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Lipidomic Analysis

The state-of-art in the lipidomic analysis is summarized here to provide the overview of available sample preparation strategies, mass spectrometry (MS)-based methods for the qualitative analysis of lipids, and the quantitative MS approaches for high-throughput clinical workflows. Major challenges in terms of widely accepted best practices for lipidomic analysis, nomenclature, and standards for data reporting are discussed as well.

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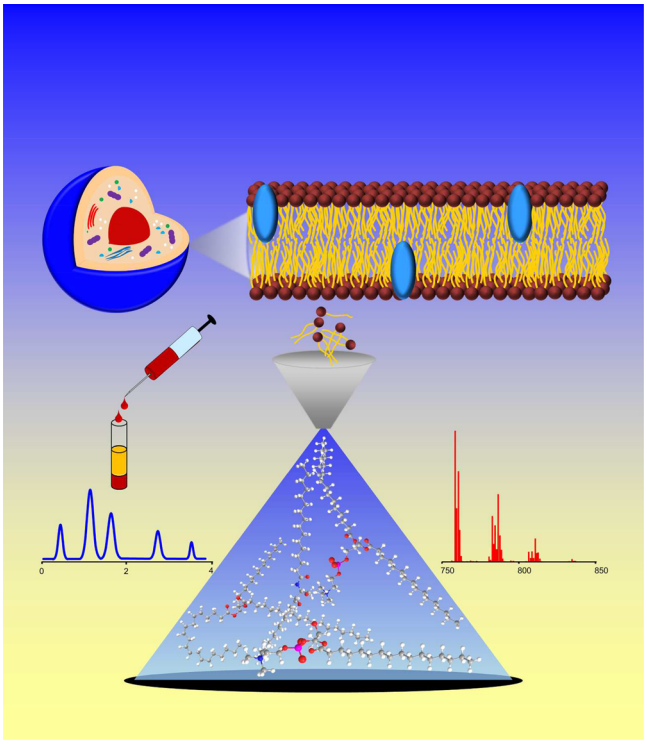

Lipids are defined as hydrophobic or amphipathic small molecules that originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units.[1] Older definitions of lipids refer them as molecules insoluble in water and soluble in organic solvents or molecules derived from fatty acids, but these old definitions do not cover all lipid molecules. The main biological functions of lipids are the energy storage, building blocks of cellular and subcellular membranes, and signaling molecules.[2] The dysregulation of lipids is related to various serious human diseases, such as cancer, Alzheimer disease, cardiovascular diseases, and lysosomal disorders.[3]

Lipids belong to the last step in the "omic" cascade (Figure 1) starting from genome, through transcriptome, proteome, and finally to metabolome.[4] The first step in this cascade, genome, represents genotype and shows the predisposition of particular subject what may happen in the future based on the genetic information. The last step is the metabolome, which is representing the qualitative and quantitative information on all metabolites occurring in a particular biological system, defining the phenotype. Unlike the genome (predicting future consequences of genetic information), phenotype is reporting on the actual state of the organism, and hence is most convenient for biomarker discoveries of pathological states of organism including serious human diseases.

The lipidome is a subgroup of the metabolome, but analytical approaches typically used in lipidomics differ significantly from methods established in metabolomics. The majority of lipid molecules contains polar/ionic head groups and nonpolar fatty acyl chain(s) (e.g., phospholipids and sphingolipids), which results in the formation of amphiphilic molecules with specific physicochemical properties, which must be taken into account during the method development including the sample preparation, chromatographic separation, and ionization in mass spectrometry (MS). The Lipid MAPS classification system is comprised of eight lipid categories (Figure 2),[1] which is described in more details in the next section.

In the past, the lipidomic analysis relied on the use of thin-layer chromatography (TLC) or gas chromatography (GC) after the derivation of polar functionalities, but nowadays the golden standard is the use of atmospheric pressure ionization MS either without separation or coupled with liquid-phase separation techniques, such as (ultra)high-performance liquid chromatography ((U)HPLC) or (ultrahigh-performance) supercritical fluid chromatography ((UHP)SFC). Electrospray ionization (ESI) is an established ionization technique for medium polar to ionic lipids and also applicable for nonpolar lipid (sub)classes due to the formation of alkali metal or ammonium adducts.[5] Atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) techniques are valuable alternatives for less polar lipid subclasses, such as triacylglycerols (TG), diacylglycerols (DG), cholesterol esters (CE), etc.[6] ESI-MS is by far the most frequently used analytical technique due to several significant advantages over other techniques, such as excellent

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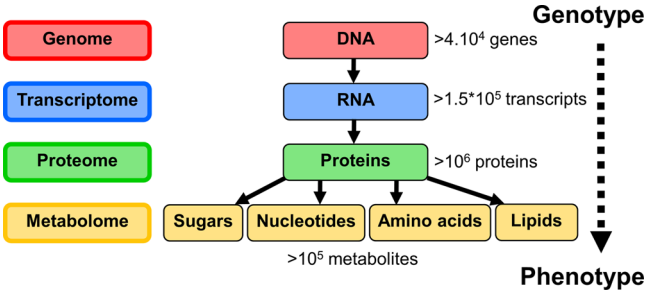

Figure 1. Overview of omics cascade in biological systems (updated according to ref 4).

sensitivity, easy coupling with liquid-phase separation techniques, structural details based on the use of tandem mass spectrometers with high mass accuracy, applicable for a wide range of lipids analyzable either in positive- or negative-ion modes, and very low sample consumption.

LIPID NOMENCLATURE

In 2005, the LIPID MAPS consortium has developed a comprehensive classification system for lipids.[1] Lipid species are placed into eight lipid categories (Figure 2), i.e., fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK), each of them containing classes and subclasses. For example, the GP category contains 21 classes and many subclasses, including glycerophosphocholines (PC), glycerophosphoethanolamines (PE), glycerophosphoserines (PS), glycerophosphoglycerols (PG), phosphatidylinositoles (PI), etc. This system is used to deposit lipid species as chemically defined structures into the LIPID MAPS database (http://www.lipidmaps.org/data/structure/index.php). Typically, lipidomic methods do not provide these structural details.[7] Therefore, a system for "Shorthand Notation for Lipid Structures Derived from MS"[8] has been proposed in 2013 based on the LIPID MAPS terminology. A key feature of this system is that only experimentally unambiguously proven structural details are annotated in this hierarchical system. When structural ambiguities are present (e.g., bond type, number of hydroxyl groups), then species annotation may be based on assumptions, but these assumptions have to be clearly visible in the annotation (Figure 3).

Here we discuss the major features for GL and GP (details for other lipid categories are described in[8]):

(A) Shorthand notation: Lipid subclass abbreviation followed by the total number of fatty acyl carbon atoms and the total number of double bonds (DB) separated by colon, e.g., PC 36:2 for phosphatidylcholine with 36 carbon atoms and two DB.

(B) Fatty acyls linked to the glycerol are known: (a) Underscore separator "_" means that *sn*-positions of fatty acyls are not known, e.g., PC 18:0_18:2 for phosphatidylcholine with 18:0 and 18:2 fatty acyls. (b) Slash separator "/" means that *sn*-positions of fatty acyls are proven (order *sn*-1/*sn*-2/*sn*-3 for GL; *sn*-1/*sn*-2 or *sn*-2/*sn*-3 for GP), e.g., PC 18:0/18:2 for phosphatidylcholine with 18:0 in *sn*-1 and 18:2 in *sn*-2 positions. (c) No FA linked (lyso) are annotated as 0:0.

(C) Other bond types than ester bonds are indicated as follows in front of the sum of carbon atoms or fatty acyl: (a) O means proven O-alkyl bond (it is important to note that letter O after the number of carbon atoms designates a keto bond, see the annotation of FA in[8]), (b) P means proven O-alk-1-enyl bond (acid-sensitive ether bond in plasmalogens).

(D) Lysophospholipid classes may be abbreviated by prefix "L", e.g., LPC for lysophosphatidylcholines.

SAMPLE PREPARATION

The sample material has to be of good quality to permit analyses of high value. Care should be taken in the sampling process covering sample collection, preservation, and extraction. These processes should always be repeated identically among samples and studies to obtain the highest reproducibility and data value. The appropriate selection of solvents, reagents, sample amounts, lipid standards, hardware, and protocols have to be carefully considered.

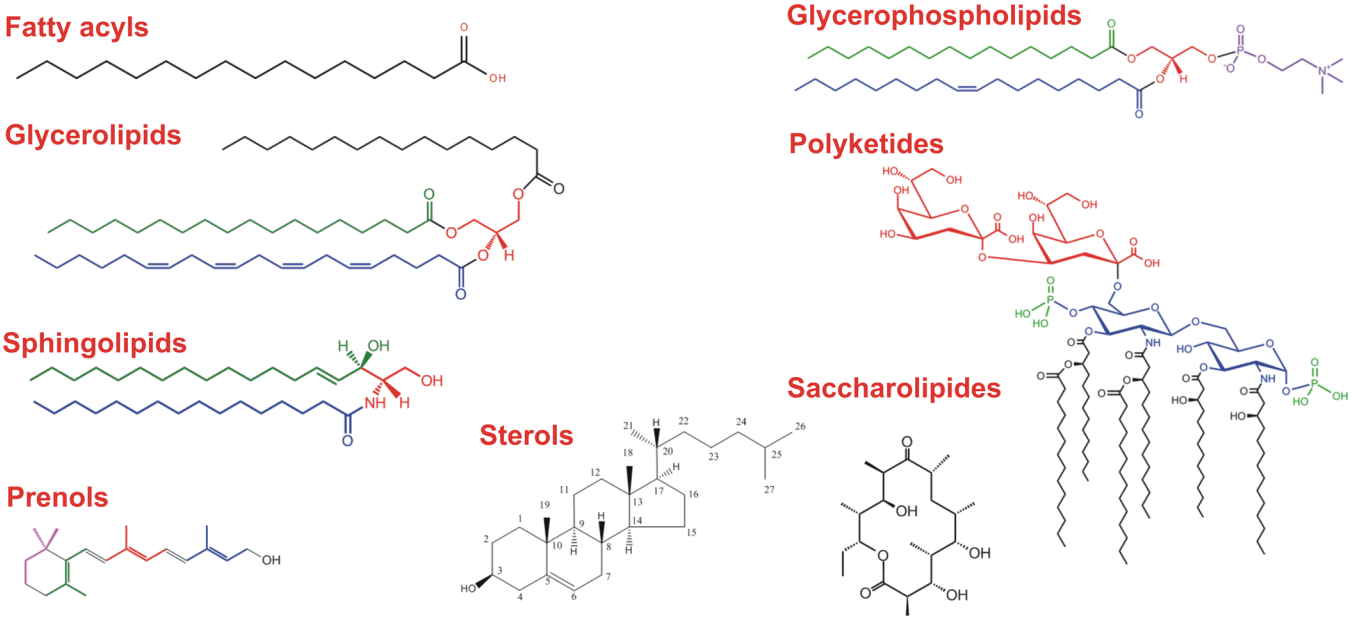

Figure 2. Lipid categories according to Lipid MAPS classification with representative structures for individual categories.

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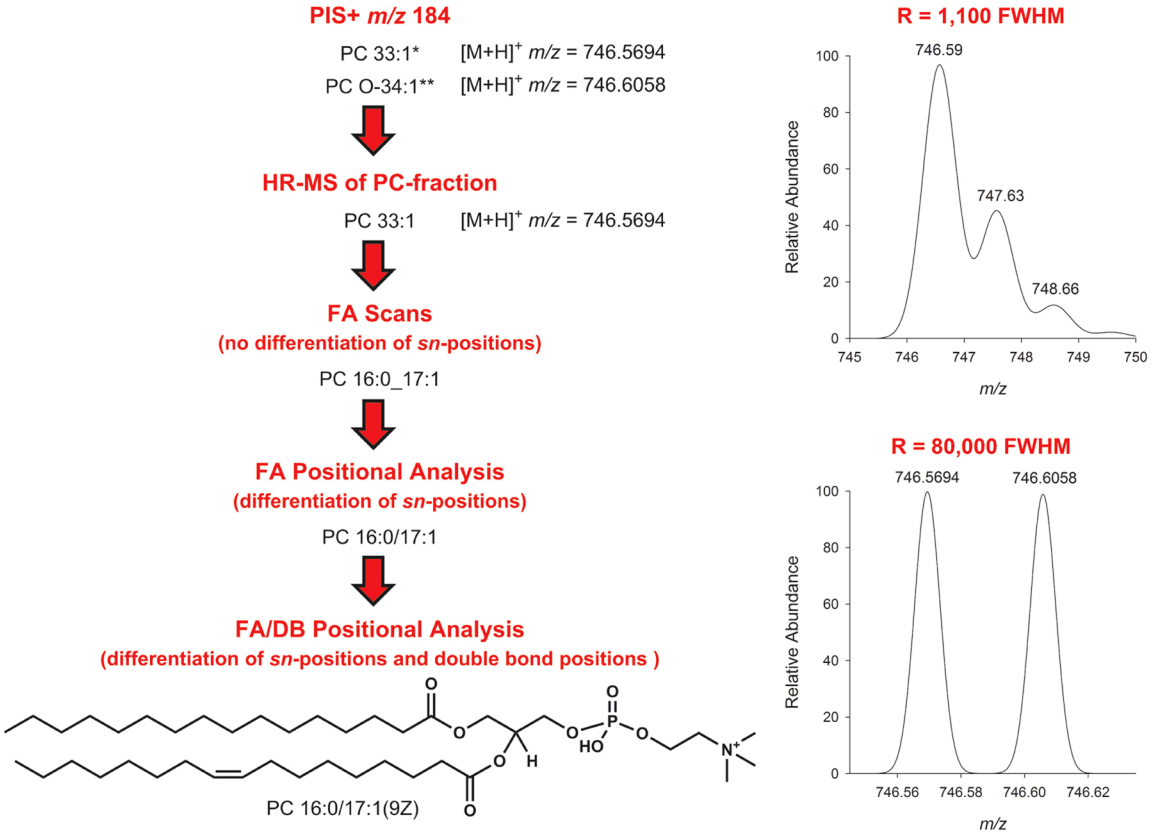

Figure 3. Example for annotation of glycerophospholipid structures: precursor ion scan of m/z 184 identifies lipid species containing a phosphocholine headgroup including PC. Low mass resolution (R = 1 100 fwhm) of precursor ions does not differentiate different bond types, i.e., isobaric diacyl and alkyl/acyl species. *Annotation is based on the assumption that ester bonds are present. **Annotation is based on the assumption of even numbered carbon chains only. High-resolution mass spectrometry (HR-MS, R = 80 000 fwhm) permits differentiation of bond types. The analysis of fragment ions (FA Scans) derived from acyl chains or their neutral loss permits annotation of acyl/alkyl chains mostly without the identification of their positions. Detailed analysis may identify *sn*-positions and positions of DB in acyl/alkyl chains.

The best sample condition is using fresh samples; however, this is typically very difficult due to the practical circumstances, e.g., collecting blood samples in a hospital and performing lipidomic analyses elsewhere. Therefore, the most common samples available are frozen stored samples. In the collection phase, it is critical that the samples are collected in the most suitable vials and maintained under conditions that will not influence the sample quality. Samples should always be kept cold and be snap frozen in liquid nitrogen and stored at −80 °C until further processing steps. The sample stability can be prolonged by maintaining samples in an environment free of oxygen, metal ions, and peroxides, and by the addition of antioxidants, such as butylated hydroxytoluene, which should suppress the degradation of lipid by (per)oxidation. Samples should be aliquoted to avoid freeze−thaw cycles that potentially stimulate the hydrolysis of lipids.[9] In this way, the potential formation of artifacts can be minimized. It has been shown that certain biological matrixes can be safely stored for years at −80 °C;[10] and in case of blood samples, ethylenediaminetetraacetic acid (EDTA) is the preferred anticoagulant.[11] Stepping outside the appropriate sample processing protocol severely increases the risk in introducing unwanted sample properties, such as the production of lysophospholipids[12] and lipid degradation by hydrolytic enzymes[13] or inappropriate storage materials.[14] Adapting proper processes for biofluids are easier than tissues. Biofluids can usually be immediately aliquoted and frozen upon collection, whereas tissues require more handling steps before freezing. For example, liver has to be perfused to remove blood components, including cells, lipoprotein particles, and albumin, followed by being sliced into smaller pieces. Although the minimal requirements for the right sample handling are known, we may still lack some experimental facts, which could help to maintain the highest sample quality. To date, the biggest gap is in tissue handling, but we also lack precise information on the wide stabilities of different lipids.

To bring the lipids from samples to the solution, nonpolar organic solvents like chloroform, methyl-*tert*-butyl ether (MTBE), and heptane are typically used for the extraction. The chloroform based extraction systems described by Bligh and Dyer[15] and Folch[16] are the most commonly used for extracting biofluids and tissues, respectively. More recently modified protocols using MTBE[17] and butanol−methanol (BUME)[18] have been developed to improve the extraction process and as less hazardous alternatives. These protocols are well established for a number of sample materials and lipid classes and can be automated using robotics.[19] However, if the sample matrix is new, the procedure should be carefully evaluated in terms of recovery, reproducibility, and generation of artifacts. The prime advantage of these partitioning systems is their efficient and broad lipid recovery combined with the greatly reduced sample complexity by removal of unwanted polar metabolites, proteins, and salts during the lipid extraction. A popular approach is to apply simple protein precipitation using organic solvents, as these methods are both simple and

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rapid. However, these should be applied with care, as they are less efficient in extracting and prone to adverse matrix effects leading to ion suppression, misidentifications and inaccurate quantitation. A correct lipidomics analysis includes the addition of internal standards prior to extraction, to facilitate monitoring recoveries and absolute quantitation. Improved standard mixtures, such as the SPLASH Lipidomix[20] for human plasma, are being developed to both improve the quantitation of lipids and simplify the working protocols. It is important to use as little sample amount as possible in order not to exceed the capacity of the organic phase permitting parallel quantitation of a broad lipidome. Therefore, no more than ∼10 μL of plasma in 800 μL of Folch lipid extraction should be used.[14,21] A similar concept applies to tissue samples, but the sample needs to be weighted and homogenized[18] prior to extraction. Here, only microgram amounts of tissue homogenate would be used. After the lipid extraction, the samples are typically dried down and reconstituted in appropriate solvents optimal for the MS analysis. In line with the sample handling, extracted samples are typically stored at −20 °C or preferably at −80 °C prior to the analysis.

■ ANALYTICAL METHODS

Three main approaches in the lipidomic research are direct infusion MS analysis (also known as shotgun lipidomics), liquid-phase separations coupled to MS (typically liquid chromatography (LC−MS)), and desorption ionization techniques MS approaches (often used for mass spectrometry imaging, MSI).

Shotgun MS. In shotgun lipidomics, a crude lipid extract is infused to the MS instrument. Direct MS scans are typically applied in high-resolution MS (HR-MS) but can also be performed in conjunction with such as ion mobility on low-resolution instruments.[22] The pioneering shotgun methods was based on low mass resolution MS/MS, typically triple quadrupole instruments.[23] The fragmentation of lipid molecules, like glycerophospholipids, yield lipid class-selective fragments that are common for lipid species belonging to the same lipid class (e.g., in the positive ion mode all PC and SM molecules provide the product ion at m/z 184, all PE species undergo the neutral loss of $\Delta m/z$ 141). In the negative ion mode, lipid molecules may be identified additionally by fragment ions matching hydrocarbon chains and can be annotated as molecular lipid species (e.g., PC 16:0_18:1).[24,25] Usually, an assignment of *sn*-positions of hydrocarbon chains in glycerolipids and glycerophospholipids is only possible with validated assays based either on monitoring ratios between fragment ions of positional isomers (direct infusion[24] and LC−MS[26]) or other more advanced methods.

Lipid species belonging to the same lipid subclass frequently differ only in one DB (i.e., mass difference of 2 Da). Thus, in a series of species with the different number of DB, the monoisotopic peak (M) may contain a substantial proportion of the M + 2 isotopic peak of the species with one additional DB (mainly due to the natural abundance of ^{13}C). For example, in the precursor ion scan of m/z 184, the M + 2 isotopic peak of PC 36:2 has an intensity of 10.5% of the M peak. This peak may contribute significantly to M peak of PC 36:1, therefore, the intensity correction has to be used. In the same spectrum, even more pronounced interference is observed for PC (even m/z values) and SM (odd m/z values) species. The M + 1 isotopic peak of PC 38:3 ($[M1 + H]^+$ m/z 813.6197) has an intensity of 46.9% of the M peak and is isobaric with SM d42:2 ($[M + H]^+$ m/z 813.6844). Hence, such overlap has to be corrected for all lipid subclasses with varying degree of unsaturation unless high mass resolution or ion mobility prior to analysis are applied.

Time of flight (TOF) instruments with the mass resolution at least 30 000 full width at half maximum (fwhm) are capable to resolve PC (M1) with SM overlap or to differentiate isobars like ether-bond PC O-34:1 ($[M + H]^+$ m/z 746.6058) from diacyl PC 33:1 ($[M + H]^+$ m/z 746.5694, see also Figure 3). In contrast, isobars resulting from $^{13}C_2$ overlap in DB series have only mass difference of 9 mDa, which requires ultrahigh mass resolution, like Fourier transform ion cyclotron resonance MS or Orbitrap instruments with resolving power >150 000 fwhm at the typical range of interest m/z 700 to 900.

Chromatography−MS. Thin-layer chromatography was frequently used for the lipid analysis in the past, but nowadays it is mainly applied for the preparative isolation of selected lipids or lipid (sub)classes. Gas chromatography/mass spectrometry (GC/MS) is a well established technique for the analysis of fatty acid methyl esters after the transesterification of all lipids, which provides the information on FA composition, but the information on intact lipids is lost. The coupling of liquid chromatography and mass spectrometry (LC−MS) is a key analytical method for lipidomic characterization together with shotgun MS. The main potential advantage of LC−MS for a lipidomic analysis is its relatively wide range of separation modes, which may be tailored for almost all existing types of lipid isomers. Reversed-phase LC is by far the most common mode, because it may provide complex separation based on the FA length and also the number and positions of DB.[6,26] On the other hand, hydrophilic liquid chromatography (HILIC) separates lipids mainly based on polar head groups, which is the most preferred mode for quantitative LC−MS lipidomics based on lipid class separation applicable for polar GP and SP (sub)classes.[27−29] The alternative lipid class separation method for nonpolar lipid classes in the normal phase (NP) chromatography.[30] Silver-ion chromatography is a special mode of NP with embedded silver ions, which can differentiate lipids differing in the DB number, positions, and cis/trans geometry and also regioisomers of TG.[31] The current trend in LC is the use of sub-2 mm particles and high-operating pressures (ultrahigh-performance liquid chromatography, UHPLC), which provide superior performance. Another popular tool is the use of two-dimensional (2D) LC, where two chromatographic modes with orthogonal separation selectivity can be coupled to provide higher peak capacities either in offline[27] or online[28] modes. The most recent advancement is a routine ultrahigh-performance supercritical fluid chromatography (UHPSFC)−MS applied for lipid class separation and quantitation both for nonpolar and polar lipid classes in extremely short analysis times.[32]

Desorption Ionization Techniques. Matrix-assisted laser desorption/ionization (MALDI) belongs to the group of desorption ionization techniques applicable for the analysis of biological tissues and cells including MSI, which provides the information on spatial distribution of individual molecules, mainly lipids, metabolites, and small peptides. Figure 4 compares MALDI image of three selected lipids (Figure 4A) with histological staining prepared after MSI experiment (Figure 4B) to illustrate the utmost combination of lateral resolution (<1.4 μm), mass resolution (>100 000 full width at half-maximum), and mass accuracy (<2 ppm) in imaging measurements.[33] MALDI can be also applied for the analysis of

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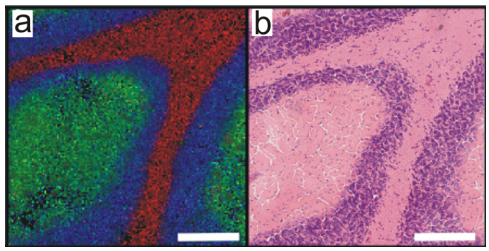

Figure 4. MALDI-MSI of mouse brain tissue section: (a) superimposed lateral distributions of three lipids: $[PC\ 38:1 + K]^+$ (red), $[PC\ 38:6 + K]^+$ (blue), and $[SM\ 36:0 + K]^+$ (green) and (b) H&E stained tissue section. Reproduced with permission from ref 33. (Springer Nature, Copyright 2017).

extracts, but the reproducibility and robustness of MALDI measurements is slightly worse in comparison to ESI. Thus, the MALDI quantitation should be rather considered as semi-quantitative only, but it can still provide valuable data on relative changes caused by particular disease, such as renal cell carcinoma.[34] In addition to MALDI, numerous other desorption ionization techniques can be used for the same purpose.

QUANTITATIVE MS APPROACHES IN LIPIDOMICS

The typical quantitative workflows in the lipidomic biomarker discovery research include the following steps. The first step is the extraction of pooled sample for particular study followed by nontargeted identification of the maximum number of lipids in the pooled sample. Then individual samples are extracted with added lipid (sub)class internal standards and quantified using targeted MS quantitative approaches, such as shotgun or separation−MS. The data processing is a rather demanding and important step to keep the integrity and quality of lipidomic data. Finally, the statistical evaluation using multivariate data analysis methods, such as nonsupervised principal component analysis (PCA) and supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) are used to differentiate healthy and disease groups and to find the most

Table 1. Typical Internal Standards Used in the Lipidomic Quantitation

		internal standards			
lipid category	lipid (sub)class	UHPSFC/MS,[32] shotgun MS, HILIC/MS[29]	shotgun MS[52]	shotgun[53,54] and HILIC/MS,[5541]	shotgun MS[20]
fatty acyls	fatty acids	D9-18:1	D8-20:4		
			D5-22:6		
			D5-20:5		
	hydroxyeicosa-tetraenoic acids		D8-5-HETE		
	octadecanoids		D4-9-HODE		
	prostaglandins		D4-PGD2		
	thromboxanes		D4-TXB2		
	epoxyeicosatrienoic acids		D11-8,9-DHET		
glycerolipids	MG	19:1			D7-18:1
	DG	12:1/12:1	17:0/17:0		D7-15:0_18:1
	TG	19:1/19:1/19:1	17:0/17:0/17:0		D7-15:0_18:1_15:0
glycerophospholipids	PC	14:0/14:0	17:0/17:0	14:0/14:0; 22:0/22:0	D7-15:0_18:1
	PE	14:0/14:0	17:0/17:0	14:0/14:0; 20:0/20:0	D7-15:0_18:1
	PG	14:0/14:0	17:0/17:0	14:0/14:0; 20:0/20:0	D7-15:0_18:1
	PS	14:0/14:0	17:0/17:0	14:0/14:0; 20:0/20:0	D7-15:0_18:1
	PI	17:0/14:1	17:0/17:0		D7-15:0_18:1
	PA	14:0/14:0	17:0/17:0	14:0/14:0	D7-15:0_18:1
	LPC	14:0	17:0	13:0; 19:0	D7-18:1
	LPE	14:0			D7-18:1
	LPG	14:0			
	LPS	14:0			
	LPA	14:0		17:0	
	BMP			14:0/14:0	
	CL			14:0/14:0/14:0/14:0	
sphingolipids	SM	d18:1/12:0	d18:1/12:0		D9-d18:1/18:1
	Cer	d18:1/12:0	d17:1/18:0	d18:1/17:0	
	HexCer	d18:1/12:0	D3-d18:1/16:0	d18:1/12:0	
	Hex2Cer	d18:1/12:0	D3-d18:1/16:0	d18:1/12:0	
	Hex3Cer		d18:1/17:0		
	SulfoHexCer	d18:1/17:0			
	S1P	d17:1	d17:1	$^{13}CD_2$-d18:1	
	SPH		d17:1	d17:1	
	GM1		D3-d18:1/18:0		
	GM3		D3-d18:1/18:0		
sterols	CE	D7-16:0	D6-18:0	17:0; 22:0	D7-18:1
	Chol	D7-Chol		D7-Chol	D7-Chol

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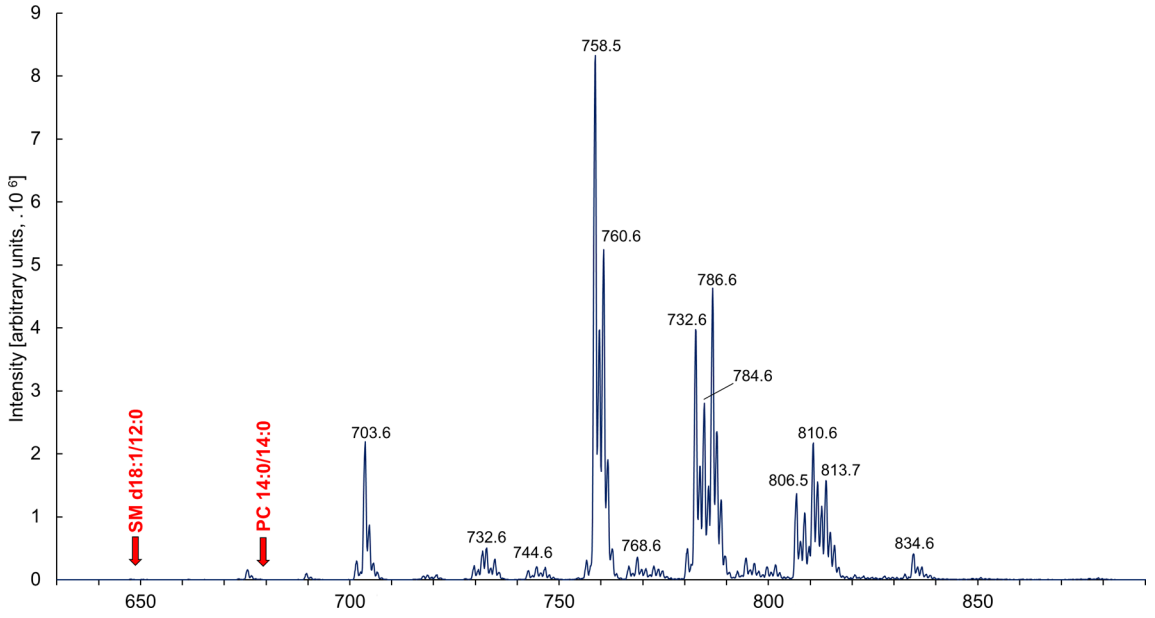

Figure 5. Selection of internal standards for PC and SM based on the analysis of pooled human plasma using shotgun MS with precursor ion scan of m/z 184.

dysregulated lipids, which could be applied as biomarkers for studied diseases.

Internal Standards and Method Validation. The basic prerequisite of any lipidomic quantitation is the use of at least one nonendogenous internal standard (IS) per each lipid subclass to be quantified. Table 1 summarizes typical IS previously used for the quantitation of lipid subclasses in individual lipid categories. The most common IS are lipids having shorter fatty acyl chain(s) (e.g., 12:0 or 14:0) and odd-carbon number fatty acyl chain (e.g., 17:0, 17:1, 19:0).[25,29,32] Recently, prepared mixtures of deuterated analogues (e.g., deuterated cholesterol or cholesterol ester) have been designed to simplify and permit prestandardization efforts by offering a common IS mixture for a particular sample matrix. For instance, the SPLASH standard mixture has in particular been designed for the quantitative analysis of human plasma.[20] For any quantitative study, the first step should be the confirmation that planned IS is absent in the extract of pooled sample for a particular study (Figure 5). In the pharmaceutical industry, the full method validation according to the requirement of approved authority (e.g., Food and Drug Administration or European Medicines Agency) is an indispensable part of any quantitation. Unfortunately, the method validation has not yet been frequently applied in lipidomic analyses, but the situation is changing, because several recent papers have used their own validation protocols, which typically includes parameters like LOD, LOQ, linear dynamic range, matrix effect, intra- and interday reproducibility, etc.[29,32] Long-term stability assessments have demonstrated that shotgun based lipidomics methods could also fulfill the regulatory environment.[21]

Shotgun MS. Shotgun lipidomics provides a simple means to quantify lipid species by the addition of IS to the lipid extraction, since all analytes and IS are present in the same sample matrix. However, the following should be considered to achieve accurate results: (a) the response of lipid species depends on the lipid concentration, solvent, and additive composition of the infusate,[35] (b) the chemistry of the lipid subclass but also lipid acyl/alkyl chains and their degree of unsaturation may contribute considerably to ionization efficiency.[36] For instance, CE 18:1 has a 2-fold higher response compared to CE 18:2 in direct infusion MS/MS analysis,[37] and (c) the isotopic overlap has to be considered (see above).

Chromatography−MS. The basic requirement for the lipidomic quantitation is the co-ionization of lipid class IS and analytes from given lipid subclass. It is automatically fulfilled in the case of shotgun MS or any technique without chromatographic separation (e.g., MALDI-MS), but the careful attention should be paid for separation−MS approaches. The goal is to separate individual lipid classes but on the other hand to suppress the separation of lipid species inside classes. The typical chromatographic modes providing lipid class separation are hydrophilic liquid chromatography (HILIC) or normal-phase (NP) modes used under HPLC or SFC conditions. The use of HILIC or NP conditions coupled to ESI-MS follows the same principles of quantitative lipidomics as for shotgun MS, where the only difference is that shotgun MS separates lipid subclasses based on a characteristic scan event (precursor ion or neutral loss scans), while HILIC/NP-MS distinguishes lipid subclasses by chromatographic separation. In both cases, we obtain lipid subclass mass spectra, which are further processed by various lipidomic softwares, and typically proprietary software solutions are used by individual lipidomic laboratories instead of commercial solutions from major MS vendors. Figure 6 shows examples the separation of lipid class representative standards obtained by the HILIC mode in UHPLC for polar lipid subclasses from GP and SP lipid categories (Figure 6A), normal-phase (NP) UHPLC separation of nonpolar lipid subclasses, typically used for acylglycerols and CE (Figure 6B), and UHPSFC separation of both nonpolar and polar lipid subclasses (Figure 6C). HILIC can be successfully applied for other lipid subclasses as well, such as lysophospholipids,[38] gangliosides,[39] bioactive SP,[12,40] and separation of isomeric bis(monoacylglycero)phosphate and phosphatidylglycerol,[41] etc.

The use of reversed-phase (RP) provides an excellent lipid species separation[42] with higher number of identified lipids (about factor of 2), but it does not follow the concept of the co-ionization of IS and analytes from particular lipid subclass. In

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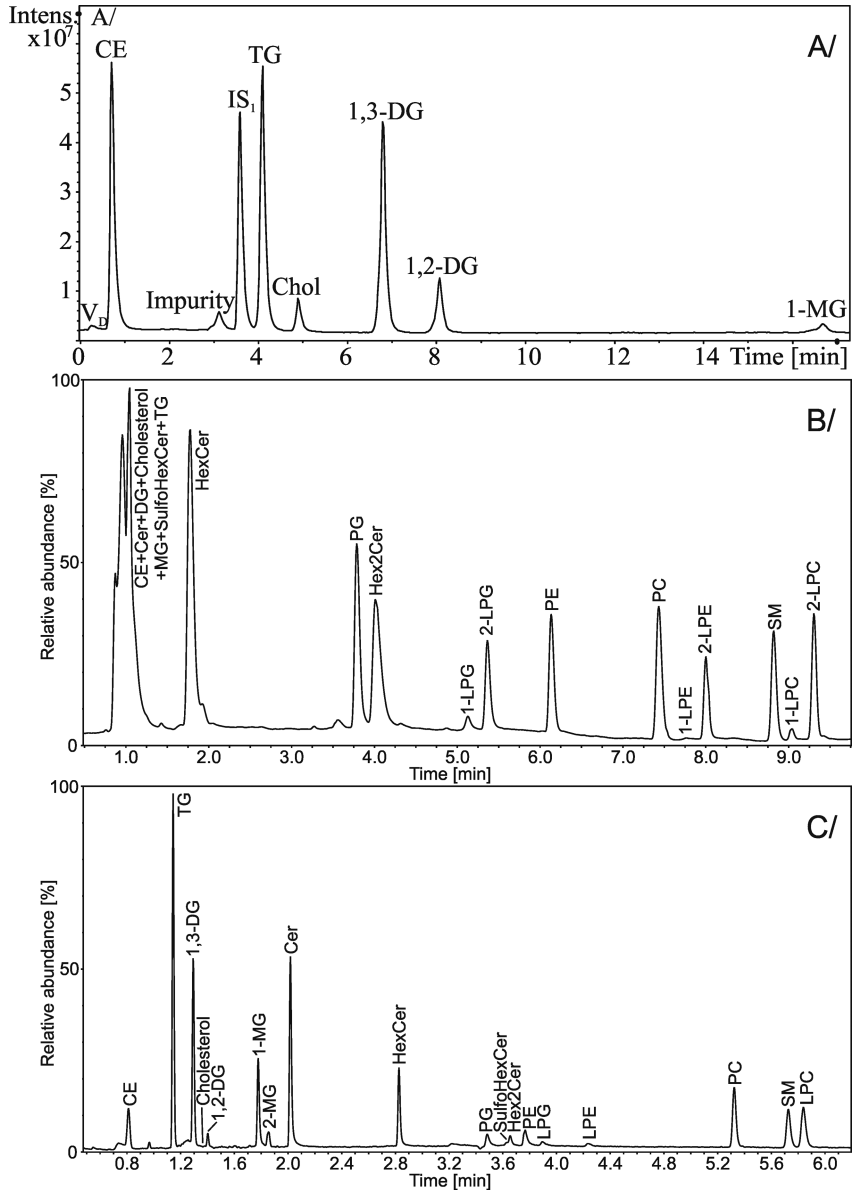

Figure 6. Separations of lipid class representative standards in various chromatographic modes: (A) NP-UHPLC/MS separation of nonpolar lipid classes.[30] Reproduced with permission from ref 30 (Elsevier, Copyright 2015). (B) HILIC/MS separation of polar lipid classes[29] and (C) UHPSFC/MS separation of both nonpolar and polar lipid classes.[29] Reproduced with permission from ref 29 (Elsevier, Copyright 2017).

general, such approach should be avoided for the lipidomic quantitation whenever possible, but there are specific examples, where RP mode has to be used due to the necessity to separate numerous isobaric lipid molecules, for example, in the case of isobaric oxylipins[43] and oxysterols.[44] In case of any lipidomic quantitation based on RP mode or other species based separation, the utmost attention should be paid to the method validation, quality control, and the use of multiple IS over the whole retention window, but the validated quantitation in RP mode is also possible.[45] The same problem with the quantitation of lipids is present in any two-dimensional (2D) HPLC separation,[27,28] because one mode typically resolves lipid classes (HILIC or NP), while the second one is based on lipid species separation (RP) or specific interactions, such as silver-ion chromatography.[31] The implementation of any type of ion mobility spectrometry results in the same situation as described for 2D that IS is not co-ionized with analytes from the same lipid subclass, but it may provide valuable additional separation dimension for various types of lipid isomers.[46]

Data Processing and Reporting. Each lipidomic workflow needs automated data processing, which is also of paramount importance to achieve accurate values. It includes the lipid species identification, the deconvolution of isotopic overlap (when necessary), and relative or absolute quantitation. Dedicated softwares are available for the data processing either in shotgun (e.g., LipidXplorer,[47] ALEX[48]) or in LC−MS mode (LIMSA,[49] LipidSearch,[50] and Lipid Data Analyzer[51]). It is essential to understand the algorithms implemented in the software packages, especially when commercial software is applied. Finally, it is important to report data using a standardized nomenclature[8] and to avoid overreporting, i.e., only proven structural details should be annotated.

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CONCLUSIONS

The lipidomics field is undergoing a meticulous development that is drawing a major attention throughout the scientific field. New technologies, methodologies, and bioinformatic solutions are continuously being developed, all targeting to improve and advance the field. Up-to-date technologies and protocols permit to perform quantitative lipidomic studies. However, the current lack of lipidomic standards hinders us from unleashing the true power of lipidomics. The issues are multifactorial and needs careful considerations in how to be resolved. Efforts have already been initiated to identify the minimum lipidomics ruleset, striving for the high flexibility to cope with unforeseen future developments in the field. We anticipate that the initial lipidomic guidelines to be ready in the near future. With adherence to lipidomic standards, we will prosper much higher successful rate of lipidomics studies, permitting for the first time complete comparability among studies and laboratories. Undoubtedly, this will foster a dramatic advancement in basic and clinical research and facilitate a powerful and transparent transit of lipid biomarkers into clinical diagnostics with the ultimate goal of improving human health.

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Kim Ekroos is the founder and CEO of Lipidomics Consulting Ltd., a consulting business providing unique services for customers globally in the field of lipidomics. He received his Ph.D. degree in biology from the Technical University in Dresden, Germany, in 2003. His expertise includes high-throughput technologies for the precise assessment of lipidomes enabled by advanced mass spectrometry, automation, and software tools towards discovery of biological architectures and of diagnostic biomarkers for clinical purpose.

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