid species might be resolved by chromatographic separation with an appropriate mobile phase and column.

A few concerns associated with LC-MS analysis of lipids should also be recognized. First, although chromatographic separation can enrich low-abundance lipid species, it also makes high-abundance lipids more concentrated (up to 1,000-fold at the peak time) leading to possible aggregates of these lipids and thus losses in their ionization efficiency (i.e., ion suppression in the same lipid class (see further discussion in Subsections IVA and IVD)), which can affect the linear dynamic range of quantification. Second, different mobile-phase compositions from gradient elution could introduce variations in ionization efficiency of analytes at different elution times in LC separation. Third, when a normal-phase LC method is used to separate lipid classes, different lipid species of a class are not uniformly distributed in the eluted peak due to their differential interactions with the stationary phase. Therefore, each individual molecular species in a class possesses its own distinct retention time and produces interactions with other lipid species in the class (i.e., ion suppression in the same class (Subsection IVD)). Fourth, the polar mobile phase used at the initial stage of the gradient in reversed-phase LC analysis can limit solubility in a species-dependent manner to lead to differential apparent ionization efficiency. Fifth, differential loss of lipids on a column that lead to carry-over effects on the column, is also usual in LC analysis (DeLong et al., 2001). Sixth, because fragmentation in tandem MS modes is lipid-species dependent, differential response factors might be present among different lipid species in SRM/MRM analysis (see discussion in Subsection IVF). Additional internal standards that represent the diversity of lipid species in a class should be used in this case. Finally, multiple steps used in the entire process of LC-MS analysis, including sample preparation, chromatographic separation, and MS analysis, could bring in experimental variations of different species of lipids in each step. These variations impact on accurate quantification of individual lipid species. Moreover, these variations are propagated during processing. These errors are unlikely fully corrected with the standard curves that are hardly established under “identical” conditions to sample analysis. Due to these practical difficulties and limitations, LC-MS (MS/MS) generally is not suitable for a large-scale, high-throughput analysis, particularly for absolute lipidomics quantification; however, there are many reports of LC-MS applications in disease-based discovery, and identification and quantification of novel

lipids, particularly those present in low- or trace-abundance on a small scale (Guan, 2009; Kingsley & Marnett, 2009; Minkler & Hoppel, 2010; Tan et al., 2009).

It should be recognized that, as an advanced technique in LC, ultra-performance LC (UPLC) achieves equal or better chromatographic resolution in much shorter time frames, and also has been widely used in analysis of different lipid classes, including glycerophospholipids, lysoglycerophospholipids, lipid isomers, sphingolipids, polyunsaturated fatty acids and their oxygenated metabolites, and TAG (Astarita et al., 2015; Cajka & Fiehn, 2014; Damen et al., 2014; Lima et al., 2014; Mapstone et al., 2014).

## B. Quantification with Direct Infusion ESI-MS (Shotgun Lipidomics)

Compared to LC-MS (MS/MS)-based approaches, shotgun lipidomics is performed at a constant concentration of the solution for lipid analysis. Maintenance of such a constant lipid concentration at the sample inlet with a constant flow for direct infusion of a lipid solution into ion source can be achieved with a syringe pump, a chip-based apparatus (e.g., NanoMate), or an autosampler loop in certain cases. Without time constraints encountered in LC-MS during its “on-the-fly” analysis, in addition to a survey full-scan mass spectrum that displays all possible molecular ions of individual lipid species of a class (this display is important for direct comparison and quantification under an identical condition), shotgun lipidomics can also be used to acquire many tandem mass spectra, including precursor ion scanning (PIS) of specific fragment ions, neutral loss scanning (NLS) of particular lost neutral fragments, and product-ion scanning of molecular ions of interest for detailed structural and quantitative analysis. These kinds of mass spectra have been widely applied in shotgun lipidomics to facilitate the high-throughput analysis of a cellular lipidome on a global scale (Brugger et al., 1997; Ekroos et al., 2002; Han et al., 2004b; Welti et al., 2002). Generally, there are three platforms that follow the principles of shotgun lipidomics: tandem MS-based shotgun lipidomics, high mass accuracy MS-based shotgun lipidomics, and multi-dimensional MS-based shotgun lipidomics. Recent advances in shotgun lipidomics have been extensively discussed (Wang et al., 2015c). It should be pointed out that shotgun lipidomics with ion-mobility has been developed and greatly advanced recently [see (Kliman et al., 2011; Mclean et al., 2010; Paglia et al., 2015) for recent reviews]. However, the application of this approach is mainly in identification of lipid species, including isomers, rather than in accurate quantification. Therefore, discussion of this approach is not included in this article.

**1. Tandem MS-based Shotgun Lipidomics—**The species in a lipid class usually contain one or more characteristic fragments that are typically associated with the head group or part of this group after collision-induced dissociation (CID). These diagnostic fragments from the precursor lipid molecular ions of a class can be used to monitor the presence of each individual species of this class of lipids through PIS, NLS, or both after direct infusion (Brugger et al., 1997). However, the distinct chemical structures, mainly the acyl chain lengths, degree of unsaturation, and location of double bonds, of individual species and the condition of CID could lead to differential fragmentation kinetics and different ion intensities of the lipids in tandem MS spectra (PIS or NLS), which is termed tandem MS factor, and is extensively discussed in Subsection IVF. Thus, the tandem MS

factor could depend on species structures and/or collision conditions, as exemplified in Figure 1 which displays variations of ion intensities of brain PS species present in a lipid extract from mouse brain cortex detected with NLS of 87 Da in the negative-ion mode (Panels A and B) or 185 Da in the positive-ion mode (Panels C and D) at different collision energies specified. The great variations indicate the differential fragmentation kinetics and/or thermodynamics of these PS species at different collision energies of CID. Therefore, for the purpose of accurate profiling and quantification with tandem MS-based shotgun lipidomics, at least two internal standards are required to correct the effects of differential fragmentation kinetics and thermodynamics. The two or more selected internal standards should represent the chemical structures that span the entire class of lipids. A calibration curve from the ion peak intensities of the internal standards is usually derived to quantify the species of the entire class from their peak intensities, as previously described (Brugger et al., 1997).

Due to its simplicity and efficiency, tandem MS-based shotgun lipidomics has been widely used to characterize, profile, and quantify lipid species. For example, Brügger and colleagues have used this approach to selectively detect individual classes of glycerophospholipids from unprocessed total cellular lipid extracts of Chinese hamster ovary cells (Brugger et al., 1997). Structures of PC were successfully characterized in the negative-ion tandem MS mass spectrum, and rat bile PC species were also quantified with this approach with PIS of *m/z* 184 in the positive-ion mode (Lehmann et al., 1997). Hsu and colleagues have applied this approach to identify class-specific fragment ions or neutral-fragment losses, and extensively characterize the fragmentation patterns of various lipid classes, including glycosphingolipids and choline glycerophospholipids (Hsu et al., 2003; Hsu & Turk, 2001). Welti and colleagues have employed this approach for numerous studies on plant lipidomics (Samarakoon et al., 2012; Welti et al., 2002; Welti et al., 2007). Stegemann and coworkers have employed this approach to profile eight different lipid classes in 685 plasma samples and reveal three specific lipid species associated with cardiovascular disease risks (Stegemann et al., 2014).

This quantification approach with tandem MS-based shotgun lipidomics is simple, efficient and suitable for high throughput analysis of lipid classes that possess one or more characteristic fragments. The tandem MS filtering process enhances the sensitivity of this approach by more than 10-times. However, some limitations of this approach should also be recognized in order to minimize limitations and improve the accuracy of quantification. The calibration curve based on selected standards might only partially represent the species of a lipid class in the sample due to complication of fragmentation kinetics/thermodynamics of various species with different aliphatic constituents. The possibility of the presence of artificial ions that do not belong to the lipid class of interest in the “so-called” specific fragment scanning of the class is relatively high due to limited mass accuracy or resolution. Information about fatty acyl substituents of the filtered ions in the lipid class is not provided by this approach. If a sensitive class-specific tandem MS profiling is lacking or the intensities of the specific fragment ions are weak, then the linear dynamic range for quantification might be limited for the lipid class.

**2. High Mass Accuracy MS-based Shotgun Lipidomics—**The high mass accuracy MS-based shotgun lipidomics uses hybrid mass analyzers (Stahlman et al., 2009) that possess high mass accuracy and high mass resolution, such as Q-TOF, Q-Orbitrap, or ion trap-Orbitrap (Chernushevich et al., 2001; Makarov et al., 2006; Zubarev & Makarov, 2013). These instruments can thus allow scientists not only to sensitively acquire full mass spectra of lipid samples of interest in the survey scan mode, but also to rapidly conduct product-ion MS analysis of lipid species in a small mass window (e.g., one or a couple of mass units) step-by-step to determine all the fragments in an entire mass region of interest (Ejsing et al., 2006; Ekroos et al., 2002; Schwudke et al., 2006; Schwudke et al., 2007). The high mass resolution and accuracy inherent in these instruments provides accurate measurement of the masses of individual molecular ions as well as fragment ions (e.g., 0.1 amu or higher). Such measurement of the fragments and molecular ions guarantees elimination of any possible false positive identification. From the acquired product-ion spectra, any PIS and/or NLS spectra of interest can be extracted with post-acquisition reconstruction to “isolate” the lipid species of a class of interest. The extracted PIS or NLS spectra also have the characteristics of high mass accuracy and high mass resolution, which can allow accurate recording of fragment ion masses to minimize artificial ions and facilitate accurate quantification

Quantification of individual species of a lipid class is conducted based on the sum of the abundance of the monitored fragment(s) of the molecular ion of interest in comparison to that of the spiked internal standard of the class (Ejsing et al., 2006; Schwudke et al., 2006). The use of sum of the fragment abundance might contribute to an increased sensitivity of detection and accuracy of quantification. The effects of different $^{13}C$ isotopologue distributions among the species of interest and the internal standard are considered. In contrast to the tandem MS-based platform, this approach identifies and quantifies those lipid classes that do not possess a sensitive class-specific fragment ion, such as TAG and ceramide. Moreover, this approach generally has a linear dynamic range of four orders of magnitude for quantification of glycerophospholipids and this dynamic range is sufficient for most biological samples (Stahlman et al., 2009). A schematic workflow and detail description of this approach for lipid analysis have been provided by Ekroos and colleagues (Jung et al., 2011; Stahlman et al., 2009).

However, quantification with this approach is still based on tandem MS analysis. Due to the differential fragmentation kinetics and/or thermodynamics of different species of a class under different collision energies of CID, two or more internal standards of a class are necessary to minimize any effects of differential fragmentation patterns for accurate quantification, especially to those species that contain polyunsaturated fatty acyl chains (Yang et al., 2009b). Moreover, identification and qualification of low-abundance species may be missed resulting from *m/z* shifts due to partially overlap with the two-$^{13}C$ isotopologue of the species with one more double bonds (Wang et al., 2014b). This issue can be resolved with a higher mass accuracy/resolution instrument, as recently described (Almeida et al., 2015). It should be emphasized that other versions of high mass accuracy MS-based shotgun lipidomics have been developed to accurately quantify lipid species with survey-MS scan mode after data-dependent or total mapping of molecular ions of lipid species (Almeida et al., 2015; Schwudke et al., 2011). In this case, one internal standard to

quantify individual species of a polar lipid class is sufficient (see discussion in Subsection IVC).

Because of high mass accuracy and resolution instruments, this approach has been broadly applied in a variety of biological and biomedical applications. For example, Papan and colleagues have used this approach to systematically screen for novel lipids (Papan et al., 2014). Searching through more than 1.5 million chemical compositions, they identified a novel class of lyso-maradolipids specifically enriched in dauer larvae (Papan et al., 2014). Jensen and colleagues have characterized ether lipid structures that carry an uncommon sulfono group and an inositol phosphate group from the archaeon *Sulfolobus islandicus*, and studied their biosynthetic pathways and physiological functions (Jensen et al., 2015). This approach has also been used to identify and quantify isomeric ceramides and more than 400 molecular lipid species in human plasma using a method that takes less than 12 minutes operating in both positive- and negative-polar modes (Simons et al., 2012).

**3. Multi-dimensional MS-based Shotgun Lipidomics—**The multi-dimensional MS (MDMS)-based shotgun lipidomics (MDMS-SL) technology maximally exploits the unique chemical properties inherent in discrete lipid classes or subclasses, including their differential hydrophobicity, stability, and reactivity, for identification and quantification (Han & Gross, 2005a; Han & Gross, 2005b; Han et al., 2012; Wang et al., 2015c). During liquid/liquid extraction, MDMS-SL uses a multiplexed approach that exploit differential hydrophobicity or differential chemical stability under acidic and basic conditions to separate or enrich differential lipid classes (Han et al., 2011; Jiang & Han, 2006; Jiang et al., 2007; Wang et al., 2015a). During sample infusion, MDMS-SL exploits differential charge properties of different lipid classes to selectively ionize a certain category of lipid classes under multiplexed experimental conditions and to separate many lipid classes in the ion source (i.e., intrasource separation), analogous to electrophoretic separation of different compounds with different pI values (Han et al., 2006b). In addition, the unique chemical properties of an individual lipid class also has been exploited to identify and quantify these lipids (Han et al., 2005; Han et al., 2006a; Wang et al., 2013).

The concept of building blocks in lipid structure is employed to identify individual lipid species with MDMS-SL (Han & Gross, 2005b; Han, 2007). Most building blocks can be determined with NLS and/or PIS in a mass-ramp fashion (Han & Gross, 2005a; Han et al., 2012), because neutral lost fragments or fragmental ions result from either the head group or fatty acyl chain building blocks. In addition to the molecular weight displayed in mass spectra, the structure of the individual lipid species is then identified from these building blocks. Some specific NLS or PIS can also be used to identify the regioisomers, including the position of fatty acyl chains (Wang et al., 2014a; Yang et al., 2009b). The PIS and/or NLS specific to head groups of most of lipid classes have been reported to identify and quantify those classes with MDMS-SL (Yang et al., 2009a).

After intrasource separation and identification of individual species with two-dimensional MS analysis, quantification of the identified individual species of a lipid class of interest is performed in the two-step procedure with MDMS-SL (Han & Gross, 2005a; Han & Gross, 2005b; Han et al., 2012; Wang & Han, 2014), which not only incorporates exogenously

added, pre-selected internal standard(s), but also includes abundant endogenous lipid species accurately quantified in the first step with exogenously added internal standard(s). Briefly, in the first step, the species in a lipid class of interest that do not overlap with species of other lipid classes, and that also are present in abundance, are quantified from the survey-MS scan acquired after intrasource separation with ratiometric comparison to the pre-selected internal standard after baseline correction and $^{13}C$ de-isotoping. The determined contents of these non-overlapping and abundant species plus the pre-selected exogenously added internal standard(s) of the class are the candidate standards in the second step to quantify the rest of the species that either are present in low abundance or overlap with species of other lipid classes with one or more class-specific PIS or NLS spectra. In this unique two-step quantification procedure, all identified lipid species are quantified by comparison to exogenous and endogenous internal standards in survey and class-specific tandem MS scans. The linear dynamic range is extended dramatically to accurately quantify those species present in overlapping and/or in low-abundance due to the use of multiple standards, filtering overlapping species that use class-specific MS/MS scan(s), and significant reduction of background noise and increases in S/N ratios of low-abundance species with tandem MS (Han & Gross, 2005a).

It should be pointed out that, although this second step of quantification is similar to tandem MS-based shotgun lipidomics to quantify individual lipid species as described above in some aspects, MDMS-SL uses combined exogenous internal standard(s) and endogenous standards determined from the first step, whereas the latter platform exclusively uses the exogenously added internal standards. One of the big advantages to use endogenous species as standards is that these standards generally provide a more comprehensive representation of the physical properties and chemical composition over the entire class of lipids in light of wide coverage of the number of chain lengths and double bonds, whereas the selections of those externally added standards are generally limited in order to eliminate any possibility to overlap with endogenous species. Another big difference between two standards for the second step quantification and tandem MS-based shotgun lipidomics is that all quantified individual species in the former are pre-identified with MDMS-SL in order to eliminate inaccuracy from any artifact peak present in class-specific tandem MS (Yang et al., 2009a). If other tandem MS scans specific to the class of lipid interest are sensitive enough, then they can also be used in the second step to quantify low-abundance or overlapped species to refine the data and serve as an internal validation of accurate quantification.

This two-step quantification approach has been applied to measure individual species of nearly 40 lipid classes from lipid extracts of biological samples with or without derivatization (Wang et al., 2015a; Wang et al., 2012; Wang et al., 2013; Wang et al., 2014a; Wang et al., 2015b; Yang et al., 2009a). Moreover, with the second step of quantification, an over 5,000-fold linear dynamic range for many lipid classes can be readily achieved (Han et al., 2008) because the second step quantification extends the dynamic range.

Two caveats should be recognized when this two-step quantification approach is applied for MS analysis. First, this two-step quantification strategy cannot be applied to any lipid class where a class-specific or sensitive tandem MS analysis is not present, such as cardiolipin, TAG, and DAG. Special approaches have been developed to identify and quantify these lipid

classes with MDMS-SL (Han et al., 2005; Wang et al., 2012; Wang et al., 2013; Wang et al., 2014a), or to exploit the unique physical and/or chemical properties of the lipid classes (Han et al., 2004b; Han et al., 2006a). Second, the experimental error for the species determined in the second step with endogenous standards might be larger than that in the first step due to the propagation and amplification of experimental errors. To reduce the propagated errors in the second step, it is critical to minimize any potential experimental error in the first step. To this end, only the high-abundance species with a large S/N ratio, which could be quantified accurately in the first step, should be selected as endogenous standards for the second step to reduce error amplification. As discussed above, the species quantified in the second step only accounts for a small amount of the total contents of the class. Therefore, the propagated experimental error in the second step only moderately affects the accurate analysis of total content of the lipid class. It should also be recognized that, in comparison to other two platforms of shotgun lipidomics, MDMS-SL is less high throughput because of the efforts involving sample preparation (e.g., derivatization) to analyze low-abundance lipid classes, as mentioned above.

**4. Advantages and Limitations of Shotgun Lipidomics—**It is a misconception consistently stated in the literature and in symposia that, due to ion suppression always present in analysis of complex lipid mixtures, accurate quantification of complex lipid species cannot be achieved with any shotgun (direct infusion) lipidomics approach, and that this approach only provides a profile comparison among different states of samples. This belief is misleading because this concept only holds true when inappropriate experiment conditions are employed in the MS analysis, such as when a high concentration of lipids in analyzed samples is directly infused into the MS, which leads to formation of lipid aggregates, or quantification performed outside of the linear dynamic range. In fact, any approach for quantification would be meaningless if the appropriate experimental conditions or requirements are not met.

With appropriate sample preparation, instrument settings, and/or experimental design, compared to LC-coupled ESI-MS analysis, shotgun lipidomics offers important advantages: (1) rapid and efficient analysis of lipid classes, subclasses, and individual species can be achieved directly from crude extracts suitable for high-throughput and large-scale quantitative analysis of lipids; (2) in the low-concentration region, there is a linear correlation over a 10,000-fold dynamic range between the ion intensities and the concentration of individual lipid in analysis of polar lipid classes; (3) for polar lipid classes with a survey MS scan, ion intensities of lipid species largely depend on the nature of polar head group instead of on acyl chain physical properties such as chain length and degree of unsaturation; and (4) the reproducibility of measurements is excellent with < 10% of experimental error for an identical sample because all measurements are normalized to the same set of internal standards (Han & Gross, 2005a).

In addition to those limitations associated with different platforms of shotgun lipidomics, as discussed above, another limitation related to linear dynamic range or ion suppression should be recognized. In order to avoid lipid aggregates at a high concentration, entire lipid extracts from biological samples must be diluted for direct infusion analysis at a low concentration. This process also reduces the concentrations of lipid species in very low

abundance, such as many of the signaling lipids (Wymann & Schneiter, 2008), and leads to the loss of quantification and detection abilities on these lipid species with shotgun lipidomics. To reduce this limitation, very low abundance lipids can be pre-enriched with a solid phase extraction column or special liquid-liquid partition, depending on their physical and chemical properties, before direct infusion (Jiang & Han, 2006; Wang et al., 2015a). Alternatively, the interfering lipids can be removed with a certain of chemical reactions (Jiang et al., 2007) or solvents (Han et al., 2011). In all these cases, the existence of internal standards is important to compensate any incomplete separation, recovery, and/or reaction for subsequent quantification of lipid species of interest.

## IV. FACTORS THAT AFFECT ACCURATE QUANTIFICATION OF COMPLEX LIPID SPECIES IN LIPIDOMICS

As discussed above, Equation 4 only holds true when the response factor for the analyte of interest in MS analysis is identical to that of the selected standard. Therefore, great efforts should be made in experimental design to achieve this goal. In this section, we extensively discuss all of the potential sources that contribute to the changes of this overall response factor so that we can understand how to deal with these issues in MS analysis through either elimination of these issues by correction factor(s) or using additional internal standards to correct for the effects caused by these factors. Addition of a number of internal standards to correct the effects of these issues is the topic of the next section.

Generally, the overall response factor $A$ could be expressed as multiple sub-terms, each of which represents a factor that contributes to the overall response factor $A$ as follows:

$$A = A_1{}^* A_2{}^* A_3{}^* A_4{}^* \ldots \ldots \quad (5)$$

where $A_1$, $A_2$, $A_3$, $A_4$, and more correspond to the components that differ between an analyte and an employed standard and such a difference could lead to inaccurate quantification of the analyte. These components include, but are not limited to, ionization efficiencies of different species within a class, concentration of lipid solution that affects different species of a class, differential collision kinetics and thermodynamics that result from tandem MS, different matrices experienced by the analyte and the standard, differential mass spectral baselines, different number of carbon atoms of different lipid species of a class that lead to differential isotopologue distributions, and so on.

### A. Lipid Aggregation

With the increase in a lipid concentration or polarity of the solvent of a lipid solution, lipids tend to form aggregates, including lipid dimers, oligomers, or micelles, due to their unique property of high hydrophobicity. More hydrophobic lipids, i.e., containing longer acyl chain or less unsaturation, could form aggregates at lower concentrations than those that contain less hydrophobic physical properties. Lipids cannot be ionized efficiently in aggregated forms, because aggregates possess very different polarity, geometry, size, etc. from lipid monomers, and thus lead to very different ionization process from individual molecular

species. In addition, the aggregates could contain a number of different lipid species and form adducts with several small ions. Thus, these aggregates could possess a different distribution of *m/z*, and some could be out of the mass-detection range of the instrument. Even those within the detection mass range are in the noise levels.

It has been demonstrated that the shorter or more polyunsaturated acyl chains in lipid species are, the higher apparent response factor they show in lipid aggregate form (Koivusalo et al., 2001). Therefore, ionization efficiency of lipids in aggregation depends on the physical properties of individual lipid species, not only on their head groups. Obviously, Equation 4 could not hold true under this situation if one species in a polar lipid class were used as an internal standard to quantify all species in the same class. This process is even more complicated with the dependence on the concentration of the solution during LC elution, so that the calibration curves were ineffective. Therefore, it is critical to keep the total lipid concentration lower than the aggregation concentration for accurate quantification of lipids by MS.

In addition to the physical properties of individual lipid class, lipid aggregation is also solvent dependent. The more polar the solvent system, the lower the concentration at which lipids start to aggregate. For instance, in a chloroform/methanol solvent system, the recommended upper limit of concentration where lipids form aggregates for shotgun lipidomics approaches is approximately 100 pmol/μL in a 2:1 (v/v), 50 pmol/μL in a 1:1 (v/v), and 10 pmol/μL in a 1:2 (v/v) system. However, non-polar lipids (e.g., TAG, and cholesterol and its esters) in a lipid solution could substantially lower this recommended upper limit. Therefore, a pretreatment with hexane or other non-polar solvents to remove most of the non-polar lipids would be ideal to quantify polar lipids if necessary, as previously described (Han et al., 2011).

In order to avoid lipid aggregates in biological samples for direct infusion into a mass spectrometer, it is important to estimate the total lipid concentration of a lipid extract prior to analysis. Some pre-knowledge of total lipids from a biological sample is very helpful. Based on our numerous laboratory studies, we found that the lipid content of the organ such as the heart, skeletal muscle, liver, kidney, or some types of cultured cells is approximately 300-500 nmol per mg protein, and the mass levels of lipids in brain samples are 1000-2000 nmol per mg protein, depending on the brain regions (Han et al., 2001; Han et al., 2002; Han et al., 2004b). Trial experiments are necessary to estimate the content of a lipid extract before analysis of an unknown sample.

The effects of lipid aggregation on quantification by LC-MS-based lipidomics have not yet been broadly recognized. Due to column enrichment, a species eluted from a column is substantially concentrated at its elution time when lipid aggregation could happen. Moreover, the mobile phase used in reversed-phase LC is typically more polar such as water, acetonitrile, methanol, or salts than with normal phase LC. Thus, lipids can aggregate at a relatively low concentration. The lipid aggregation formed in LC-MS-based lipidomics potentially affects their response factors and consequently their quantification, especially if only one standard is used. Therefore, stable isotope-labeled internal standards are always ideal for accurate quantification in LC-MS-based lipidomics.

The concentration where lipids start to aggregate is estimated through determination of the linear dynamic range for quantification of lipid of interest as described previously (Jiang et al., 2009). In the linear dynamic range, the upper concentration where regression deviates from linearity represents the concentration where the lipid species begin to form aggregates. Collectively, elimination of potential lipid aggregation (i.e., analysis at low concentrations) is essential to accurately quantify lipid species if a limited number of internal standards were employed. It should be emphasized that an alternative approach to avoid the molecular species-dependent manner under aggregation states for lipid quantification could also be employed by using their isotopologues of lipid species as internal standards to quantify these species if practical.

## B. Effects of $^{13}C$ Isotopes on Quantification

It has been shown a decade ago that, when an equal molar mixture of polar lipid species with different acyl chains in the same class are analyzed with shotgun lipidomics at low concentration (i.e., below an aggregation concentration), the species with longer acyl chains have lower monoisotopic peak intensities whereas species with shorter acyl chains show higher monoisotopic peak intensities due to differential distribution of isotopologues in these species (Han & Gross, 1994; Han & Gross, 2005b; Koivusalo et al., 2001). Therefore, inaccurate quantification occurs if only monoisotopic peak intensities between an analyte and its internal standard are ratiometrically compared without considering differential distribution of the isotopologues of these species. The isotopologue distribution is generally based on the total number of carbon atoms of each species because carbon atoms contribute to most of the isotopologue pattern of a lipid species, regardless that hydrogen atoms count for the largest number of atoms of a lipid species because a hydrogen atom has very low natural isotopic abundance (e.g., deuterium, 0.0115%) in comparison to carbon (e.g., $^{13}C$, 1.07%). It should be recognized that the contribution of other atoms (e.g., O, N, and P) to the isotopologue distribution is equal due to their identical numbers in each species of the entire class of interest if they are not modified lipid species. Therefore, only differential carbon isotopologue distribution must be considered for accurate quantification (discussed below). It should be emphasized that, if desired or necessary, the isotopologue effects of other atoms on the distribution can also be corrected with a more comprehensive calculation, as described previously (Eibl et al., 2008; Liebisch et al., 2004). However, if atoms such as Cl or S, which both have large nature isotopologue abundance, are present in a species and the atom numbers are different between the analyzed species and its internal standard, then their isotopologue distribution must be considered for accurate quantification.

Generally, two types of $^{13}C$ isotope corrections must be performed for quantitative comparison. The first one is to sum the intensities of all the isotopologues for each species of interest, including internal standards, before quantification is performed by ratiometrically comparing the sum of the isotopologue intensities of a species to that of the internal standard. Because the monoisotopic peak is the most-abundant one compared to the rest of the isotopologues of a lipid species for the majority of all lipids, its intensity can be determined more accurately than that of other isotopologue peaks of this species; whereas those isotopologue peaks can be easily deducted from the determined monoisotopic peak

intensity and the natural abundances of $^{13}C$. Therefore, the total ion intensity $I_{total(n)}$ of an isotopologue cluster of a lipid species is:

$$I_{total(n)}=I_n\left(1+0.0109n+\frac{0.0109^2 n\,(n-1)}{2}+\cdots\right) \quad (6)$$

where $I_n$ represents the monoisotopic peak intensity of the species that contains $n$ carbon atoms and 0.0109 is the natural abundance of $^{13}C$ when the abundance of $^{12}C$ is defined as 1. The dots represent the contribution of the following isotopologues that contain more than two $^{13}C$ atoms, which usually contributes less than 5% of the total ion intensity for the majority of lipid species and could be ignored. If their response factors are identical, then quantification of this species with an internal standard that contains $s$ carbon atoms can be expressed as:

$$\begin{aligned} C_n &= \frac{I_{total(n)}}{I_{total(s)}} * C_s \\ &= \left[I_n\left(1+0.0109n+\frac{0.0109^2 n(n-1)}{2}+\cdots\right)\right] / \left[I_s\left(1+0.0109s+\frac{0.0109^2 s(s-1)}{2}+\cdots\right)\right] * C_s \\ &= Z_1 * \frac{I_n}{I_s} * C_s \end{aligned} \quad (7)$$

where

$$Z_1=\left(1+0.0109n+\frac{0.0109^2 n\,(n-1)}{2}+\cdots\right) / \left(1+0.0109s+\frac{0.0109^2 s\,(s-1)}{2}+\cdots\right) \quad (8)$$

$I_n$ and $I_s$ are the actual monoisotopic peak intensities of the analyzed species and the internal standard, respectively; and $C_n$ and $C_s$ are the concentration of the species and the internal standard, respectively. $Z_1$ has been defined as a type I $^{13}C$ isotope correction factor (Han & Gross, 2005a)

The other type of $^{13}C$ isotope corrections is only needed for quantitative analysis with a unit-resolution mass spectrometer, where the monoisotopic peak of the species of interest overlaps with the second isotopologue of a species with only one more double bond than the species of interest. If these two isobaric peaks are resolved with high mass resolution MS, then this type of correction is obviously not needed. If these two peaks are partially resolved, the peak intensity of species of interest can be extracted with the aid of its first isotopologue peak (Wang et al., 2014b). If the overlap from these two peaks cannot be resolved, then correction on the apparent overlapped peak intensity $I_n'$ is necessary to obtain its actual intensity $I_n$ for quantitative analysis prior to performing the type I correction by Equation 6.

$$\begin{aligned} I_n &= I_n' - I_N{}^*\left(0.0109^2 n\,(n-1)\,/2\right) \\ &= \left(1-\left(I_N/I_n'\right)\left(0.0109^2 n\,(n-1)\,/2\right)\right)^* I_n' \\ &= Z_2{}^* I_n' \end{aligned} \quad (9)$$

where

$$Z_2 = 1 - (I_N / I_n') \left(0.0109^2 n (n-1)/2\right) \quad (10)$$

$I_N$ is the monoisotopic peak intensity of the species with one more double bond than the species of interest; and $n$ is the total carbon number in the species of interest. $Z_2$ has been termed as type II $^{13}$C isotope correction factor (Han & Gross, 2005a). This type II correction factor can be negligible if $I_N << I_n'$.

A few points must be noted when the two types of $^{13}$C isotope correction factors are used for quantitative analysis. First, in tandem MS-based lipidomics, because the monitored fragment is the monoisotopic peak and only contains $^{12}$C atoms, both types of correction factors should be modified by subtracting the carbon number in the monitored fragment from $n$ and $s$ in Equations 8 and 10. Second, if two or more internal standards are used to cover a wide mass range, then a type I correction factor ($Z_1$) can be largely covered by the added internal standards and do not need to be considered, whereas a correction for factor II must still be performed. Third, with LC-MS, if individual lipid species of a class are totally resolved by LC and a calibration curve is established for each analyzed species, then neither correction factor is required. Otherwise, one or both of the correction factors, or another alternative deisotoping approach, must be considered for accurate quantification.

### C. Ionization Efficiency

We discussed in the last subsection the relationship between monoisotopic peak intensities of individual lipid species of a polar class and the number of carbon atoms present in the species. The question now is what should be done in the next step after correction for the $^{13}$C isotopic effects. It has been extensively discussed that the ionization response factors of individual lipid species of a polar class are essentially identical after $^{13}$C de-isotoping (see above), if the analysis is conducted in a concentration range less than that at which lipid aggregates begin to form (Subsection IVA). Figure 2 shows an example of such a relationship before and after correction for a type I $^{13}$C isotopic effect. When this principle is applied to other previously reported observations, an identical conclusion can also be drawn. For example, Koivusalo and colleagues showed that the peak intensities of equimolar mixtures of different PC species not only depend on the molecular species in a manner of decreased peak intensity as carbon atoms of the species increase, but also remarkably depend on the solution concentration (Koivusalo et al., 2001). However, after correction for the differential $^{13}$C isotopologue distribution, we found that only the top two lines, which correspond to 0.1 and 0.4 pmol/μL of each species with a total of 14 PC species in 1:2 chloroform/methanol, show essentally identical intensities whereas other lines with higher concentrations do not (Koivusalo et al., 2001). This outcome is in great agreement with those shown in Figure 2 and suppports what we have discussed in the last two subsections. Taken together, if MS analysis of lipid species of a polar class is conducted in a low concentration range (i.e., below an aggregation concentration) without other variables further discussed below, then the mass spectrum displays essentially identical peak intensities of these species (within 10% variations) after correction for $^{13}$C isotopic effects.

This conclusion also indicates that we can employ any species of a polar lipid class as an internal standard to quantify other species of the class under the specified conditions, including (1) a polar lipid class, (2) a low concentration of a lipid solution, (3) correction for $^{13}C$ isotopic effects, and (4) acquisition in the survey-scan mode.

However, for lipid species of a non-polar class, no predominant charge center occurs in these species. The contribution of fatty acyl chain length and unsaturation to the polarity of the species becomes apparent. Logically, the ionization efficiencies of different species even under aforementioned experimental conditions become different. This point has been previously demonstrated with measurement of different TAG molecular species at different concentrations (Han & Gross, 2001). Collectively, one internal standard is not sufficient for accurate quantification of other individual species of the non-polar lipid class; additional internal standard(s) are needed to correct for differential ionization response (a variable). Alternatively, a correction factor for this variable should be pre-determined and implemented in the developed method in the case where a single internal standard is used (Han & Gross, 2001).

### D. Ion Suppression

Ion suppression happens when ionization efficiency or ion counts of a compound or a group of compounds are significantly reduced due to the presence of other compounds, changes in the matrix components, or the formation of lipid aggregation that result from dramatic changes of its (or their) concentrations. This phenomenon is basically caused by decreases in efficiency of droplet formation or droplet desolvation, and it is species- and concentration-dependent. Therefore, ion suppression might affect ion-formation efficiency, detection precision, and quantification accuracy.

Ion suppression happens in shotgun lipidomics and LC-MS analysis, although their formation mechanisms are likely different. In shotgun lipidomics, low-abundant or less-ionizable species are always suppressed by co-existence of abundant or readily ionizable species. In LC-MS lipidomics, the varied mobile phase composition always affects ionization efficiency; the increased lipid-lipid interactions between same lipid species or classes due to the column enrichment and concentration changes might also lead to ion suppression. Although the mechanism of ion suppression is still unknown, there are many possible reasons, including endogenous compounds from the sample itself as well as exogenous substances from contamination during sample preparation. A high lipid concentration, mass overlap, basicity, and simultaneous elution in LC are some factors that induce ion suppression (Annesley, 2003).

When an entire class of lipid species is suppressed in the presence of other lipids in the survey-scan mode in shotgun lipidomics, which is performed in the region of low concentration of total lipids, the suppressed order to each of the species is usually identical. Therefore, the linear dynamic range in the survey-scan mode could be narrowed due to the reduced detection limit and lipid aggregation effect, as discussed above. However, the linear dynamic range after direction infusion can always be improved with tandem MS analysis, and this kind of ion suppression is not a severe issue for shotgun lipidomics approaches.

Moreover, the rapid advances in sensitivity of new-generation mass spectrometers could further relieve these of ion-suppression effects.

Another complication of ion suppression exists in LC-MS-based lipidomics when species either within the same class or between classes cannot be completely resolved. The varied concentration of each individual lipid species during elution from the column leads to competition for either space or charge during ionization. This type of ion suppression is harmful due to its unpredictability, because the concentrations of co-eluted lipid species constantly change. Therefore, total resolving between species or between classes is the key to minimize this complication.

Diluting samples for direct infusion or reducing the volume and concentration of a sample loaded into an LC system is one way to efficiently reduce ion suppression. It has been verified that nano-ESI could significantly reduce ion suppression due to its smaller and more highly charged droplets, which are more tolerant to nonvolatile salts (Gangl et al., 2001). Other ways to lower ion suppression are to reduce matrix-ion intensities in the infusion solution or mobile phase, and to improve chromatographic resolution.

### E. Dynamic Range

To establish a new quantification method, the linear dynamic range must be determined and validated. In lipidomics, the upper limit of a dynamic range is usually the concentration at which the lipids form aggregates, as discussed in Subsection IVA, because the detectors used in modern mass spectrometers generally possess a much wider range and should not limit lipid quantification. The lower limit of a dynamic range is usually the concentration of quantification limit, which is generally defined as the concentration with an intensity at 5-times the noise level. It is largely dependent on instrument sensitivity, method optimization, matrix effects, and others. For example, SRM/MRM improves S/N ratios through an increase in duty cycles and selectivity, and consequently possesses a lower quantification limit and an extended dynamic range in comparison to SIM (see Subsection IIIA).

Measurement of a dynamic range could be expressed in different forms. One is to investigate the linear range of concentration of the analyst of interest, which is defined as the linear relationship between absolute ion counts and its corresponding concentration of a species. The linear range could reach over 10,000 fold at the low concentration region (Han & Gross, 1994; Hermansson et al., 2005; Koivusalo et al., 2001; Lehmann et al., 1997). However, the absolute ion counts of lipid species vary with even very minor alterations in sample preparation or instrumental conditions, and therefore, not meaningful for quantitative analysis of lipids. Alternatively, the concentration dynamic range could be plotted as the peak intensity ratio of the analyte to an internal standard in a solution *vs.* the concentration of the solution that spans a wide range by different folds of dilution. The plot should be a horizontal line within the linear dynamic range of concentration (Han & Gross, 2001; Han et al., 2006a; Wang et al., 2013).

A dynamic range can also be expressed as the linear range of ratios of an analyte to its internal standard, where the concentration ratio of the analyte to its standard is plotted against the peak intensity (or area) ratio in a mass spectrum from shotgun lipidomics or the

extracted peak area ratio from LC-MS-based analysis (Han & Gross, 2001; Wang et al., 2013). In a survey-MS scan spectrum, this type of dynamic range is usually less than 100-fold, due to the possible presence of baseline drift and background noise, which substantially reduces the S/N ratio of low-abundance species. The same issues arise in the SIM approach with LC-MS analysis, although the baseline drift and background noise of mass spectra cannot be viewed directly from the spectra. However, the use of tandem MS extends the dynamic range to 1,000-fold or more, depending on its sensitivity due to the reduced baseline drift and background noise by the double filtering of tandem MS, whereas an even wider dynamic range is observed in a two-step quantification or with multiple standards at different ratios (Han et al., 2004b; Han et al., 2008; Han et al., 2012). This dynamic range is very dependent on the mass levels of internal standard(s) added to the samples. Therefore, it is important to select an appropriate amount of internal standard for quantification. This topic is discussed in the next section.

A dynamic range must be investigated in the presence of sample matrices instead of in standard solutions with either shotgun lipidomics or LC-MS to account for possible matrix suppression. Especially for low-abundance species or classes, the matrix effects could be more severe in the presence of abundant species or classes. Therefore, the optimized conditions for accurate quantification should be developed similar to the sample conditions to account for matrix effects (Jiang & Han, 2006; Wang et al., 2014a).

### F. Tandem MS Factor

Using tandem MS enhances detection sensitivity, thereby greatly extending the linear dynamic range for quantification. Therefore, quantitative methods based on tandem MS (i.e., PIS, NLS, and MRM) are commonly developed in LC-MS-based lipidomics and shotgun lipidomics. However, fragmentation of individual species of a lipid class during CID is species-dependent. This dependence is due to the differential kinetics to produce the product ions and/or the varied thermodynamic stability for the yielded product ions from individual species of a class. These differences consequently produce differential product ion intensities from different molecular species of the class. The relationship of molecular species-dependent ion peak intensities with fragmentation conditions has been extensively discussed (Han & Gross, 2005b), and is clearly illustrated in Figures 1 and 3. Such a differential physical property of individual molecular species has even been explored to determine the location of double bond(s) in fatty acyl substituents (Yang et al., 2011b).

However, this relationship makes selection of internal standard(s) and optimization of tandem MS conditions complicated. It is clearly indicated that measures have to be taken to minimize this tandem MS factor on accurate quantification. These measures include (1) use of stable isotopologues as internal standards for quantification; (2) employing as many internal standards as possible to cover the effects of fatty acyl chain length, degree of unsaturation, and location of double bonds on accurate quantification; and (3) selection of an optimized CID energy to balance fragmentation dependency of all species of the entire class, or even ramp CID energies in a certain range to minimize the dependency for quantification in the case of shotgun lipidomics. Similarly, optimization of an SRM/MRM condition should focus on an entire class of lipids instead of individual species if the goal is to

quantify all species of the class with a limited number of internal standards, because optimization for individual lipid species could lead to a different response factor of the species from that of the selected internal standard(s). These differences could lead to substantial errors in quantitative analysis if different collision energies are applied for different species in a class of lipids in either shotgun lipidomics or LC-MS/MS analysis (Han & Gross, 2005a).

It should be kept in mind that the fragment ion in each pair of transition for quantification by SRM/MRM should be highly specific to the lipid class. Therefore, a fragment ion that corresponds to a fatty acid chain should be avoided because a fatty carboxylate anion could arise from many different lipid species or even different classes to negate the advantage of SRM/MRM in specificity to analyze species in a given class. Moreover, the fragment ion intensity of a fatty acyl chain also depends on regioisomeric position (Han & Gross, 1995). A huge experimental variation or inaccurate quantification might be resulted if these factors are not considered.

### G. In-source Fragmentation

In-source fragmentation occurs in the ESI ion source instead of during CID. ESI is usually considered as a 'soft ionization' technique, because there is very little fragmentation produced during this process. Unfortunately, many lipid classes, subclasses, and individual molecular species can easily fragment, even under soft ionization conditions. Currently, such a process and/or its severe consequences on accurate quantification have not been widely recognized. This process reduces ionization efficiencies due to the loss of a certain amount of lipids, artifactual identification due to the presence of resultant fragment ions, and species-dependent ionization at a low concentration due to the species-dependent fragmentation as in CID. For instance, the loss of serine from phosphatidylserine (PS) to yield phosphatidic acid (PA) species is very facile and common; observation of lysoPA species resulted from lysoPC species has been reported due to in-source fragmentation (Zhao & Xu, 2009). Accordingly, in-source fragmentation should be avoided, if possible, for quantification when a lipidomics method is developed. In-source fragmentation results from high ionization temperature, high ionization voltage, an extremely high electrical capacity due to a very short distance between the spray tip and the inlet, and an inappropriate setting of the gate voltages.

In order to eliminate in-source fragmentation, instrumental conditions must be finely tuned with a solution of lipid standard mixture following vendor-recommended standard procedures. A few common fragmental patterns in negative ion MS can be used to judge if in-source fragmentation happens during ionization. PA species are generated from their corresponding PS species, and PC species produce its dimethyl PE counterparts. Although endogenous PA and dimethyl PE species both exist in low abundance, particularly the latter, these endogenous species must be considered when one determines the in-source resultant PA or dimethyl PE species. Usually, the standard(s) of PS or PC species, which do not yield endogenous counterparts of PA or dimethyl PE, should be used. An alternative criterion to examine a minimal in-source fragmentation is to maximally display chlorinated PC species in a spectrum instead of the corresponding in-source generated dimethyl PE from

chlorinated PC. This factor could also be corrected through addition of internal standards after considering the effects of aliphatic chain length, and double bond numbers and locations, as described in the last subsection.

### H. Matrix Effects

It is well known that the matrix of a solution greatly impacts the ionization efficiency of an analyte. This topic for analysis of lipid species has been extensively discussed in a previous review article (Han & Gross, 2005a). Briefly, minor changes of the matrix in a lipid solution such as the solvent composition, pH value, and ion strength affect ionization efficiency of individual lipid species. Following this line of reasoning, the matrix effects on shotgun lipidomics and LC-MS based lipidomics are very different, so different methods should be used to overcome or minimize these effects.

In the case of shotgun lipidomics, all molecular species of a class of interest experience an identical matrix condition. Thus, the effects of the matrix on the ionization efficiency of individual lipid species are identical. Although different samples contain different matrices and lead to different ionization efficiencies of a lipid class between the samples, the internal standard(s) of the class used should be able to cancel the differences between the samples. Therefore, quantitative results of individual species in a sample obtained through comparison to the internal standards should be essentially identical to those obtained from the other sample in the same way. However, it should be recognized that different matrices in different samples could affect the linear dynamic range for analysis of a class to a certain degree, as discussed in Subsection IVE. The matrix effects could be minimized through the efforts on sample preparation with the measures such as minimize inorganic contaminants, dilute a small volume of a lipid extract to a large volume of a solvent mixture, etc.

In the case of LC-MS based lipidomics, a different matrix effect from shotgun lipidomics is present, and results from a mobile phase gradient (if employed), assuming the matrices from sample preparation such as inorganic contaminants can be efficiently eliminated with the column. When a gradient of solvents with or without modifier(s) is used in LC-MS analysis, individual lipid species might experience different matrices from their internal standard(s). This difference leads to different ionization efficiencies of individual species in comparison to their internal standard(s). To correct for this matrix effect, at least one internal standard plus a few pre-established external standard curves must be employed. The internal standard is used for normalization of any batch effects for comparison between analyses. The external standard curves are designed to eliminate the matrix effects caused by the gradient. The number of standard curves needed depend on the changes of solvents, pH values, ion strengths, etc. in the gradient.

## V. SELECTION OF INTERNAL STANDARDS

At this point, the readers should have the knowledge about the principles of accurate quantification by MS (including MS/MS), the fundamentals of different platforms for quantification of lipid species, and all the factors that might lead to inaccurate quantification. The rest of this article extensively discusses the topics that we should know about internal

standard(s), and suggests the number of (internal) standards to be used in different platforms.

### A. Why Internal Standards are Required for Accurate Quantification

As discussed in Section II, there is no definitive rule between the absolute intensity (i.e., counts) of a molecular ion and the concentration in a solution of the analyte that yields the ion with ESI-MS. The ion intensity of an analyte measured with ESI-MS could be affected by many factors related to sample preparation, ionization conditions, tuning conditions, the analyzer and detector used in the mass spectrometer, etc. Minor changes of these factors could lead to significant alterations in ion intensity from one condition to another. As the sensitivity of MS instruments continues to improve, these factors also more and more influence the ion intensities measured with ESI-MS. Thus, it is essentially impossible to replicate a measurement of absolute ion counts of an analyte in a biological sample with ESI-MS from time to time, from an instrument to another instrument, from one laboratory to with others, or even one operator to another in the same laboratory. Although scientists frequently measure the ion intensities of a compound in a biological sample for profiling, quantification or even semi-quantification cannot be achieved with this approach. Therefore, to minimize the variations introduced at any step of analysis, addition of some kind of controls (e.g., analogs to the analytes of interest) between the samples or between analyses becomes essential. If these controls are appropriately selected (see below), then they can serve as internal standard(s) for accurate quantification.

An internal standard is preferably added during sample preparation at the earliest step possible and analyzed at the same time as the sample. An external standard is analyzed separately, but should be analyzed under “identical” conditions with the sample of interest. A calibration curve must be established by using the external standard also under “identical” conditions. Because it is hard to achieve the so-called “identical” conditions for use of external standards, a combination of external standards with an internal standard for quantification is possible and popular (Castro-Perez et al., 2010; Masukawa et al., 2009; Popendorf et al., 2013; Sparagna et al., 2005). Therefore, any unexpected changes of ion counts during analysis can be either controlled internally or normalized. Results obtained from sample analysis without any internal control can only be used for qualitative comparisons.

### B. What Kind of Lipid Species can be Used as Internal Standards

The lipid species that can be used as internal standards for analysis of a sample depend on the existence of endogenous lipid species in the sample. As extensively discussed above and broadly practiced, if accurate quantification is the goal, then it is better to use internal standards that have identical chemical and physical properties to the analytes and to experience the identical experimental conditions from sample preparation to MS analysis. Thus, the ideal internal standard to quantify an analyte of interest is its stable isotope-labeled analog, which is nearly identical to the analyte and exhibits exactly the same response factor in Equation 3. However, it is currently impractical to globally quantify lipids in a complex cellular lipidome with thousands of stable isotope-labeled internal standards, although

quantification of a few lipid species can be achieved with their stable isotopologues as internal standards (Harrison et al., 1999).

Luckily, it has been broadly demonstrated that the ionization efficiencies of polar lipid species predominantly depend on the electrical properties of their head groups, and the effects of acyl chains are negligible in the low-concentration region in the ESI-MS survey scan (See Section IV). Therefore, one species in a polar lipid class could be employed as an internal standard to quantify individual species in the same class of lipids within a reasonable accuracy (> 95%) in the MS survey scan mode as long as the measurement is made in the low concentration region and different $^{13}C$ isotopologue distributions is corrected.

Another primary requirement for selection of an internal standard is the absence of any overlap of internal standard(s) with the endogenous species, or the overlapped endogenous species in very low abundance (e.g., << 1% of the most abundant species of the class) in lipid extracts. This condition must be pre-determined with a lipid extract without any internal standards to ensure that the overlapped peak intensity with the selected internal standard in the mass spectrum is much less than 1% of the most-abundant species in the class of interest.

### C. How Many Internal Standards are Necessary and Why

In this subsection, we discuss the minimal number of internal standards necessary to quantify the species of a class with different approaches. In fact, the number of internal standards that should be employed really depends on the number of the variables present in the developed methods, which are outlined below.

In Subsection IVC, it has been concluded that any species of a polar lipid class can serve as an internal standard to quantify other species of the class under the specified conditions, including (1) a polar lipid class, (2) a low concentration of a lipid solution, (3) correction for $^{13}C$ isotopic effects, and (4) acquisition in the survey-scan mode. Therefore, if quantification is conducted with shotgun lipidomics in the survey-scan mode, then all these specified conditions are achievable and one internal standard of a class is adequate. The first step of MDMS-SL quantification is an example, and thus one internal standard is the minimal number used to quantify other abundant and not overlapped species of a class (Yang et al., 2009a).

Because tandem MS depends on the subclass linkage and individual molecular species (two variables), applying tandem MS adds a variable to the analysis of individual species of each subclass in comparison to that conducted in the survey scan mode. Therefore, any method based on tandem MS in shotgun lipidomics should employ at least an extra internal standard to cover each variable to quantify individual lipid species of each subclass. In other words, two or more internal standards are needed for relatively accurate quantification of individual species of a subclass. This conclusion has previously been demonstrated (Brugger et al., 1997). The second MDMS-SL quantification step, which employs multiple quantified endogenous species from step 1, is another example of this category. As discussed in Subsection IVF, this tandem MS factor due to differential fragmentation kinetics and

fragment ion thermodynamics of different molecular species of a polar lipid class can be minimized through ramping and/or balancing the collision energies. In this case, the internal standard for correction of tandem MS factor could be omitted. High mass accuracy MS-based shotgun lipidomics has been developed based on the principles of tandem MS (Ejsing et al., 2006; Schuhmann et al., 2011; Schwudke et al., 2006; Schwudke et al., 2007; Stahlman et al., 2009). Accordingly, it would be better to have two or more internal standards employed for relatively accurate quantification of individual species of each (sub)class. However, for those newly-developed versions of high mass accuracy MS-based shotgun lipidomics (Almeida et al., 2015; Schwudke et al., 2011), quantification of lipid species is conducted with a survey-MS scan. In this case, one internal standard to quantify individual species of a polar lipid class is sufficient.

With SIM after LC-MS with isocratic elution, the additional variable in comparison to that conducted in the survey-scan mode after direct infusion is the changes in the concentration of individual species. Therefore, two or more internal standards at different elution times are necessary in this case. If the isocratic elution is replaced with a gradient of mobile phase, then one extra variable due to the solvent gradient (i.e., matrix effects (Subsection IVH)) is introduced, and at least one additional internal standard must be employed.

With SRM/MRM after LC-MS, another additional variable is introduced in comparison to those present in the SIM method after LC-MS, namely the tandem MS process. Therefore, as in the case of shotgun lipidomics, an extra internal standard should be employed relative to the corresponding SIM methods with or without a solvent gradient.

In SIM and SRM/MRM methods after LC-MS, the necessary number of internal standards employed for accurate quantification could be compensated for with a combination of an internal standard with a few external standard calibration curves.

Taken together, the minimal number of internal standards that must be used for accurate quantification varies from method to method, and largely depends on the existence of the variables in each method (see a flowchart (Figure 4) leading to the minimal number of internal standards required for different methods). Table 1 summarizes the easily-recognizable variables present in each method, and therefore lists the minimal number of internal standards necessary for the methods. As mentioned before, some alternative approaches could be used to reduce the minimal number of internal standards for quantification. It should be pointed out that any method that uses a number of internal standards much less than the variables present in the method will not provide accurate quantification, but can still be used for relative comparisons between the samples. If this situation is the case, it is advisable that the investigators not overstate the results as quantitative.

So far, all the discussions are for quantification of individual species of a polar lipid class. For quantification of non-polar lipid classes, due to the differential ionization response factors of individual molecular species (a variable) (Subsection IVC), additional internal standards in comparison to those of a polar lipid class (Table 1) must be used. Alternatively,

a correction factor for this variable must be pre-determined and implemented during method development (Han & Gross, 2001).

### D. How Much Individual Internal Standard Should be Used

For accurate quantification (e.g., > 90% accuracy) of lipid species in a biological sample, or comparison of lipid profiles between lipid extracts of biological samples, we know the minimal number of internal standards that must be added during lipid extraction. The next question is how much of these internal standards is needed. To answer this question, scientists should first decide which endogenous parameter is used for normalization. In other words, we must select an endogenous parameter that is relatively stable under the states of interest and readily assessable under the laboratory conditions so that they can be used to normalize the determined lipid amount. To this end, the protein, DNA, or RNA content in tissue or cell samples, the phosphorus content in the lipid extract, the tissue wet or dry weight, the cell number, and the volume of the biofluids are parameters frequently used by investigators in the field to serve the purpose.

We should recognize that each of these “normalization” parameters has benefits and detriments that depend on the physiological or pathological system. In terms of detriments, for example, determination of phosphorus content might carry a large experimental error, and the phosphorus content in lipid extracts might also vary under different physiological or pathological conditions. It is somewhat difficult to make all tissue samples that contain the same amount of water, which makes the ratio of lipids to tissue wet weight inconsistent, whereas it takes too long (at least overnight) to incubate tissue samples to dryness. The volume of biofluids might be influenced by the fluid and/or food intake, whereas counting cell numbers becomes difficult when the cells are clustered. In contrast, the total protein, DNA, or RNA content of a biological sample are more stable, and can be readily determined in a high-throughput fashion. Therefore, use of one of these contents as a normalization parameter is highly recommended. It is worth noting that, although the levels of many proteins might change from one state to the other, the amounts of the structural proteins, which account for the majority of the protein content of a sample, are usually quite stable. It should be recognized that determination of total protein, DNA, or RNA content could readily have an error/reproducibility of ~ 10% if the measurement is not performed carefully and this type of error/variation would propagate to the final quantitative results.

The amount of individual internal standards that must be added must be optimized to a certain range. The reason is that too much or too small of an amount of internal standards could lead to large experimental errors. If too little is added, compared to the endogenous species of the class, then any small error carried with the internal standards will be amplified, and a small variation of the abundant species between the samples of a group could result in a large variation of the content of this species and lead to a large variation of the final results. If too much internal standard is added, which will lead to an ion-suppression effect on endogenous species, then quantification of low abundance species of the class will fail because the upper limit of a linear dynamic range is capped with a concentration at which lipids aggregate (See subsection IVD). These arguments also hold true for LC-MS analysis, although the requirement of the amount of internal standards is not

as strict as for shotgun lipidomics. Generally, we must optimize the added amount of internal standards to make the relative intensity of the internal standard peak in the range of >20 to 500% in comparison to the ion peak that corresponds to the most-abundant species in the class. Accordingly, the optimal amounts of internal standards that are necessary for lipid quantification could vary largely for different kinds of samples.

Below is a list of typical internal standards currently used in the authors' laboratory to analyze lipid species present in mouse cortical samples by MDMS-SL (All these standards can be purchased from Avanti Polar Lipids, Inc., except those specified): di15:0 phosphatidylglycerol (PG) (3), 17:0-20:4 phosphatidylinositol (PI) (4.5), di14:0 PS (19), tetra14:0 cardiolipin (1.5), di14:0 PA (0.5), di16:1 PE (26), di14:1 PC (26), 17:0 lysoPC (1.5), 14:0 lysoPE (1.2), 17:1 lysoPG (0.004), 17:1 lysoPI (0.08), 17:1 lysoPS (0.08), 13:0 lysoPA (0.045), *d*18:1-N12:0 sphingomyelin (3), *d*18:1-N15:0 cerebroside (8, Matreya), *d*18:1-N16:0 sulfatide (3, Matreya), *d*18:1-N17:0 ceramide (1), 17:0 sphingoid base (0.1), 17:0 sphingoid-1-phosphate (0.05), N,N-dimethyl psychosine (0.05, laboratory-synthesized), tri17:1 TAG (0.2, NU-CHEK-PREP, Inc.), di15:0 DAG (0.05, NU-CHEK-PREP, Inc.), 17:1 monoacylglycerol (0.05, NU-CHEK-PREP, Inc.), $^{2}H_{4}$-16:0 non-esterified fatty acid (5, Cambridge Stable Isotope Laboratories), $^{13}C_{4}$-16:0 acylcarnitine (0.05, Sigma Chemical Co.), 17:0 acyl CoA (0.05, Sigma Chemical Co.), $^{2}H_{3}$-4-hydroxynonenal (0.8, Cayman Chemical), $^{2}H_{7}$-cholesterol (170, Cambridge Stable Isotope Laboratories), and $^{2}H_{5}$-16:0 cholesteryl ester (1, Cambridge Stable Isotope Laboratories). The levels used for mouse cortical lipid quantification are given in parentheses in nmol/mg protein, which can be adjusted appropriately for different tissue, cell type, or other biological samples. To this end, the purity as well as the content of the species used as internal standards should be determined with a kind of classical method after they are purchased commercially. Many different classical methods such as HPLC, NMR, phosphorous assay, and GC can be employed for this purpose. The protocols of these methods can be found from many technique books specific for classical lipid analysis (Christie & Han, 2010; Kates, 1986). It should also be recognized that when stable isotopologues of lipid species are used as internal standards, the impurity of isotope (usually at the level of 2%) might cause a large systematic error if a large number of atoms (e.g., > 5) are labeled and the monoisotopic peak of the internal standard is still used for ratiometric comparison or ion intensity extraction.

The given amounts for mouse cortical lipid analysis (see above) can also be used as references to estimate the internal standards used for a particular class of cellular membrane lipids present in other biological samples. Here, the assumption is that the levels of cellular membrane lipids are similar for different cell types. For example, the aforementioned levels of internal standards are not suitable for quantification of lipid species in the liver. However, we can estimate the levels of the internal standards to quantify hepatic lipids based on the amount of internal standards for the cortex. Specifically, the content of an internal standard for quantification of liver PC species can be estimated as follows: the total content of lipids in the liver is ~400 nmol/mg of protein; the total content of lipids in cortex is ~1000 nmol/mg of protein; the internal standard for quantification of PC species in the cortex is 26 nmol/mg of protein (see above); thus, the level of internal standard for PC species in the liver is approximately 26 × 400/1000=10.4 nmol/mg of protein. This number is consistent with that used in experiments (Han et al., 2004b), particularly considering that the flexible

range of peak intensity ratios varied from 20 to 500%. These estimated levels of internal standards could then serve as a starting point for a pilot experiment. Moreover, it should also be recognized that the different lipid compositions occur among lipid classes in different samples are present. For example, only minimal amounts of sulfatide and cerebroside, as well as a greatly reduced amount of ceramide species, are present in non-neuronal samples such as the heart or the liver. For analysis of lipid extracts from non-mammalian tissue samples such as plants, yeast, and mycobacterium, the levels of internal standards used for quantification of complex lipid species are different from that of mammalian samples (Ejsing et al., 2009; Samarakoon et al., 2012; Shui et al., 2007), but the procedures for estimation of these levels should be similar. Specifically, a review of previous work for the employed levels of internal standards is very useful. Based on the knowledge obtained from literature, a trial for one's own experiments should be performed to determine the right levels of internal standards for quantification of lipid species.

It should be pointed out that alternative approaches exist for semi-quantitative or qualitative comparisons between biological samples. In those approaches, only a limited set of, or no, internal standards are used during the analysis and/or only the peak intensities (or areas) of the detected ions are compared. These ions might or might not be well-characterized and/or identified. A normalized composition relative to a selected ion among the detected ions is usually used for comparison. These approaches are generally referred to as lipid profiling. Although lipid profiling does not provide direct information on the stoichiometric relationship among lipid species and might be prone to poor analytical reproducibility, it can provide a comprehensive comparison between biological samples. High-throughput analysis is a major goal for such profiling, whereas substantial statistical analysis (e.g., PCA) is always required.

## VI. SUMMARY

It is well known that the most-prominent and successful approaches in lipidomics are the methods by ESI-MS to quantify lipid classes, subclasses, and individual molecular species. In order to achieve accurate analysis of the mass levels of individual lipid species, no matter whether chromatography is used or not, it is critical to understand the principles of lipid quantification with MS, learn the advantages and limitation of each platform, and most importantly, know how to select correct internal standards to overcome all the variables inherent in the methodology. Such an accomplishment could provide deep insights into molecular mechanisms that underpin the changes of lipid class, subclass, and species and could facilitate to draw meaningful conclusions. We sincerely hope that this review article provides some degree of foundation for readers to successfully improve and/or establish their methods to accurately quantify lipids with ESI-MS in lipidomics.

Lipidomic analysis impacts biomarker discovery, drug development, and biosystem knowledge. Accurate quantification is continuously being researched to further promote these processes of lipidomic analysis and to broaden our knowledge on any single biological systems. With the state-of-the-art analytical techniques and the accurate quantification approaches of lipid molecular levels, lipidomics, integrated with other omics sciences (including genomics, transcriptomics, proteomics, and metabolomics), medicine, and

bioinformatics, will lead us to a comprehensive understanding of the biological functions of organisms under health and disease states.

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## VII. ABBREVIATIONS

| | |
|---|---|
| **CID** | collision-induced dissociation |
| **DAG** | diacylglycerol(s) |
| **ESI** | electrospray ionization |
| **LC** | liquid chromatography |
| **lysoPC** | lysophosphatidylcholine(s) |
| **MDMS-SL** | multi-dimensional mass spectrometry-based shotgun lipidomics |
| **MRM** | multiple reaction monitoring |
| **MS** | mass spectrometry |
| **NLS** | neutral loss scanning |
| **PA** | phosphatidic acid(s) |
| **PC** | phosphatidylcholine(s) |
| **PE** | phosphatidylethanolamine(s) |
| **PIS** | precursor ion scanning |
| **PS** | phosphatidylserine |
| **S/N** | signal to noise |
| **SIM** | selected ion monitoring |
| **SRM** | selected reaction monitoring |
| **TAG** | triacylglycerol(s) |

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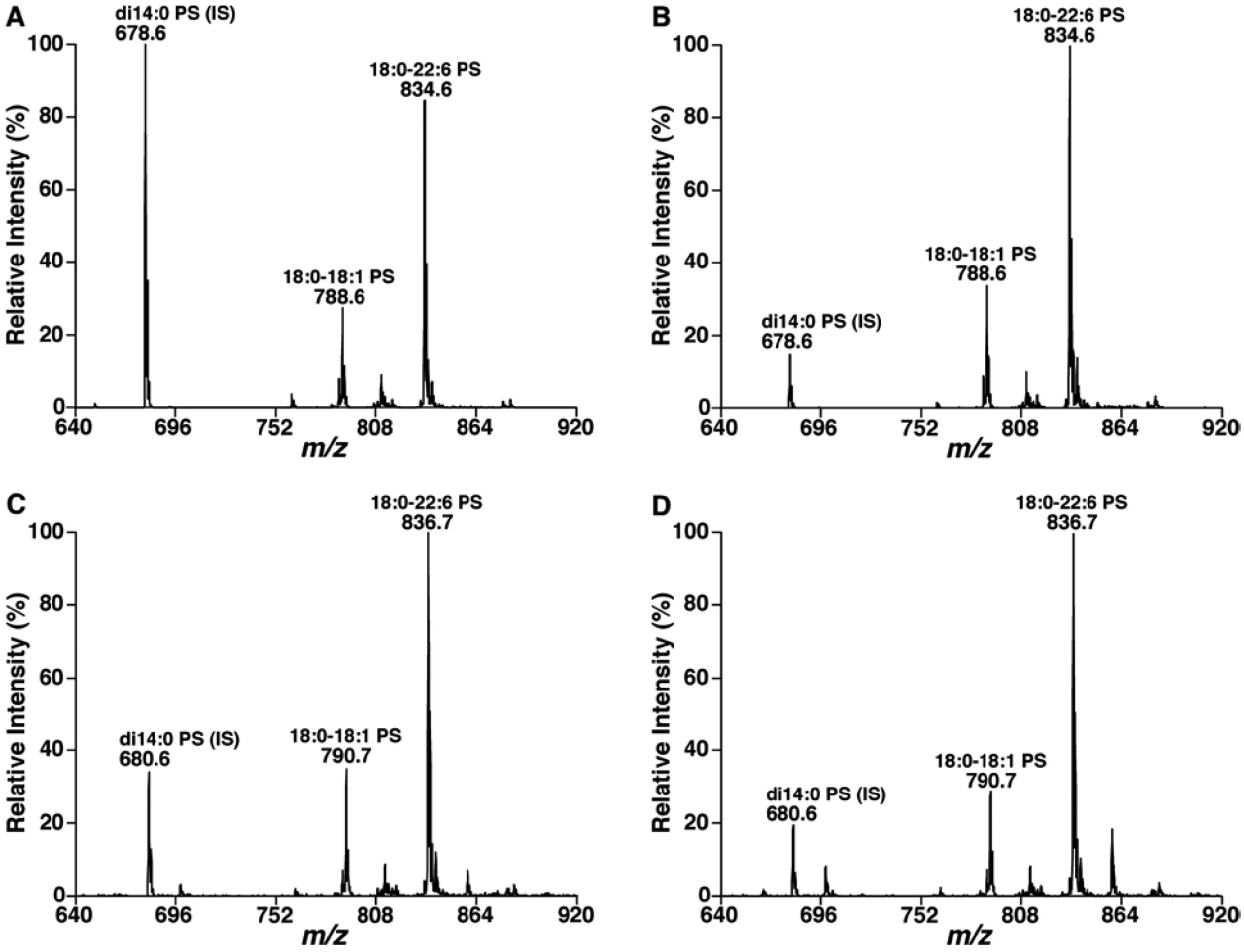


**Figure 1.**

Representative tandem MS mass spectra of brain phosphatidylserine species from lipid extracts. Tandem MS mass spectra of neutral loss of 87 Da at collision energy of 20 (Panel A) and 35 (Panel B) eV in the negative-ion mode and of neutral loss of 185 Da at collision energy of 20 (Panel C) and 30 (Panel D) eV in the positive-ion mode, respectively, were acquired at collision gas pressure of 1 mTorr with a triple quadrupole mass spectrometer (Quantiva, Thermo Fisher Scientific) equipped with a NanoMate device.

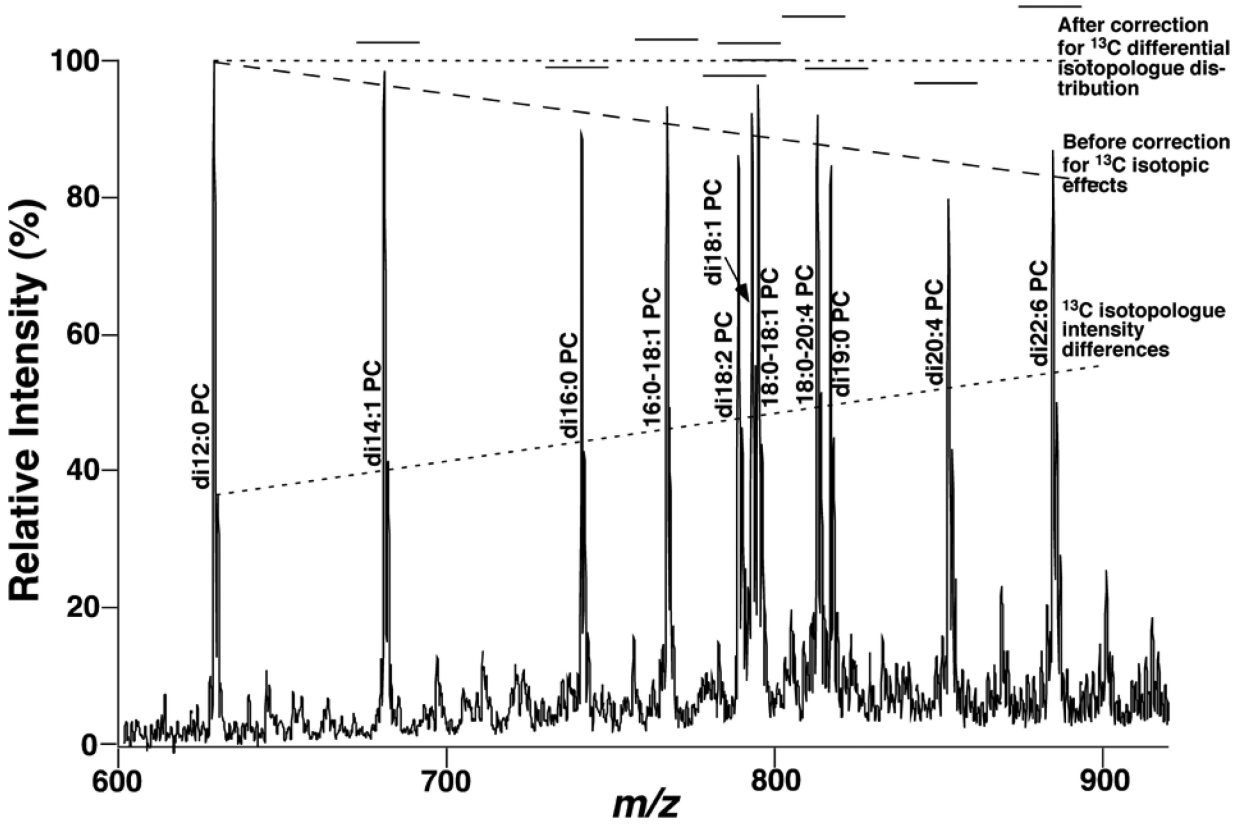


**Figure 2.**

A positive-ion mass spectrum of an equimolar mixture of phosphatidylcholine molecular species before and after corrections for $^{13}$C isotopologue distributions. A positive-ion ESI mass spectrum of an equimolar mixture (1 pmol/μL each) of di12:0, di14:1, di16:0, 16:0-18:1, di18:2, di18:1, 18:0-18:1, 18:0-20:4, di19:0, di20:4, and di22:6 PC in the presence of a small amount of LiOH shows eleven intense ion peaks corresponding to their lithiated adducts (as indicated). Essentially equal intensities of these ion peaks were obtained within experimental errors of 10% after correction for differential $^{13}$C isotopologue distributions relative to di12:0 PC as indicated with horizontal lines.

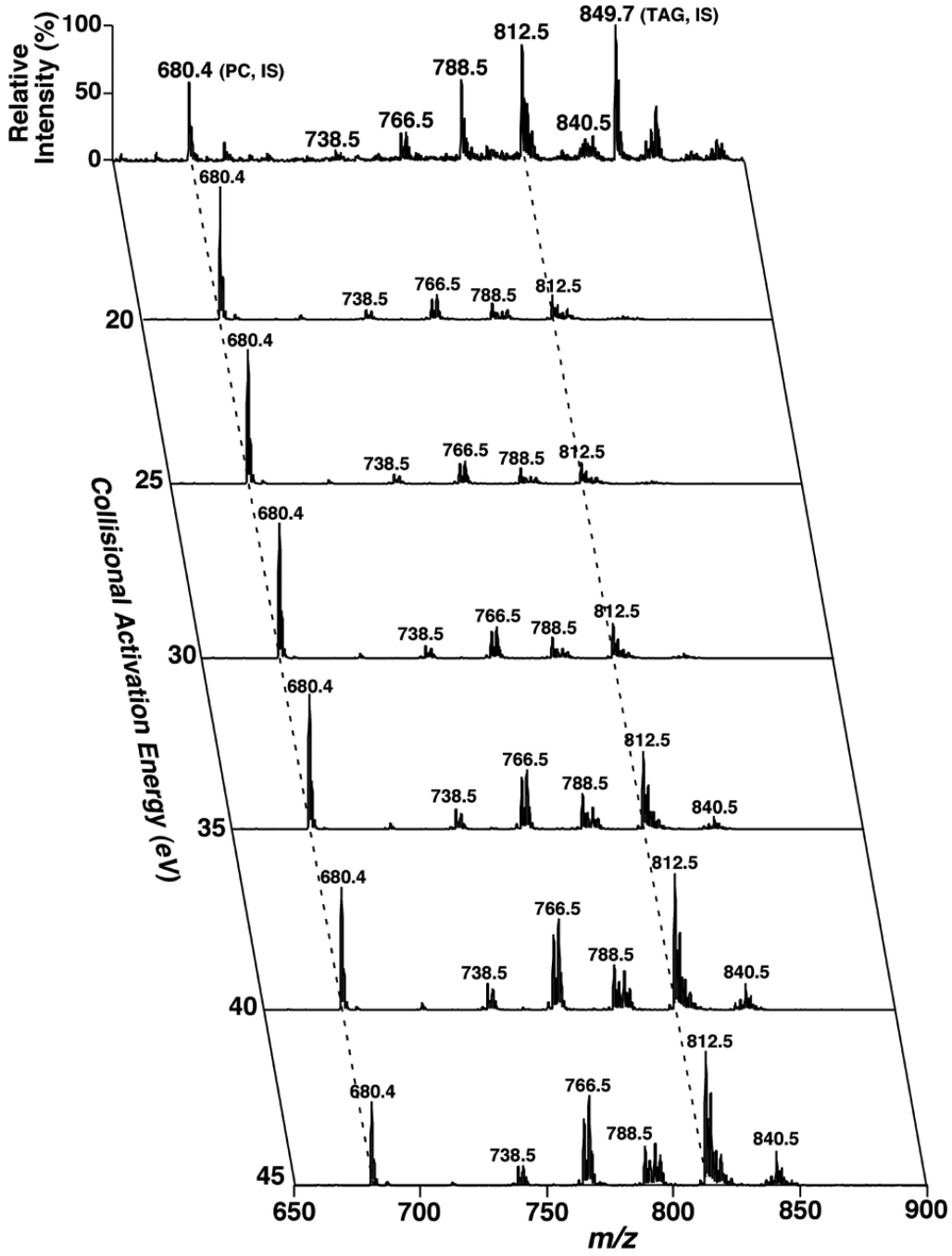


**Figure 3.**

An example of two-dimensional electrospray ionization mass spectral analysis of a mouse liver chloroform extract under different collisional activation energy conditions in the positive-ion mode in the presence of LiOH. A conventional ESI mass spectrum was acquired in the positive-ion mode directly from a diluted hepatic lipid extract prior to performance of neutral loss scan of 189.1 amu (i.e., a loss of lithium cholinephosphate from lithium adducts of phosphocholine-containing molecular species) from a crude hepatic lipid extract under

conditions with a variety of collision energies (as indicated) in Y-axis. All mass spectral traces are displayed after normalization to the base peak in each individual spectrum.

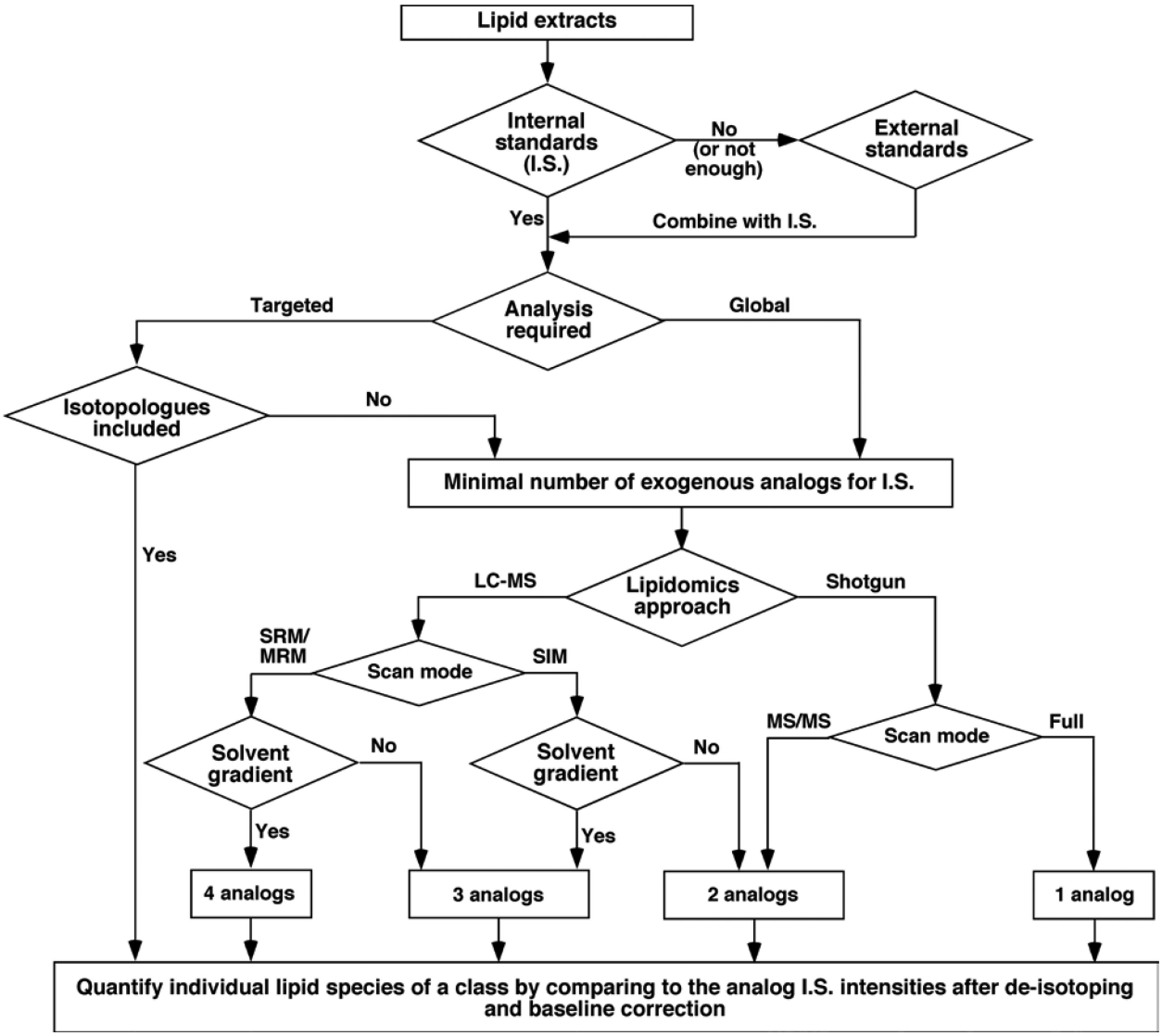


**Figure 4.**

A flowchart that leads to the minimal number of internal standards required for different methods. I.S. denotes internal standard(s).

## TABLE 1

Summary of Variables present in Lipidomics Approaches and Their Required Minimal Number of Internal Standards for Accurate Quantification of a Polar Lipid Class

| Platforms[a] | Variables | Minimal number of internal standards |
|---|---|---|
| MDMS-SL | - | 1 (endogenous species can be used as additional internal standards) |
| Tandem MS-based SL | MS/MS | 2 |
| High mass resolution MS-SL | - | 1 |
| High mass resolution MS-SL with product ion scans | MS/MS | 2 |
| SIM with isocratic elution | Concentration | 2 or 1 with external calibration curves |
| SIM with solvent gradient | Concentration, gradient elution | 3 or 1 with external calibration curves |
| SRM/MRM with solvent gradient | MS/MS, concentration, gradient elution | 4 or 1 with external calibration curves |

[a]MS, MDMS, and SL stand for “mass spectrometry”, “multi-dimensional mass spectrometry” and “shotgun lipidomics”, respectively.