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Lipidomics and recent applications in clinical and medical research with ultrahigh performance liquid chromatography-mass spectrometry – A review

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Abstract

Lipids are organic biomolecules, which contribute to energy storing, cellular and subcellular membrane assembly, functionality, signalling, gene expression regulation and surfactant formation. Lipidomics comprises of identification and quantitation of organic lipids, their derivatives and variants.

The present review article combines the literature on glycero-, glycerophospho- and sphingolipids in lipidomics from the years 2017-2019. In addition, a few papers which have promoted advancement in lipidomics are discussed. The primary focus is lipid profiling of biological lipidomic systems with ultrahigh performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS, tandem MS) detection, data handling, and calculations with analytical tools and current statistical methods. Research on *in vivo* samples includes matrix categories, such as primary blood derivatives (plasma, serum, erythrocytes, and blood platelets), faecal matter, urine, and liver tissues.

Keywords: Lipids, liquid chromatography, tandem mass spectrometry; clinical profiling, urine, blood.

Highlights:

Compilation of the recent literature on clinical samples; presentation of the accurate methodology. Evaluation of sample preparation techniques in lipid analyses.

Information on separation and identification of neutral, hydrophobic and hydrophilic lipids. Compilation of current statistical methods used in mass spectrometry of glycero-, glycerophospho- and sphingolipids.

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Contents

Abbreviations

ACP acyl carrier protein; Cer ceramides; Cer-PC ceramide – phosphatidylcholine; Cer-PE ceramide – ethanolamine; CEs cholesteric esters; DG diglycerols; DB double bond; ESI electrospray ionization; FA fatty acid; GluCer glucosylceramide; GPs glycerophospholipids; GLs glycerolipids; HDL/LDL/VLDL high/low/very low density lipoproteins; HILIC hydrophilic interaction chromatography; LC liquid chromatography; LacCer lactosyl ceramide; LPC lysophosphatidylcholines; LPE lysophosphatidylethanolamine; MS mass spectrometry; MALDI matrix-assisted laser desorption ionization; MG mono glycerols; MGDG monogalactosyldiacylglycerol; MUFA monounsaturated fatty acids; NADPH nicotinamide adenine dinucleotide phosphate; nanoLC nano-liquid chromatography; NEFA/FFA non-esterified fatty acids/free fatty acids; NP normal phase chromatography; PA phosphatidic acid; PG phosphatidylglycerol; PI phosphatidylinositol; PKs Polyketides; PUFA polyunsaturated fatty acids; RP reversed phase chromatography; PRLs prenol lipids; S sphingosine; SA sphinganine; SA1P sphinganine-1-phosphate; SFA saturated fatty acids; SLs saccharolipids; SM sphingomyelin; SPs sphingolipids; SQDG sulfoquinovosyl diacylglycerols; ST cholesterol-based sterols; S1P sphingosine-1-phosphate; PC phosphatidylcholines; PE phosphatidylethanolamine; TG triglycerols;

TGDG trigalactosyldiacylglycerol; UHPLC-MS ultrahigh performance liquid chromatography-mass spectrometry.

1 Lipidomics

Lipidomics is a subgroup within the field of metabolomics with which lipid metabolites can give valuable information about organisms and their biological functions. Lipidomics may be divided into membrane-lipidomics and mediator-lipidomics, [1] which include either comprehensive or quantitative description of membrane lipid constituents or structural characterization and quantification of low abundant bioactive lipid species, respectively. As a sector of metabolomics devoted to the qualitative and quantitative analyses of the lipidome, lipidomics has met an utmost expansion to various biochemical areas. [2] Lipids may be involved in explaining the physiology of multiple interlinked organs, a single organ, biological tissues, individual cells, and biofluids.

In broad terms, lipidomic research covers all aspects of individual lipid species including their structure, function, connection with other cell constituents such as protein, lipid, and metabolite in both health and disease conditions. Details of cell biology obtained from different pathogens (viruses, bacteria, and parasites) provide a great amount of data on molecular structure of host pathogen relations and, consequently, on infection processes [3]. Novelty of lipid analytics is related to reliable, accurate, and sensitive analytical methods for observing new lipid information and correlation with diseases. Abnormalities in lipid metabolism may be an indicator for the stage of a lipidome-related disease, a correlation factor which may be observed when comparing a patient with a healthy individual [4], [5].

The current review describes identification and determination of lipids in biological, clinical and medical samples with ultrahigh performance liquid chromatography – mass spectrometry (UHPLC-MS) in 2017-2019. The publications discuss about determination of biological samples based on (1) chromatography/separation, (2) identification with various types of mass spectrometry, (3) matrices, (4) lipid groups, and (5) mathematical data handling.

1.1 Classification of lipids

According to the definition of lipids made by Fahy et al. [6] lipids are "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units". [6] Recently, the newest recorded updates of the definition was published by the lipidomics gateway LIPID MAPS® consortium (*https://www.lipidmaps.org/data/classification/LM_classification*, March 20th 2017). Thus, lipids are characterized according their backbone structure. Lipids include glycerophospholipids (GPs), glycerolipids (GLs), sphingolipids (SPs), polyketides (PKs), cholesterol-based sterols (ST) such as cholesteric esters (CEs), prenol lipids (PRLs) and saccharolipids (SLs). In addition, high-/low-/very low-density lipoproteins (HDL/LDL/VLDL) are frequently studied alongside conventional lipids, though lipoproteins are excluded from this study.

1.2 Lipid types

Polar fatty acids (FA) contain fundamental elements of more complex lipids (e.g. for triacylglycerols). They are heteroatom-substituted and esterified conjugates, e.g. acyl carnitines [AcCA] [7]-[10] or bis(monomyristoylglycero)phosphate [BMP] [11], which may include branched or cyclic functionalities [4]. In comparison to the lipid headgroups and hydrocarbon backbones, FA chain function and diversity is less understood. Antonny et al. [12] report several FA saturation gradients: saturated fatty acid glycerophospholipids (SFA GPs) increase the further they are processed in the mammalian cell, namely from the endoplasmatic reticulum (ER) to Golgi apparatus to plasma membrane. [12] Conversely, neural cells have rare sightings of poly-unsaturated fatty acid phosphatidylcholines (PUFA PCs) around the soma, increasing towards the C20:4- and C22:6-rich PCs axon end. Higher saturation is often present in uptake-based long FA chains like C22:6-n3, synthesized from C18:3-n3 plant oil (*Figure 1*). [12]

Lipids get their FA chains either *de novo* via the Kennedy pathway synthesis [13] or through re-acylation (i.e. FA substitution) with available components via the Lands cycle [13]. [12] This

substitution is enabled through acyltransferase activity whose rate of specificity affects acyl chain diversity.[12] More research on SPs with >C18 FAs is needed. For instance, counter to common belief, SM and Cer include a large abundance of C24:1 and C24:0 FAs. [15]

Glycerophospholipids (GPs) are derived from glycerolipids by substituting a phosphate-linked end-group to the sn3-carbon of glycerol. GPs have also passive roles as the lipid bilayer cell membrane components. The most abundant lipids in eukaryotic cells are phosphatidylcholines (PC), phosphatidylethanolamine (PE), and their derivatives. [4] Other GP sub-groups include phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerols (PG), and PG-derived cardiolipins (CL). [4]

GPs in FAs may include ester or ether groups, of which the latter group is defined as plasmalogens (PLs). They cover approximately one fifth of the glycerophospholipids in eukaryotic organisms. [4] PLs are divided into two categories: plasmanyls and plasmenyls. Plasmanyls are noted to have an oxygen bridge (e.g. PI[O-18:0/17:0]) or due to a phosphorus bridge in a case of a plasmenyl group with an ester bond conjugated to a double bond of the FA (e.g. PI[P-18:0, 17:0]). [4] GL and GP structures are depicted in *Figure 2* with a list of typical FAs in the mammalian lipidome.

Sphingolipids (SP), characteristic for eukaryotic cells, [15] have a sphingosine base as the backbone (*Figure 3*). One of these sub-classes are ceramides (Cer), which are lipidic sphingoid bases having nitrogen bonds. One of the SPS is sphingomyelin (SM), which is an interrelated [15] phospho-SP structure that combines ceramide with phosphatidylcholine (Cer-PC) or -ethanolamine (Cer-PE).

1.3 Lipid synthesis in organisms

Most lipids are either synthesized in the endoplasmatic reticulum, in Golgi apparatus, and in the mitochondria. [11] Glycerolipids (GL) are mono- (MG), di- (DG) or trisubstituted (TG) glycerols, which belong to common fatty acids having esterifies substituents. [18]

Lipids are primarily formed in FA synthesis with excess of acetyl coenzyme A in presence of NADPH [18] in a cell's cytoplasm. [19] In a model proposed by Nelson and Cox, [18] the synthesis

is a six-step process that involves most notably derivatives formed by acetyl coenzyme A and the acyl carrier protein (ACP). As for the reactants, an initial thioester carbon chain is modified to a malonyl body, which is further lengthened through a catalysed loop of acetyl group addition. The loop includes catalysed reduction steps of thioesters keto-groups (and the leftover double bonds) with NADPH and concludes with releasing of the synthesized FA conjugate base from ACP (either fully saturated or unsaturated). [20] According to Dennis, [18] the formed FAs are typically esterified further near hydroxyl groups in other biomolecules (e.g. glycerol), since the process stabilizes reactive FA. [18] The FA synthesis is biological basis for complicated lipids.

GP metabolism is considered to originate from an enzyme-assisted combination of a FA-metabolism based metabolite (acyl coenzyme A) combined with modified glycolysis products (Dihydroxyacetone phosphate and glycerol), which produces lyso-PA (LPA) further synthesized into PA. [21] As a sidenote, another pathway not going via LPA and PA synthesis is known for synthesizing plasmalogens (PL) [22] and the platelet activating factor (PAF) [23].

For SP synthesis, Cer produced in the ER is translocated and further processed in the Golgi apparatus to produce other SPs. [11] The often identified and quantified sphingosine (S), sphinganine (SA) and their phospholipid metabolites (S1P, SA1P), both of which are important in signalling and central processes alongside LPA and other lipid mediators [24], are considered to be highly potential biomarkers in disease and cancer research. [24]-[26] Pukolski-Gross et al. [26] and Haynes et al. [27] report S1P as the crossroads between reversible dephosphorylation and irreversible catabolism of SP-originated sphingosines into phosphoethanolamine and hexadecenal (in the case of SA1P, hexadecanal), catalysed by sphingosine phosphate lyase. [28] Other sources for more SP metabolism can be found from Kita et al. [24], Sahu et al. [29] and Merrill Jr. et al. [30]. More specifically, articles on Cer metabolism is available by Zheng et al. [31], t'Kindt et al. [32], Castro et al. [33] and Manni et al. [15].

1.4 Cellular functions of lipids

Intra- and intercellular lipid metabolism must be understood in its localized context to understand their function in more complex biological systems, e.g. tissue and biofluids. Lipidomic studies often discussed the matters of single cell metabolism and tissues separately, the only link between them being studies on cell signalling [84]. For appropriate tissue analysis, lipid cell function must be understood before drawing conclusions of in-depth lipidomic mechanisms. According to Han[16], more lipidomic pathway flux studies (i.e. reaction rate, metabolic hierarchy and interrelation determinations) are necessary for better understanding lipidomic processes in living organisms[16].

For example, the quality of lipids (e.g. lipid class, FA saturation and chain length) in a plasma membrane promote specific lamellar phase properties such as intermolecular order, translational diffusion and elasticity of a cell [11]. A change in these properties is strongly proposed to enable cellular processes like cell budding and tubulation [11] as well as cell fusion and fission. [11], [12], [16]

Van Meer et al.'s [11] extensive reviewed compiled lipidomic observations of cell organelles and membranes in mammals and yeast. Cytoplasmal and extracellular leaflets of double layer membranes have different lipid concentrations and permeabilities: where ER leaflets allow free lipid translocation along its double layer with non-specific lipid transporters, Golgi, plasma membranes and endosomes have restricted lipid mobility [11]. This leaflet asymmetry of specific lipids is proposed to promote special properties for the plasma membrane in question. For translocation of some "trapped" subclass species, lipids are modified enzymatically to enable leaflet flipping (e.g. cytoplasmic leaflet PC is converted to DG in the Golgi apparatus) [11].

Van Meer et al. [11] lists out mechanisms for various means of lipid transportation: lipids can diffuse either within an organelle via the double membrane-specific translocation between leaflets or moved between organelles via tubule, vesicles or nonspecific/specific transport proteins (e.g. the ATP-binding cartridge (ABC) exporters for GP/SP and CERT for Cer-specific transportation) [11]. Basically, eukaryotic membranes are most abundant in PCs (>50%) and PEs as well as PSs, PIs and

PAs [11]. For major component SPs, SMs are characteristic for mammalian membranes.[16] However, this trend in GPs and SPs is not the case in yeast or other microorganisms since their metabolic pathways differ from eukaryotic [11]. For topical reasons, mammalian metabolism is primarily discussed in this review.

Relative to other organelles, the plasma membrane has a large abundance of SPs, [11] most of which contribute to cell signalling. These lipid classes are either synthesized in the Golgi (SPs) [11]. In addition to its barrier function, the plasma membrane is responsible for cell signalling. [11] Most of the lipids are imported The endoplasmatic reticulum (ER) is the main cell organelle for the bulk synthesis of lipids, especially GPs and cholesterol. [11] Thus, ER is interconnected with many other organelles that synthesize specific lipids [11], [16], [29], [35]: for example the Golgi apparatus. [11] Mostly known as a lipid- and protein-sorting organelle, Van Meer et al. [11] reports the Golgi to be the biggest producer of SPs [11] which are synthesized from ER-produced intermediate Cer. [11] Mitochondria are known for the unique production of cardiolipins (CL), a lipid class proposedly taking part in oxidative phosphorylation. [11], [16] Furthermore, mitochondria are also able to produce LPA for TG, PA and PG synthesis. [11]

Lipid droplets (LDs) are monolayered organelles responsible of TG and cholesterol supply (bound with deployable FFA) [36] for lipid regulation [36] and synthesis as well as energy storage [35],[37],[36], [11]. In addition, LDs are proposed to store lipid signalling precursors and and hydrophobic vitamins. [37] Histologically, LDs consist of a TG and steryl/retinal ester core[36] and phospholipid (PC, PE, PI, LPC, and LPE) membrane. [36] Furthermore, PC availability and lack of its key regulatory enzyme, phosphocholine cytidylyltransferase-α (CTP, Kennedy pathway) [13], are considered integral regulators in LD-growth and -fusion. [35] Since some proteins (articles listed by Fujimoto and Parton) [35] are shared between ER and LDs, [11] not to mention the metabolic regulation between ER-synthesized PA and LD-stored TG usage for lipid synthesis, [37] the two cell organelles are often observed very close to each other. LDs are either positioned in

proximity, intercalated or membrane stalk-connected with the ER. [35] Similar proximity with LDs has been observed with other lipid-producing organelles [35], [36]: Welte [36] proposes nuclear LDs to affect lipid synthesis via the nucleus, suggesting that LDs regulate transcription factor, enzyme and chromatin availability [36]. Welte [36] also reports LD interaction with mitochondria and peroxisomes [16], [35], both of which are capable of β-oxidation and FA breakdown [36]. Other studied cell organelle lipidomes include endocytic pathways, namely early and late endosomes, [11] and lysosomes [38].

2 Interpretation of lipidomic profiles

2.1 Structural identification

Isobaric interference, i.e. co-isolation [39] by co-elution of species with identical or nearly the same molar mass, affects both identification and quantitation in LC-MS [40]. Thus, lipidomic structure profiling and species validation with LC-ESI-MS is mostly done with tandem mass spectrometry (MS^n) where more information on class- and species-specific fragmentation can be attained.

For instance, Ivanova et al. [41] reported the characteristic fragmentation of three major lipid classes in positive (*Figure 4*) and six major lipid classes in negative ESI mode [41]. In positive ESI mode, PC, PS and PE species experience characteristic neutral loss of headgroups as well as fragmentation into lysophosphatidic species. [41] Since negative ESI mode fragmentation seems more complex -maybe more of it is known compared to positive mode- we suggest to read further into GP fragmentation literature by Pi et al. [42] and Ivanova et al. [41] and SP fragmentation by Sullard et al. [43] and Merrill Jr et al. [153] (SP fragmentation depicted in *Figure 5*).

As a charged particle is moving against an inert gas with known velocity, its movement can be rotationally averaged. Ion mobility spectrometry (IMS) observes this by recording the drift time of an ion species in an electric field. [44] Hence the theoretical CCS value of the analytes can be measured via their drift times with no calibration standards necessarily needed. [44] Some QTOF-instruments are equipped with an IMS module, [45]-[48] enabling further identification tactics with

another molecular property dimension. However, identifying CCS values of molecules in ion mobility-mass spectrometry mode (IM-MS mode) decreases the amount of ions ending up at the TOF, which in turn decreases sensitivity in the analyzer. IM-MS may provide valuable information that is otherwise difficult to obtain, since that ion mobility is affected by head group geometry (which is impacted by phosphorylation and glycosylation). [49]

2.2 Profiling of homeostatic systems

Determining an organism's lipidome in homeostasis may both act as a fingerprint to the species in question, a distribution of traits in a population as well as a baseline or reference for possible changes of the organism's state. All articles concerning such research were listed in *Table 1*. As an example, Meulebroek et al. [50] developed a method which may cover all eight lipid classes via faecal matter, using a polarity-switching UHPLC-interfaced Orbitrap. With this, healthy controls and type 2 diabetes patients were monitored and their lipidomic profiles compared with each other. [50] Instead of interpreting as many lipid classes as possible, Manni et al. [15] focused selectively on multiple tissue's and cell culture's 12 Cer:s [51] and sphingomyelins (SMs) in positive mode. Tissue types in this targeted approach included adipose tissue (human, rat), liver (dog, human, rat, mouse), brain (rat, mouse) and serum (human, rat, mouse). [15] For more exotic lipid classes, Drotleff et al. [52] identified and quantified ST hormones from plasma, and, similarly, Gobo et al. [53] cerebral prostaglandins from brain tissue.

Mostly, profiling of human-based biological materials use *in vitro* cell culture samples and *in vivo* clinical samples. According to literature, solid and liquid biomatrices, as well as synthesized lipids [54], such as nanoscale liposomes have been studied. The *in vitro* samples included conditioned cell culture medium (CM, a suspension acquired from cells), and *in vivo* clinical samples target to e.g. to human amniotic fluid and plasma.

Ghosh and Nisala [5] showed that human tears include at least 600 identified lipids. [5] Thus, for identification and characterization of tears the samples need to be dilute and less diverse than the

aqueous bioliquids from a multi-cells and complex organisms. In their project, CMs of cell cultures (e.g. from cancer cell lines) were isolated and characterized to evaluate single lipid components in the context of a multivariate tissue. [5] Furthermore, Ghosh and Nishala showed that plasma is a complicated matrix with a large group of GPs, SLs, CEs and TGs. This is understandable, since the bloodstream of an organism is linked to the most of its individual cells, able to transport nutrition, lipids, hormones, cell metabolites, and e.g. dead blood cells. However, Hyötyläinen and Orešič [55] reported that they found a more stable lipid profile in serum than plasma. Hence, a potential alternative for a more reliable analysis could be developed. For plasma, their review suggests differences within sample types, such as lower lipid content in citric acid containing plasma compared to EDTA modified plasma, which might influence the stability of lipids. [55]

2.3 Profiling of metabolic alterations

Metabolic profiling of lipids included a discrete comparison of two different diets or other similar analyses between controlled base and altered states of an organism's lipidome (*Table 2*). In profiling of homeostasis the profiling of metabolic lipids utilizes the main analysis (UHPC-MS/MS), investigates the lipid compounds, and studies the dependence and correlation between lipid species and their alterations. Equilibria between lipids in a lipidome may give insight to an organism's metabolic response to its environment. As an example, Zalloua et al. [62] noted a correlation between serum sphingomyelins (SMs) and plasma cholesterol (i.e. LDL, HDL and total cholesterol). [62] metabolite features (m/z 750-810, associated with SMs) expressed a strong correlation to cholesterols.

2.4 Profiling of diseases

When triggers concerning a specific lipidomic pathway achieve an extended time to stress the organism, an allostatic load will accumulate (early phase of a clinical condition) until the organism either recovers from the load or fails in the process. Upon failure, a breakdown of the allostatic

adaptation can be observed as an over- or decompensation of the metabolic pathway biomarkers; a time/concentration threshold essential for clinical diagnostics [78]. Biomarkers studied between 2017-2019 and in the fashion of this disease model were listed in *Tables 3-4.*

The field of lipidomics has been widely studied for both pathological diagnostics and identification of biomarkers [4]. Mass spectrometry in lipidomics enables discovery, identification, and quantification of lipids in mycobacteria and applications to unravel novel functions of the metabolites. The mycobacteria [81] genus is a group of acidic species characterized by a lipid-rich waxy cell wall, which is much thicker than that found in most other bacteria. Mycobacteria have a high lipid content and complex lipid profile including several unique classes of lipid. Notable members of the genus include *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium ulcerans*, causative agents of tuberculosis (TB), leprosy and Buruli ulcers, respectively. In recent study, MS was used to detect mycolic acids in archeological bone samples. Fourteen mycolic acids were detected in a skeleton from the Neolithic period with bone lesions characteristic of skeletal TB. In this case, identification of lipids unique to *M. tuberculosis* offered extra evidence of TB infection to complement diagnoses based on anatomical features and analysis of bacterial DNA. [79] According to Daffe and Draper the amounts lipids in the cell of mycobacteria make up 30–60% of the dry weight. They have important biological roles, notably in determining virulence and drug resistance. [82] Lipids have importance in mycobacterial infections and they have renaissance in these bacteria.

For ovarian cancer, Perrotti et al.'s [80] review on multiple large studies (n>40) strongly suggests an increase of LPLs (specifically LPAs, which have been proposed as early-detection plasma biomarkers) and a change in the FA profile. One explanation for LPL increase was suggested [80]. In addition, Zhang et al. [83] found potential biomarkers in the groups of TGs (decreased), PCs and LPCs (increased). [83] The increased LPC levels caused by "deregulation of phospholipase A2" mentioned by Perrotti et al. [80] Qadir and Malik also reported decreases in TG levels as well as

HDL-cholesterol, cholesterol and LDL-cholesterol of ovarial cancer patients [84]. Furthermore, Perrotti et al. [80] agreed the research of Zhao et al. [86] that GP LPL-profiling can diagnose ovarial cancer in patients [80].

Hu et al. [85] also listed lipidomic studies concerning epidemic disease, cancer, inflammation, dysfunction, genetic disease and drug treatment. Lipidomic data on diseases can also be compared with supporting results of other analyses, as is done for instance with so-called Mendelian Randomization (MR) [87], [88], a statistical-epidemiological method where an risk factor's (biomarker, exposure) causality to an effect (disease) is evaluated. Since genetic influences are not affected by confounding factors (i.e. life choices, environment), a large population of patients can be grouped according to their biomarker-associated genetic variants (i.e. variation in genetic code) and compared with the frequency of a disease in that population. [88] For solid evidence of causality, all the genetic variants must be associated with the biomarker that is studied. [87]

3 Sampling and storage procedures

Sample preparation needs specific care, since decomposition of chemically stable lipids is considered mainly in the biological way [30]. Directly after isolation from organisms, self-degradation of lipid compounds initiates via enzyme activation at room temperature. To avoid this, proteins need to be precipitated before analysis which is usually done with methanol or isopropanol [30]. Alternatively, snap freezing of samples is used for storing the authentic samples [114]. Protein precipitation is utilized especially for liquid matrices for improved sensitivity in detection [115].

Double bonds of unsaturated fatty acids are susceptible to peroxidation, hydrolytic degradation, and oxidation. Especially, after isolation and extraction, antioxidative properties of biological compounds may be reduced or lost during storing. Major factors contributing to degradation are formation of ice crystals combined with osmotic shock when freezing samples, which decreases the volume of aqueous solution, breaks structures (e.g. cell membranes) between lipids and enzymes which consequently hastens lipid breakdown. [114]

Jurowski et al. [114] reported drastic changes in lipid composition of plasma, when the sample was stored for 1-3 years at -20 ˚C. Furthermore, Jurowski et al. [114] observed an increase in concentrations of saturated FAs and decrease of unsaturated polyFAs. The reason for that were enzymes and activity of antioxidants. [114] Furthermore, Hyötyläinen and Orešič [55] informed that a drop in lyso-phosphatidylcholine (LPC) concentrations is detected in less than two hours (storage temperature 4 ˚C). In total, LPC concentrations increased within 24 hours, potentially due to activity of the phospholipid-splicing phospholipase A2. On the other hand, most PCs and sphingolipids seemed to stay stable for this time even at room temperature. [55] Thus, erythrocyte FAs in plasma are already degraded within one week at storage temperatures between 4 ˚C and -20 ˚C. Jurowski et al. [114] informed that samples stored at -60 ˚C do not change in one year of storaging. [114] Hyötyläinen and Orešič [55] agreed the stability of lipid containing samples stored at -80 ˚C for at least six months, but a change was seen at a time span of five years. [55] Sampling, sample storage and pre-analysis lipid degradation has been discussed in greater detail by Hyötyläinen and Orešič [55], Patterson et al. [115], Tumanov et al. [116], Jurowski et al. [114] and Monnin et al. [30].

4 Sample preparation for lipid analyses

The main limitations of lipidomics analysis are the chemical complexity of the lipids, the range of concentrations at which they exist, and the variety of samples usually analyzed. [117] Especially, sample preparation is needed to prevent lipids at high concentration to suppress ionization of low concentrated lipids in mass spectrometry detection.

Here, only few examples are discussed to show the complexity of the matrices, when HPLC-MS methods are used for information collection of lipids. Altered lipid metabolism plays a key role in the pathogenesis of common diseases. Most of the bioactive mediators originate from the cleavage of lipid constituents of cellular membranes under the activity of phospholipases. In amniotic fluid research, lipids identified were used as indicators for stages of pregnancy or pregnancy

complications. [5] Total lipid and phospholipid concentrations were observed to increase from 24 weeks of gestation until labour. Thus, routine tests for fatal development evaluation of lecithin/sphingomyelin-ratios could be used for long-term monitoring. Furthermore, lipidome profiles of amniotic fluids were observed to be different between infants born in term and those of pre-term children. [5]

Primary ascites sample matrices have discovered to have abnormal formation of intra-peritoneal fluid in a patient's abdominal cavity. [118] Two types of lipid samples could be used according to formation: non-inflammation induced (e.g. hydrostats- or osmosis-produced) transudates matrix and inflammation-accompanied substances secreted by an organism. Transudates are suspect of de-compensation of blood circulation, kidneys, and liver, whereas exudates are formed in oozing of ruptured or otherwise damaged cell tissues formed in complications of cysts in ovarian cancer tumors. In contrast to commonly clear transudates, in intraperitoneal space exudates are noticed to have higher protein-concentrations due to the less filtrated nature. [118]

In the Folch-method [119] about 5% of proteins are left in the organic phase, when lipids are isolated. That may contribute to matrix effects and higher background noise. [115] In comparison to the Folch method, BUME method [120] is an extraction technique considered superior from the environmental, economic and preparative standpoints, since extraction needs no chloroform and needs less solvent. The method allows to extract all matrices into millilitre volumes with comparatively lowered safety hazards. [121]-[124]

For samples with low lipid concentrations, solid phase extraction (SPE) [58], [125] or lyophilization (i.e. freeze-drying) [125] can be considered to concentrate lipids in a sample for the analysis. Teo et al. [125] introduced a concept of energy-based extraction methods (i.e. pressurized liquid, microwave- and ultrasonic-assisted extraction) alongside polarity-based extraction methods (i.e. single solvent, liquid-liquid, solid phase extraction combined with supercritical fluid extraction which was developed by Jurovski et al. [114] The latter techniques are used more frequently in

lipidomics studies. [125] Energy-based techniques warm the extraction system to achieve a faster and more efficient removal of lipids from the matrices. [125] *Table 5* lists extraction and sample purification methods used in lipidomics.

Bang et al. [127] conducted solid-phase extraction (SPE) in a superabsorbent polymer (SAP) device for small sample sizes to minimize carry-over of aqueous solvent. In the procedure water was gelated with the SAP polymer, and organic solvent was adsorbed and collected lipids with recoveries similar or greater than in the Folch extraction [127]. Other extraction methods [58], [114], [119]-[125] as well as single solvent and liquid-liquid extractions with acetonitrile or chloroform-methanol 2:1 (v/v) [51], [128] or with methyl-tert-butylether (MTBE, i.e. Matyash) extraction [55], QuEChERS methodology (Quick, Easy, Cheap, Effective, Rugged, and Safe) [127], and the "Bligh and Dyer" method with chloroform-methanol [129]. The use of a chloroform-methanol solution for lipids extraction was observed to isolate all major lipid classes, which were enriched in the medium polar chloroform phase. [126] The use of MTBE as the solvent led to higher extraction efficiency for unsaturated fatty acids, GPs and Cer, while chloroform-methanol mixture favoured the isolation of saturated fatty acids. [117] However, the recommended sample preparation for isolation of polar lipids in adipose tissue should be combination of liquid–liquid extraction and an SPE to enhance detection of GPs in MS.

5 Liquid chromatography in lipidomics

Some analytical techniques are specifically better used to identify lipid classes and sample types than individual lipids, why prior knowledge about targeted analysis is to choose the suitable lipid method. According to Hu et al. [85], NMR techniques can identify and quantify (isolated) lipid fractions as well as provide lipid-associated disease profiles. However, NMR can easily falter in the face of complex sample matrices and their analysis. [85] Apart from desorption and probe MS techniques, imaging techniques lack selectivity for lipid species analysis (though they do have the advantage of nonspecific lipid localization). [132]

5.1 High-performance liquid chromatography of lipids

High-performance liquid chromatography (HPLC) is a method using a liquid mobile phase and solid stationary phase. Analytes are separated depending on the strength of their interaction between stationary phase and mobile phase. Reversed phase liquid chromatography (RP-LC) dominates the chromatography in lipid research and, for that reason, mostly nonpolar and medium-polar lipids are studied. In RP-LC most of the lipid species and internal standards elute at different times, thus experience different matrix effects and different solvent composition, which influences their ionization and may result in inaccurate quantification. [133] However, recent studies with normal phase (NP) stationary packings and with the newest technology in hydrophilic interaction chromatography (HILIC) provides lipid class-specific separation. This has great advantages in terms of quantification, since usually analytes and internal standards show similar retentions. [134] The ultimate advantage is that identification of lipid species with lipid classes is straightforward. Especially, HILIC separation has gained active development recently, since the method separated lipids by distribution, adsorption, ion exchange, and exclusion chromatography. [135] The literature about lipids research with HILIC have slightly increased during the recent years.

Since there is no separation based on the chain lengths of FAs in normal phase chromatography separation, which is the phenomenon in reverse procedures in RP-LC, the precursors affect the retention in the NP column and identification of their MS fragmentation patterns. Full scan MS spectra are usually collected either in negative ionization or in positive ionization mode. Data dependent tandem-MS (MS^2) was performed in parallel with ESI-Orbitrap-MS scanning on a Fusion mass spectrometer, which is accurate enough for lipid class confirmation. [141] However, identification of chain lengths and saturation states of lipids need high resolution MS instrumentation, when ion resolution is expected further to improve separation efficiency obtained in LC. That was the reason in the study by Jeucken et al. [70], who used a HILIC procedure to observe retention of inorganic ions in lipid matrices. Inorganic ions lead to formation of distorted

lipid adducts in identification with MS. In addition, inorganic salts are shown to decrease repeatability of quantitation, but they are known to co-elute with lipids. [136]

Typically, additives are used, since they are dissolved into water or into water-organic solvent mixtures. They allow stabilization of adduct ions concentrations. Additives may form complexes with neutral species due to adduct formation during MS ionization. In lipid analyses, additives have shown to quench and suppress counter-ion species. Furthermore, they show to control the formation of predictable adduct ions, when weaker ligands are present or concentrations of the main ligands are negligible. Moreover, as Erngren et al. [136] have demonstrated with positive mode ionization, inorganic ions improve separation in LC, since the ions are retained onto a normal phase separation column material (such as in HILIC), and they are not co-eluted nor form complex adducts or clusters, thus resulting in decreased repeatability in quantification. [136] This was also the reason for using additives in post-column derivatization by Monnin et al. [30] They compared their LC method performance by using acetic acid and ammonium hydroxide as the additives in the eluent, when detection was done with negative ionization MS. Generally, the study showed that additive ions could form pseudo-neutral adducts with charged lipids by utilization of interaction with the stationary phase via adduct-driven polarity manipulation.

5.2 UHPLC systems in lipid analyses

Ultra-high-performance liquid chromatography (UHPLC) was introduced to use fast chromatography with nano-columns [131] Fekete et al. [138] showed that good separation efficiency of lipids at abnormal slow eluent flow through less than 2 µm sized particles was possible. The success of the discovery was followed by a near exponential growth of UHPLC and UHPLC-MS publications in the following ten years. Based on Danne-Rasche et al. [140] a Venn diagram is a useful tool to visualize similarities between data from normal and ultra-high LC systems. They discovered that UHPLC and capillary-packed nano-LC (contrary to conventional LC) is necessary for the detection of over half of yeast's lipidome.

Jeucken et al. [70] used UHPLC to separate HeLa-cell lipid metabolites. In their one-step protocol, proteins and other macromolecules were removed and the protein-free chloroform-methanol mixture was injected directly to LC-MS. The extracted lipids were loaded onto a HILIC column [141] During a run time of only 4 min, they achieved separations based on end-groups of lipids (*Figure 6*). In the study sample preparation of HeLa cells [70] was done on a 96-well plate, on which the caves were filled with the cells and cultivated in the presence and absence of lipid metabolic inhibitors. The studies were done to observe changes in lipidome expression, when the process was altered at the metabolic state.

5 Mass spectrometry in lipidomics

The first reports of mass spectrometric (MS) analyses of complex lipid mixtures via soft ionization techniques (matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI)) were developed in the 1990s. [142] In particular, the use of atmospheric pressure ionization (especially electrospray ionization, ESI) and the coupling of liquid chromatography (LC) to MS have allowed the identification and quantitation of ever-increasing numbers of lipid species. Han and Gross [142] showed with ESI-MS that they could make structural determination and even quantitative analyses of individual phospholipid molecular species at sub-picomole level from chloroform extracts of biological samples. In lipidomics mass spectrometry is the only reliable tool for structural identification. Nevertheless, lipidomics is challenging with the many compounds, which cannot yet be expressed as fully as the compounds in genomics and proteomics, which are well represented in various forms at leading research institutions worldwide. [49]

6.1 Mass spectrometric analyses of lipids

Lipid analyses from simple profiling to complex metabolite interaction mapping is primarily made with various kinds of ionization techniques in mass spectrometry. Holcapek et al. [143] divided mass spectrometry into three major approaches in lipidomics: direct infusion MS (DIMS, i.e.

shotgun lipidomics, flow injection MS), chromatographic methods on-line coupled with MS, and desorption techniques in MS. [143], [144]

DIMS was recommended to be used as a fast-diagnostic method. [186] The direct sample infusion method to MS analyses uses one sample per one run at the time. Thus, it is a very appealing method with multiple precursors and fragments. With DIMS, very high ion resolution and resolving power are needed. Because of many fragment ions continuously present lipid samples are diluted before injection to MS to avoid detector saturation. Furthermore, knowledge on spectral interference is good to be available for quantitation data and considering identification of similar compounds, like identical isomer fragments. [144] Furthermore, matrix effects may play a large role in analyses. [146] Anyhow, desorption techniques are ideal mass-spectrometric imaging of tissues and cells. [143] When locations of the origin of lipids are not possible in conventional LC-MS, desorption DIMS offers a fast solution. For this localization of lipids, multiple approaches have been attempted such as mass spectrometric imaging (MSI) [111], [143], [144], [147] liquid extraction surface analysis (LESA) [140], [148] and probe research [147]-[149]

Solvents are very important in MS analyses of lipids (Monnin et al. [30]). Ammonium hydroxide – acetic acid composition were studied in aqueous solution to optimize degree of ionization of lipids in negative ESI-MS. The tests were made for lipids in the classes PA, PE, PC, LPC, LSM, Cer, LPA, and PS. [30] Usually, in positive ESI-MS water-methanol and water–acetonitrile solutions are used with modification of formic acid or acetic acid. [150] Lorenzen et al. used water, acetonitrile, and isopropanol as the eluents in UHPLC-MS/MS studies and identification with both negative and positive electrospray ionization modes. The eluents (pH 5.5) contained also ammonium acetate and formic acid as a buffering additives in order to maintain the lipids in the same ionic state during the separation process. [151] To gain all metabolites of a subclass, Griffiths and Wang [188] single-extract STs with ethanol to acquire oxysterols esterified with sulphuric acid and linked sugars. [188]

Table 6 lists examples of lipid classes and molecular species formed during electrospray ionization in negative ESI-MS mode. The fragments used for lipid class identification were $[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M–H_2O+H]^+$ in the positive mode and $[M–H]^–$, $[M + HCOO]^–$, and $[M + CH_3COO]^–$ in the negative mode. [152] Cajka et al. [152] noticed in their project that PEs were the only detectable glycerophospholipid species, which were distinguished by the $[M-141+H]^+$ MS^2 fragment resulting from the neutral loss of the polar end-group. Isolation of lipids from cells was made according to Bligh and Dyer [129] and with reverse-phase liquid chromatography for sample introduction to MS.

6 Method optimization protocols

For understanding a metabolic-lipidomic system, the lipidome or the relevant lipid group should be mapped out. Merrill Jr. et al. [153] designed a workflow for sphingolipid (SP) analyses of a lyophilized cell culture by using multiple LC-MS/MS protocols for specific subclasses. [153] However, performing comprehensive analyses of lipid polarity ranges is still not sought for until today.

To help method optimization, there are four protocols that inform distinct target groups in analyses. They deal with (1) the extraction procedure for the preparation of sphingolipids for analysis by LC MS/MS, (2) reverse-phase LC, (3) normal-phase LC, and (4) the order of steps to follow in setting up an LC–MS/MS for a new biological sample. Furthermore, they mentioned the relation between sphongolipids (SPs) and their metabolites, which remind the biochemical equilibrium present in a biological system.

6.1 Lipid classes studied with liquid chromatography

Teo et al. [125] studied various biofluids and tissues that were analyzed with different chromatographic and ionization methods with mass spectrometry. LC-MS is necessary, when comprehensive screening of biomarkers is required (e.g. in cancer research[137]). Primarily, GLs, GPs, SPs, and STs in liquid-type biological materials (e.g. blood and secretions) and solid tissues

548 (e.g. eyeball, fibroblasts and skin) were studied with LC-MS. [125] Later, Jurovski et al. [114]
549 showed that FAs and CEs are also important lipids to study clinical samples, when there is a need to
550 identify the lipid profiles.
551 Since variation of lipid classes is enormous, it is not surprising that the polarity of lipid species
552 varies a lot. Figure 7 demonstrates the lipid-subclass ranges of four distinct chromatographic
553 approaches, namely lipids separated in reverse phase and normal phase LC separation strategy. The
554 subclasses include to analyse separately both nonpolar and polar lipids with liquid chromatography
555 separation. Based on the structural differences, the lyso-forms are more polar than their
556 counterparts, which have an extra fatty acid chain in the structure. Particularly, polar and mid-polar
557 (PS, PG, PI, Cer) lipids seem to be species that are often identified with negative mode in MS,
558 though more species are primarily found with positive mode identification. As to the GLs groups,
559 only MG has a fatty acid chain that is small enough to be rather polar. As a thumb rule, SPs are
560 mid-polar lipids, since they are amines and have nitrogen-bound fatty acid chains.

561 **7 Lipids important in clinical and medical samples**

562 Lipids have important roles in the profiling of cell types (e.g. cancer cell lines or bacteria [114]) as
563 well as directly from various biological fluids and biotargets (e.g. plasma [45], [112] and human
564 amniotic membrane [154]). When cells are the examined matrices, they are usually harvested by
565 ultracentrifugation, washed with water, resuspended, incubated, extraction phase isolated with
566 centrifugation, re-extracted, filtered, dried under nitrogen, and dissolved in methanol-
567 dichloromethane-water mixture (35:70:5, v/v/v) [151], though sample preparation methods vary.
568 *Tables 7, 8, and 9* list samples analyzed between 2017 - 2019 in lipidomic UHPLC/MS research.
569 Cell lipids are important biomolecules for diseases due to metabolism.
570 The range of unconventional biological extracts and tissues, as described by Jurovski et al. [114], is
571 very broad. Furthermore, *in vitro* biological cell culture analyses reached a high variation of studies

among themselves, which lead to include them separately in *Table 9***.** The multi-matrix approach was a pool of plasma, urine and faecal matter of the same patient. [155]

Tables 10 and 11 list all lipids in the articles where identification of various lipid groups was discussed. Just like fatty acyls (FAcyls, including FAs, BMPs and CLs) were reported for physiological profiling [50], [53], [58], [107], lipido-metabolomic [67], [69], [70], and disease biomarker studies [5], [9], [95], [98]-[100], [102], CEs were frequently found in method development [46], [108], [130], [155], [189] and cancer research. [46], [108], [110], [113] Also, sub-groups, the totality of identified lipids, and biomarker lipids were included. In total, a range of 5-700 total lipids and 5-87 proposed biomarkers were discussed in the articles. Typical GLs in articles included DGs and TGs, with MGs or BMP. [69]

Profiling lipid metabolites of hepatocellular carcinoma revealed down-regulation of TGs having less than two double-bonds in their structures, which was also observed in ceramides. Conversely, up-regulation was noticed for only GPs and SM. Particularly, new information on TG, PC, PE, and PI trends was uncovered. [111] Lipid profiles differ a lot from sample to sample, though some consistencies remain. During negative mode ESI-ionization, Tumanov et al. [116] claimed to have observed a bias in LPA quantitation. Noteworthy is that the concentration of LPAs may be easily overestimated due to typical over-abundance of LPCs combined with the choline moiety which is lost during ionization process in MS. [116]

9 Metabolite identification with MS and data analysis

The experiments with both positive and negative ionization modes in MS are useful in lipid analyses, since the data can be combined in order to identify the maximum possible number of lipids. [151] Usually, lipid standards are needed in order to evaluate the chromatographic profiles and fragmentation behavior of lipid species. Comprehensive characterization and quantification of molecular lipid species was shown to be achieved by spiking the lipid extracts with unique lipid

596 standards and using selective ionization conditions for sample infusion to have structure-specific
597 mass analyses by mass spectrometry.
598 Liebisch et al. [39] proposes two ways for solving the lack in identification: Either mass accuracy
599 must be increased to separate two nearly identical species or fragmentation of MS^n must be
600 developed. Thus, fragmentation behaviour of PI-Cers (GIPC-Cer) have been described previously
601 and structures were characterized to have a polar head group, fatty acid, and sphingoid base [151],
602 [162], [163]. Ceramide molecules form distinct domains in the cell membrane, which may serve to
603 re-organize cellular receptors and signalling molecules [164]. ESI-MS data for identification and
604 characterization of the structure of PGs, PEs, CLs, and glycerolipids (DGs and TGs) are also
605 available. [164]-[168] The presence of PGs, PEs, and C_{26}-dihydro Ceramide (Cers), as well as the
606 presence of distinctive α-hydroxylated FAs together from complete FA profiles of strains have also
607 been published. [169]-[172]
608 When lipids were studied with UPLC-ESI-MS with eluents containing ammonium acetate and
609 formic acid, PGs, PEs, and CLs were detected as [M-H]− ions, and Cers being both hydroxylated
610 and no hydroxylated as [M+HCOO]− ions in negative ion mode. In positive ionization mode PEs,
611 Cers, MGs, DGs, and TGs could also be detected, but the former two were detected as [M+H]+ ions
612 and the latter two as [M+NH4]+ ions. [151], [173]
613 Research on routine *in vivo* analyses of samples included six categories of matrices, primarily blood
614 (plasma, serum, erythrocytes and blood platelets), faecal matter, and urine. One of the revised
615 papers even proposed a multimatrix analysis by pooling plasma, faecal matter, and urine in one
616 sample to increase method repeatability and lipid coverage. [156] Unconventional biological
617 materials, i.e. harder-to-get as *in vivo* samples of 18 different biofluid and tissue types, of which the
618 most frequent topic of interest was liver tissue. [15], [63], [111] [127], [155], [160] In addition,
619 experiments with 18 different cell lines were found to be cultured *in vitro*.

Moreover, a new way for improved identification and quantitation in lipidomic studies was found: The utilization of the lipidome isotope labelling of yeast (LILY) standard to produce (non-radioactive) isotopically labelled eukaryotic lipid standards in yeast for normalization and quantification in mass spectrometry. [174] When looking at analytes of low concentrations, higher mass accuracy is often preferred to exchange lower resolution and to increase sensitivity.

A slight trend of multiple studies on big lipid structures (e.g. cell cultures, exosomes, [98], [110] lipid droplets, [60] and liposomes [159]) can be observed. Partially, this can be explained with new trending asymmetric flow field flow fractionation (AF4) [96], [97], [110] instruments, which has ability to separate precisely biomolecules by mass into smaller fractions before primary analysis. In UHPLC-MS lipidomics, the use of a pooled quality control (QC) sample in the analysis sequence has been standardized, which mean that according to the literature search in the year 2017 11 papers from 25, in 2018 14 papers from 22, and in 2019 8 papers from 10 showed the trend of increased pooled sample usage (even in qualitative analyses). [150]

10 Untargeted vs targeted approaches

It is currently not possible to comprehensively measure the lipidome of a cell or tissue in a single experiment. Either the precise alteration in lipids to expect is not known in any given case. Thus, the first studies are often exploratory, which means that they are done using untargeted profile monitoring. Thus, the MS methods need high mass accuracy and resolution. Analyses of fragmentation pathways has led to a detailed understanding of the connections between different building blocks found in lipids (such as fatty acids, sphingoid bases, and head groups**.** Precursor lipids are determined based on characteristic fragment ions, as seen in *Figure 8*. Other targeted approaches based on tandem mass spectrometry are now available for analysis of many different classes of lipids and in complex mixtures. [175], [176]

Lipids have some unique advantages and challenges for mass spectrometric analyses. The application of electrospray ionization to crude lipid extracts without prior fractionation is one such

example, as it has perhaps been more successfully applied in lipidomics than in any other discipline. The diverse molecular structure of lipids means that collision-induced dissociation alone may be limited in providing unique descriptions of complex lipid structures. [176], [177]

A conventional targeted approach needs a lot of calibration standards in lipidomics. [50] It is not feasible to calibrate all lipids individually. Thus, a simpler compromise on class-representative lipid standards with optimally chemical similarities are often applied in lipidomic studies. Ideally, however, standards and analytes should be chemically equivalent.

Though expensive, the most used internal standards are deuterated standards, most often by Avanti Polar Lipids (Alabaster, AL, USA). Typically, the protons are deuterated at the FA end of the carbon chain with either 7 or 9 deuterium atoms. For representative standards in analysis, a self-made or commercial standard mixture is typically used (e.g. a SPLASH mix [130] or well-known organic standard [porcine brain, chicken egg, e. coli] [161]). The molecular ion and its fragments that still include the deuterated FA chain experience a noticeable shift in their m/z value compared to their non-deuterated counterparts. Since fragmentation is identical for both variants, this m/z differentiation is ideal for ISTD normalization via EICs. [174]

11 Current statistical methods used in mass spectrometry

Targeted and untargeted methods are two distinctively different approaches, as well as the means of data processing. Targeted data processing of metabolites was divided to five phases: (1) the acquisition of raw data, (2) the contemplation of which database should be used when considering the research question and analytes, (3) the pre-processing and identification of these metabolites, (4) the normalization and quantification of the identified species, and (5) the reflection of the results on the biochemical/physiological context of metabolic pathways [179]. In contrast, untargeted approaches need more careful experimental MS data and more data modifications, but also pre-processing for detection. In addition, resolution in screening and identification of the relevant

biomarkers from the totality of features/peaks need to be considered, although the non-targeted steps include the same pathway elucidation as in the targeted analyses [179].

In lipidomic research, statistical methods have enlarged into a broad variety of numerical tests and visualization techniques. Typically, statistical methods include a null hypothesis to test similarities by using one dataset. However, the p-value parameter in the Student's t-test, [56], [57], [64], [65], [67]-[69], [72], [73], [75], [76], [92], [97], [98], [94], [102], [104], [105], [127], [156], [159] the non-parametric Mann-Whitney U-test, [56], [74], [76], [93], [99], [103], [104]-[106], and the analysis of variance test (ANOVA) [63]-[65], [98], [107], [109], [113], [156], [157] are for comparison of two or more datasets. These methods are often used for validation of analytical data and for detection of changes between controls and authentic samples. Correction methods such as the Benjamini-Hochberg test [180] are used to calculate the false discovery rate (FDR), i.e. minimizing false positive data from the dataset. [74], [75], [76], [105], [106], [181] An unequal variance test like the Storey [74], [182] or Welch [87] t-test may also be used. [74], [87]

The Mann-Whitney U-test [183] is used in mathematical data handling, when all parameters are variables to evaluate similarities of two independent dataset medians with a null hypothesis. For two sets with same or different sample sizes, the values of both datasets are sorted from the smallest to the largest ones. A Mann-Whitney U-test can be fitted for non-Gaussian distribution data. It is typically combined with a Benjamini-Hochberg (or Bonferroni-Holm [69]) test to exclude false positive values, thus giving the false discovery rate (FDR). These methods were applied in multiple studies to limit uncertainty in the results of lipids. Furthermore, Paepe et al. [156] and Gong et al.[105] used cross-validated ANOVA test (CV-ANOVA) to improve reliability of the identified analytes.

Lately, Tietz-Bogert et al. [93] calculated the FDR value in a lipido-metabolomic study by searching significant biomarkers of primary sclerosing cholangitis, [93] which is a disorder of lipids in the bile duct of unknown origin. The concentrations of lipid species in control samples of healthy

individual's and the clinical samples of sick patient's blood and bile were compared to find changes with a statistical significance of 0.01. [93]

Machine learning and software advancements for multi-ionic identification have allowed to evaluate MS spectra produced with UHPLC-MS *in silico*, which is only demonstrated in a few recent articles. [47], [57], [90], [113], [161] Due to machine learning techniques and their discovered use in omics, automated lipidomic analyses, ROC/AUC cross-validation analyses, [90], [105], [181] random forest studies, neural network applications, [184] *in silico* spectra evaluation [161], and CCS value [47] generation algorithms have become in use. More detailed information is available in ref. [132].

LipidBlast methodology has been created for generating MS/MS spectra (Figures 9 and 10). Lipid analysis of polar lipids can be performed with tandem mass spectrometry and mass spectral library search. LipidBlast was mostly developed with ion trap tandem mass spectra, but it can be used with data from other platforms such as QTOF mass spectrometers.

The LipidBlast library works with low-resolution and high-resolution instruments. [185] An *m*/*z* precursor-ion filter first filtered the data, and a subsequent product-ion match generated a library hit score that reflects the level of confidence for compound annotation. [185] It must be noted that a "closest-match" bias can be apparent when identifying an analyte with a library search. [187], [188] As the library may be incomplete, possible isomers must be considered when validating data.

12 Discussion

Lipid function on the tissue-level is rarely explained via cell-level mechanisms, which are the only frequent mechanisms mentioned being lipid signaling. In speculations, lipids may be distributed between tissue cells via vesicles or other means of expulsion depending on the tissue. Do the well-established tissue types (epithelium, muscle, connective/protective and nervous tissue) have inter- or intra-class uniformity or variations in lipid function, which could be supported by cell-level observations?

Due to the structural complexity of lipids, their quantification is not easy to perform. Different strategies and techniques should be addressed depending on the targeted analytes. The difference of ionization efficiency between lipid species, depending on their structure, is indeed a real limitation of this technique.

An increasing number of studies report about poor quality of lipidomics data with misidentification and inaccurate or inappropriate quantification of lipid molecules. These studies primarily use standardless [145] and untargeted metabolomics approaches. [170] To all intents and purposes, the reasons for the poor data quality seem to be analytical, bioinformatics, and educational aspects. Therefore, it is necessary to implement reporting standards for lipidomics data to share with the scientific community [39], [134]. These standards need to cover both shotgun and HPLC–MS approaches. Only applying both approaches in a complementary and confirmatory way permits a comprehensive and accurate coverage of the lipidome, as earlier was documented by G. Liebisch [134].

Plasma membrane asymmetry of two phospholipid leaflets could have implications for membrane potential as well as phase properties (e.g. signalling, diffusion and biochemical activity). Possible mechanisms, such as change in lipid composition or shift in leaflet asymmetry by local synthesis or lipid transportation could be an interesting subject for lipidomic research. Evidently, in real time this would need improvements in the selectivity of in situ lipid imaging techniques, which may possibly be desorption MS [111], [143], [144], [147], or Raman [190], [60], [150] technology in the future.

Some interpretation of the data in the low-abundance field may lead to over speculation. Han [16] proposes small amounts of lipids to be originated from leftover reactants of imperfect biochemical reactions, since virtually no perfect thermodynamic conversion from precursors to products exists. For example, when LPE and LPC is observed in a matrix, PA-derived GP species must be present from de novo plasmanyl synthesis. [16]

Antonny et al. [12] reminds that FA chain function of lipid classes is less understood, which would insinuate more separation by reversed-phase chromatography. Traditionally, nonpolar and medium-polar lipids are studied with non-polar phases and two-component mobile phases made of water or buffered water and organic solvents in RP-HPLC. The advantage of ternary mobile phase compositions made of medium polar organic solvents, water or buffered water, and methanol or acetonitrile mixtures would be needed to separate and elute also polar lipids. In addition, the possibility of sequentially performed NP-HPLC and RP-HPLC coupled with MS should be more studied for lipids in biological samples. On the other hand, Liebisch et al. [39] promotes NP-HPLC and HILIC quantification over RP-HPLC for providing polar group selectivity by class selective separation and, thus, internal standard co-elution. [39]

13 Conclusions

The paper shows the fact that new methods for lipids and applications in clinical and medical research are still challenging in life science. To obtaining a truly complete overview of all lipids in a sample has remained very challenging due to their enormous structural diversity. A combination of sample extraction and separation procedures is required. Given the central role of lipids as key metabolites with remarkably diverse biological roles, the field of lipidomics may follow a trajectory comparable to the developments seen in genomics and proteomics over the past decade.

This review compiles recent lipidomic studies from the angle of UHPLC-MS research from the past three years (2017-2019). Identified lipids by subclass, biomarkers, clinical and in vitro sample matrices (i.e. biological materials) as well as specified topics on homeostatic, metabolic and disease-metabolic profiling are represented alongside quintessential analytical, biological and chemical insight for data interpretation. As an example, cell tissue data in research has rarely been put into context with cell-level observations. So far, signaling is seen as an only connection between these levels of biological organization.

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Figure 7. Structures of (a) nonpolar and (b) polar lipid subclass in separation techniques. Reprinted with permission from ref [116], DOI: 0958-1669/ 2016. S Tumanov and JJ Kamphorst. Published by Elsevier Ltd, an open access article under the CC BY license.

Figure 8. Product ion scans of negative mode HILIC-separated lipids from a standard mix (molecular ion is marked with an arrow). Reprinted from ref [178] © 2019, Springer Nature MMPE: monomethyl-phosphatidylethanolamine, DMPE: dimethyl-phosphatidylethanolamine

Figure 9. Creation, validation, and application of *in-silico* generated MS/MS spectra in LipidBlast. (a) New lipid compound structures were generated using *in-silico* methods. Lipid core structure scaffolds were connected *via* a linker to fatty acyls with different chain lengths and different degrees of unsaturation. Asterisks denote connection points. (b) Reference tandem spectra (top) were used to simulate mass spectral fragmentations and ion abundances of the *in-silico* spectra (bottom). The compound shown is PC(16:0/ 16:1) at precursor *m/z* 732.55 $[M + H]^+$. (c) For lipid identification, MS/MS spectra obtained from LC-MS/MS or direct-infusion experiments were submitted to LipidBlast. Ref. [185]

Figure 10. LipidBlast was mostly developed with ion trap tandem mass spectra but can be used with data from other platforms such as QTOF mass spectrometers. a) The Cardiolipin example shows that even in the in the case of the non-matching but abundant precursor ion at *m/z* 1239.8355 $[M-H]^-$, the correct result is obtained with LipidBlast. **b)** The standard reference compound with precursor *m/z*=793.4841 $[M-H]^-$ is correctly identified as phosphatidylinositol PI(17:0/14:1) as first hit in a library search with LipidBlast. Ref. [185]

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Table 1. Research on homeostatic profiling (10 articles)

Year	Citation	Theme	Subject
2017	[50]	Homeostatic profiling	Human gut phenotype profiling
2017	[56]	Homeostatic profiling	Buttermilk profiling
2017	[57]	Homeostatic profiling	Distinguishing between canine breeds
2018	[58]	Homeostatic profiling	Oxylipin analysis in human patients
2018	[15]	Homeostatic profiling	SM and Cer in multiple tissues/cell cultures of humans, dogs, mice and rats
2018	[59]	Homeostatic profiling	Larvae profiling (*Lysphlebia japonica*)
2018	[52]	Homeostatic profiling	Steroid hormone quantification in human plasma
2018	[53]	Homeostatic profiling	*In vivo* prostaglandin identification and quantitation in human brain tissue
2019	[60]	Homeostatic profiling	Coherent Raman scattering (CRS), non-destructive lipid/metabolite profiling
2019	[61]	Homeostatic profiling	Mouse plasma analysis with matrix-matched calibrants and SWATH* MS/MS

*SWATH: sequential window acquisition of all theoretical fragment ion mass spectra

Table 2. Metabolomics research topics. (16 articles)

Year	Citation	Theme	Subject
2017	[63]	Applications / Metabolomics	Nonalcoholic fatty liver disease profiling, improved data-analysis
2017	[64]	Metabolomics	Radiation countermeasure mechanism, GT3 inhibitor test
2017	[65]	Metabolomics	Fertilization of plants
2017	[66]	Metabolomics	Inflammatory macrophage characterization
2018	[67]	Metabolomics	Ketogenic diet, consequent metabolic perturbations, odd carbon lipids
2018	[68]	Metabolomics	Bioaccumulation & metabolomic response to chiral PCB 91
2019	[62]	Metabolomics	Correlation between serum SMs and plasma cholesterol
2019	[69]	Metabolomics	Oxalobacter profiling for oxalate-based disease research
2019	[70]	Metabolomics	High-throughput 96-well cell culture assay
2019	[71]	Metabolomics	Elevated CO2 concentration, leaves & berries of the black elder plant
2019	[72]	Metabolomics	Lipido-metabolic changes upon PCB153/PC12 exposure
2019	[73]	Metabolomics	Lipidomic perturbations of HEK 293 cells and exosomes under oxidative stress
2019	[74]	Metabolomics	Keratinocyte lipidomics affected by botulin
2019	[75]	Metabolomics	Analysis on the cellular functions of liver and skeletal muscle mitochondria
2019	[76]	Metabolomics	Targeted bile acid analysis for validation of liver cholestasis-associated protein
2019	[87]	Metabolomics	Skeletal muscle analysis of urban industrial waste water-exposed fish*

**B. meridionalis* and *S. laietanus*

Table 3. UHPLC-research on lipidomic pathway-based diseases and potential biomarkers (19 articles)

Year	Citation	Theme	Subject
2017	[89]	Diseases and Biomarkers	**Diabetic cardiomyopathy**
2017	[90]	Diseases and Biomarkers	**Lethal ventricular tachyarrhytmia** induced by **myocardial ion channel diseases** & **infarction**
2017	[91]	Diseases and Biomarkers	**Atheroslerotic dyslipidemia** via a high-fat diet on mice
2018	[92]	Diseases and Biomarkers	*In vitro* **Coronavirus-infection** of cell cultures
2018	[93]	Diseases and Biomarkers	**Primary sclerosing cholangitis**
2018	[94]	Diseases and Biomarkers	**Lethal ventricular tachyarrhytmia** induced by **myocardial ion channel diseases**
2018	[95]	Diseases and Biomarkers	Glyoxylate-induced **nephrolithiasis**
2018	[96]	Diseases and Biomarkers	**Acute coronary syndrome**
2018	[97]	Diseases and Biomarkers	**Alzheimer's disease** and amnestic **mild cognitive impairment**
2018	[98]	Diseases and Biomarkers	Plasma derived exosomal biomarkers, **radiation exposure**
2018	[99]	Diseases and Biomarkers	Dysregulation in **respiratory syncytial virus pneumonia** (mouse)
2018	[100]	Diseases and Biomarkers	Regulation of rosuvastatin in **lipidemia** patients
2019	[101]	Diseases and Biomarkers	**Obesity** biomarkers (rhesus monkey)
2019	[102]	Diseases and Biomarkers	VLDL, LDL and HDL CEs during **lipidemia** (golden hamster), unsaturation correlation with logarithmic mathematical model
2019	[103]	Diseases and Biomarkers	GL-induced **acute lung injury** (lipopolysaccharides, mouse)
2019	[9]	Diseases and Biomarkers	Blood platelets in **coronary artery disease**
2019	[104]	Diseases and Biomarkers	**Premature progesterone rise** affecting women’s endometrium
2019	[105]	Diseases and Biomarkers	Plasma-analysis of **Metabolic syndrome**
2019	[106]	Diseases and Biomarkers	Alveolar lavage fluid analysis, **acute lung injury**

Table 4. Lipidomic UHPLC-research focusing on cancer

Year	Citation	Theme	Subject
2017	[46]	Applications / Cancer research	Kidney cancer, human patients; Comparison of LC/MS;SCF/MS;DIMS
2017	[107]	Applications / Cancer research	Bee pollen anti-inflammatory properties on cancer cells
2017	[108]	Applications / Cancer research	Lipid extraction comparison with pancreatic cancer cell line
2017	[109]	Applications / Cancer research	Cancer cell lines, Quantitative analysis, PC/SM PIS-184 optimization
2017	[110]	Cancer research	Urinary exosomes in prostate cancer patients
2017	[111]	Cancer research	Hepatocellular carcinoma in cancer patients
2018	[112]	Cancer research	Review comparing plasmalipid profiles of liver, lung, gastric, colorectal and thyroid cancer, nanoflow UHPLC
2018	[113]	Cancer research	Non-small cell lung cancer serum biomarker identification
2019	[8]	Cancer research	Colorectal cancer, Validation of colon cell/tissue analysis

Table 5. Extraction and sample purification methods used in lipidomics.

Class	Technique	Citation
Polarity-based	Single solvent extractions	[51], [128], [130]
Polarity-based	Liquid-liquid extractions	[51], [128], [130]
Polarity-based	Single solvent mixtures	[51], [128]
Polarity-based	Bligh and Dyer	[129]
Polarity-based	BUME	[120]
Polarity-based	Folch	[119]
Polarity-based	MTBE	[55]
Polarity-based	QuEChERS	[127]
Polarity-based	Supercritical fluid extraction (SFE)	[114], [125]
Polarity-based	Solid phase extraction (SPE)	[58], [125]
Polarity-based	Superabsorbent polymer extraction (SPE)	[127]
Energy-based	Microwave-assisted extraction (MAE)	[114], [125]
Energy-based	Ultrasonic-assisted extraction (UAE)	[114], [125]
Energy-based	Pressurized liquid extraction	[114], [125]

Table 6. Molecular species formed during electrospray ionization of lipids. Ref. [152]

Lipid class	**Positive mode**	**Negative mode**
LPC, PC	$[M + H]^+$, $[M + Na]^+$	$[M–H]^–$, $[M + HCOO]^–$, $[M + CH_3COO]^–$
LPE, PE	$[M + H]^+$, $[M + Na]^+$	$[M–H]^–$
PG	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$
PI	$[M + H]^+$, $[M + NH_4]^+$	$[M–H]^–$
PS	$[M + H]^+$	$[M–H]^–$
PA		$[M–H]^–$
CE	$[M + NH_4]^+$	
SM	$[M + H]^+$	$[M + HCOO]^–$
Cholesterol	$[M–H_2O+H]^+$	
Cer, GluCer, LacCer	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$, $[M + HCOO]^–$
MG, DG, TG	$[M + NH_4]^+$, $[M + Na]^+$	
MGDG, DGDG, SQDG	$[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$
Fatty acids		$[M–H]^–$
CL	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$
Cer	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$

Table 7. Classical biological material types. Extension to Jurowski et al.'s Table (Ref. [114]).

Biological samples		**Lipid classes**	**Reference(s)**
classical biological materials (conventional)	Urine	AcCa, MG, DG, TG, PC, PE, LPC, Cer, SM, CEs	[5], [110]
	faecal matter	FA, GPs, STs, SPs, PRs, PKs	[50], [156]
	blood derivatives:		
	Plasma	FA, MG, DG, TG, PA, PC(+PLs), PE(+PLs), PI, PS, LPA, LPC, LPE, Cer(+Hex/Hex2/sulfoHex), SM, S, SA, cholesterol, CEs, SLs, STs	[5], [46], [52], [57], [58], [61], [62], [64], [67], [189], [91], [96], [99], [100], [102], [103], [105], [155], [157]*, [158]
	Serum	MG, DG, TG, PA, PC(+PLs), PE(+PLs), PG, PI, PS, LPA, LPC, LPE, Cer(+HexCer/Hex2Cer), SM, S, CEs, STs, PRs	[5], [15], [62], [76], [90], [94], [95], [113]
	Erythrocytes	PC(+PLs), PE(+PLs), LPC, LPE, Hex2Cer, SM	[46]
	blood platelets	AcCa, FA, DG, TG, PA, PC, PE, PG, PI, PS, LPA, LPC, LPE, Cer, SM	[9], [48]**

*NIST SRM 1950 plasma, ** Salmonella enterica serovar Typhimurium-infected murine bonemarrow-derived macrophages and thrombin activated blood platelets (human)
Hex: for hexosyl, **Hex2**: dihexosyl, **S**: sphingosine, **SA:** sphinganine

Table 8. Alternative bioloical materials. Extension to Supplementary Table 1 by Jurovski et al. [114] Hex is an abbreviation for hexosyl, Hex2 for dihexosyl

Biological samples		**Lipid classes**	**Reference(s)**
alternative biological materials (unconventional)	Aqueous humor		[5], [127]
	Bile		[93]
	Bronchoalveolar lavage fluid	FA, GLs, GPs, OxGPs, LPLs, SLs	[106]
	Cerebrospinal fluid (CSF)		[5]
	Colon derived suspension		[8]
	Milk	DG, TG, PC, SM	[47]
	Tear drops		[5]
	Cells and cell lines:		
	x exosomes	FA, GLs, OxGPs, LPLs, SPs, HexCer, CEs, STs	[5], [73], [98], [110]
	x liposomes	FA	[159]
	x alveolar cells	PC, PE(+PLs), PS, LPLs, SM, Cer, HexCer	[160]
	x lipid droplets		[60]
	x ceratinocytes	FA, GLs, GPs, OxGPs, LPLs, SPs, HexCer, CEs	[74]
	Tissues:		
	x adipose tissue	Cer, SM	[15]
	x lung tissue	AcCa, FA, TG, PC(+PLS), PE(+PLs), PG, PI, PS, LPC, LPE, LPI, LPS, Cer, SLs	[99], [103]
	x renal tissue	FA, GLs, GPs, SLs, STs, PRs	[95]
	x myocardial tissue	FA, CL, DG, TG, PA, PC, PE, PG, PI, PS LPC, LPE, Cer, cholesterol	[89], [90]
	x brain tissue	Cer, SM, thromboxane, prostaglandins	[15], [53], [155]

	x endometrium	GLs, GPs, LPLs, SPs	[104]
	x liver tissue	DG, TG, PA, PC(+PLs), PE(+PLs), PG, PI, PS, LPA, LPC, LPE, LPG, LPI, LPS, Cer(+HexCer), SM, STs	[15], [63], [76], [111], [127], [155], [160]
	x colonic tissue		[8]
	x tumor tissue	PC(+PLs), PE(+PLs), LPC, LPE, Hex2Cer, SM	[46]

Table 9. Cell culture matrices analyzed with UHPLC-MS techniques.

Hex is an abbreviation for hexosyl, Hex2 for dihexosyl

Biological samples		**Lipid classes**	**Reference(s)**
Cell cultures	HC1*	DG, CL, BMP, PE(+OxPE), PG, PS, LPE, PRs	[69]
	HCC827 (+HCC827-GR)		[109]
	HEK 293	FA, GLs, GPs, PLs, OxGPs, HexCer, CEs, STs	[73]
	HeLa	CL, DG, TG, PC, PE, PG, PI, PS, LPC, LPE, Cer(+HexCer), SM	[70], [130]
	Huh7	LPC, LPE	[92]
	HTC-116		[109]
	Huh7	LPC, LPE	[92]
	MDCK (+MDCK-GR)		[15], [109]
	OVCAR-3		[109]
	OxWR*	CL, DG, BMP, PE, PG, PS, LPE, PRs	[69]
	PANC-1	CE, DG, TG, PC, PE, PI, PS, LPC, Cer, SM	[108]
	PC-9 (+PC-9-GR)		[109]
	PC12	PC, PE, PI, PS	[72]
	RAW 264.7 (macrophages)**	GLs, PA, PC, PE, PG, PI, PS, LPLs, SPs, CEs, cholesterol	[66], [107]
	SKOV-3		[109]
	Bladder cancer cell		[155]
	Melanoma B16***	Cer, SM	[15]
	Primary CD4+T lymphocytes***	Cer, SM	[15]
	Yeast	PA, PC, PE, PG, PI, PS	[140]

*anaerobic, *oxalobacter formigenes* ; **widest lipid range ; ***mouse

Table 10. Common lipids according to research topic and identification approach. When "Identified lipids" are indicated as a sum without any explanation, it means that the first number is the amount of lipids found in positive and the second in negative mode (positive+negative).

Citation	Theme	T/relT/UT		GPs				Hex	Identified lipids	Bio-markers
[47]	Method development	UT	2	1				1		429	
[161]	Method development	relT	1	4				1			
[160]	Method development	UT	2	3	*		2	1	1		
[160]	Method development	UT	2	4	*		2	2			
[160]	Method development	UT		3	*		2	2	1		
[140]	Method development	UT		6	*					436	
[127]	Method development	T/UT	2	6			5	2		207	
[130]	Method development	UT	2	4			2	*		292+206	
[157]	Method development	T	1	4			2	1		22	
[155]	Method development	PT	2	2	*		2	2	2	515+630+640 [D]	
[189]	Method development	UT	2	3			1	2		104	
[159]	Method development	UT								403	
[48]	Method development	UT								85 [E]	
[108]	Method development / cancer research	UT	2	4			2	2		226-414	
[63]	Method development / Metabolomics	UT	*	*						83	8
[46]	Method development / cancer research	T/UT	3	5			3	2	1	132	
[110]	Cancer research	UT	2	1	*			2	1	286	34
[111]	Cancer research	UT	2	2							50

[113]	Cancer research	T/UT	3	5	*			3	2	493	14+10+2 [B]
[92]	Diseases and Biomarkers	UT					2			24	
[101]	Diseases and Biomarkers	UT		4			2	1		129	
[94]	Diseases and Biomarkers	UT	3	6			3	3		749	16
[89]	Diseases and Biomarkers	UT	1	2						12	9
[95]	Diseases and Biomarkers	UT	*	*				*		179	
[95]	Diseases and Biomarkers		*	*				*		196	
[90]	Diseases and Biomarkers	UT	1	2			1	1		746	11
[90]	Diseases and Biomarkers		2	2			1	1			
[91]	Diseases and Biomarkers	UT	3	5				4		261+39	7
[96]	Diseases and Biomarkers	UT	1	2	*		2	2		365	19
[97]	Diseases and Biomarkers	UT	2	2	*		2	2	3	363	28
[98]	Diseases and Biomarkers	UT	1	1						Features	Features
[5]	Diseases and Biomarkers		2	4				1			>25
[102]	Diseases and Biomarkers	relT/T								81 [A]	17 [A]
[103]	Diseases and Biomarkers	UT									77
[9]	Diseases and Biomarkers	UT	2	5			3	2			77
[99]	Diseases and Biomarkers	UT	1	5	*		3	1		188+62	87
[100]	Diseases and Biomarkers		2	1			1	1			
[104]	Diseases and Biomarkers	UT	1	4			1	1		1026	25
[105]	Diseases and Biomarkers	T/UT	2	2	*		1	2		55	55
[106]	Diseases and Biomarkers	UT	1	3	*		2			38+31	11+14
[45]	Drug-testing	UT	1	1			1	1		155	
[56]	Foodstuff profiling	T	1	4			2	1		81	
[67]	Metabolomics	UT	1	5			1	*		61	
[68]	Metabolomics	UT					4				
[62]	Metabolomics	UT	2	4			*				

[65]	Metabolomics	UT	2	5				1		178	
[69]	Metabolomics	UT	1	4	*	1	1			97	
[69]	Metabolomics		1	3	*		1				
[158]	Metabolomics	UT	1	2			2	1		226	
[70]	Metabolomics	UT		5				1		249+451	
[71]	Metabolomics	UT	1	1						7	
[72]	Metabolomics	T		4						22	5
[66]	Metabolomics	UT	3	6			6	7		523	
[73]	Metabolomics	UT	1	7	*	*	5	2	1	642	53
[74]	Metabolomics	UT	2	5	*		4	2	1	611	440
[75]	Metabolomics	UT		6	*		3	2		N/A	≥43
[76]	Metabolomics	T	1	4	*		1	2			126+124 [E]
[87]	Metabolomics	UT		7	*		1	2		119	38
[50]	Physiology	T/UT		*				*		Features	
[58]	Physiology	T									
[15]	Physiology	T						2		45	
[59]	Physiology	UT	1	6	*		2	2		283	
[52]	Physiology	UT								12	
[53]	Physiology	T								5	
[107]	Physiology	UT			*			1		184+150	
[57]	Physiology	UT	2	1							35
[61]	Physiology	UT	3	5			2	1		529+179	

*: Lipid subgroups could not be determined

T/relT/UT: targeted/relatively targeted/untargeted

[A] for hyperlipidemia: plasma(74, 57 biomarkers), VLDL(74, 52 biomarkers), LDL(76, 42 biomarkers), HDL(73, 41 biomarkers)

[B] non-small cell lung cancer+lung benign disease+healthy controls

[C] metabolites

[D] in plasma+tissue+cell

[E]: serum+liver

Table 11. Extension of lipid classes analyzed in different research articles (2017-2019)

Citation	Theme	T/relT /UT/PT	FAcyls	CEs	SLs	STs	PRs	PKs	HDLs	LDLs	VLDLs	Identified lipids	Bio-markers
[47]	Method development	UT										429	
[161]	Method development	relT											
[160]	Method development	UT											
[160]	Method development	UT											
[160]	Method development	UT											
[140]	Method development	UT										436	
[127]	Method development	T/UT			*	*						207	
[130]	Method development	UT		*								292+206	
[157]	Method development	T	1									22	
[155]	Method development	PT		*								515+630+640 [D]	
[189]	Method development	UT		*								104	
[159]	Method development	UT	*									403	
[48]	Method development	UT	*									85	
[108]	Method development / cancer research	UT		*								226-414	
[63]	Method development / Metabolics	UT			*							83	8
[46]	Method development /cancer research	T/UT		*								132	
[110]	Cancer research	UT		*								286	34
[111]	Cancer research	UT											50
[113]	Cancer research	T/UT		*								493	14+10+2 [B]
[92]	Diseases	UT										24	
[101]	Diseases	UT										129	
[94]	Diseases	UT		*	*							749	16
[89]	Diseases	UT							1			12	9
[95]	Diseases	UT	*		*	*	*					179	
[95]	Diseases		*		*	*	*					196	
[90]	Diseases	UT										746	11
[90]	Diseases												
[91]	Diseases	UT				*						261+39	7
[96]	Diseases	UT				*						365	19
[97]	Diseases	UT										363	28
[98]	Diseases	UT	1									Features	Features
[5]	Diseases		1	*									>25
[102]	Diseases	relT/T	1						*	*	*	81 [A]	17 [A]
[103]	Diseases	UT			*								77
[9]	Diseases	UT	2										77
[99]	Diseases	UT	2		*							188+62	87
[100]	Diseases	UT	1									188 [C]	
[100]	Diseases		1										
[45]	Drug-testing	UT										155	
[104]	Diseases	UT										1026	25

[105]	Diseases	T/UT		*	*							55	55
[106]	Diseases	UT	*		*							38+31	11+14
[56]	Foodstuff profiling	T										81	
[67]	Metabolomics	UT	*									61	
[68]	Metabolomics	UT											
[62]	Metabolomics	UT		*									
[65]	Metabolomics	UT			3							178	
[69]	Metabolomics	UT	1				1					97	
[69]	Metabolomics		1				1						
[158]	Metabolomics	UT							*	*		226	
[70]	Metabolomics	UT	1									249+451	
[71]	Metabolomics	UT				1						7	
[72]	Metabolomics	T										22	5
[66]	Metabolomics	UT		*								523	
[73]	Metabolomics	UT	*	*		*				*		642	53
[74]	Metabolomics	UT	2	*								611	440
[75]	Metabolomics	UT	*									N/A	≥43
[76]	Metabolomics	T										126+124	
[87]	Metabolomics	UT										119	38
[50]	Physiology	T/UT	*			*	*	*				Features	
[58]	Physiology	T	*										
[15]	Physiology	T										45	
[59]	Physiology	UT			1							283	
[52]	Physiology	UT				**						12	
[53]	Physiology	T	***									5	
[107]	Physiology	UT	2									184+150	
[57]	Physiology	UT											35
[61]	Physiology	UT	*									529+179	

*Lipid classes not counted

**15 steroids

*****5 prostaglandins**

[A] for hyperlipidemia: plasma(74, 57 biomarkers), VLDL(74, 52 biomarkers), LDL(76, 42 biomarkers), HDL(73, 41 biomarkers)

[B] non-small cell lung cancer+lung benign disease+healthy controls

[C] metabolites

[D] in plasma+tissue+cell

[E]: serum+liver

Figure 1

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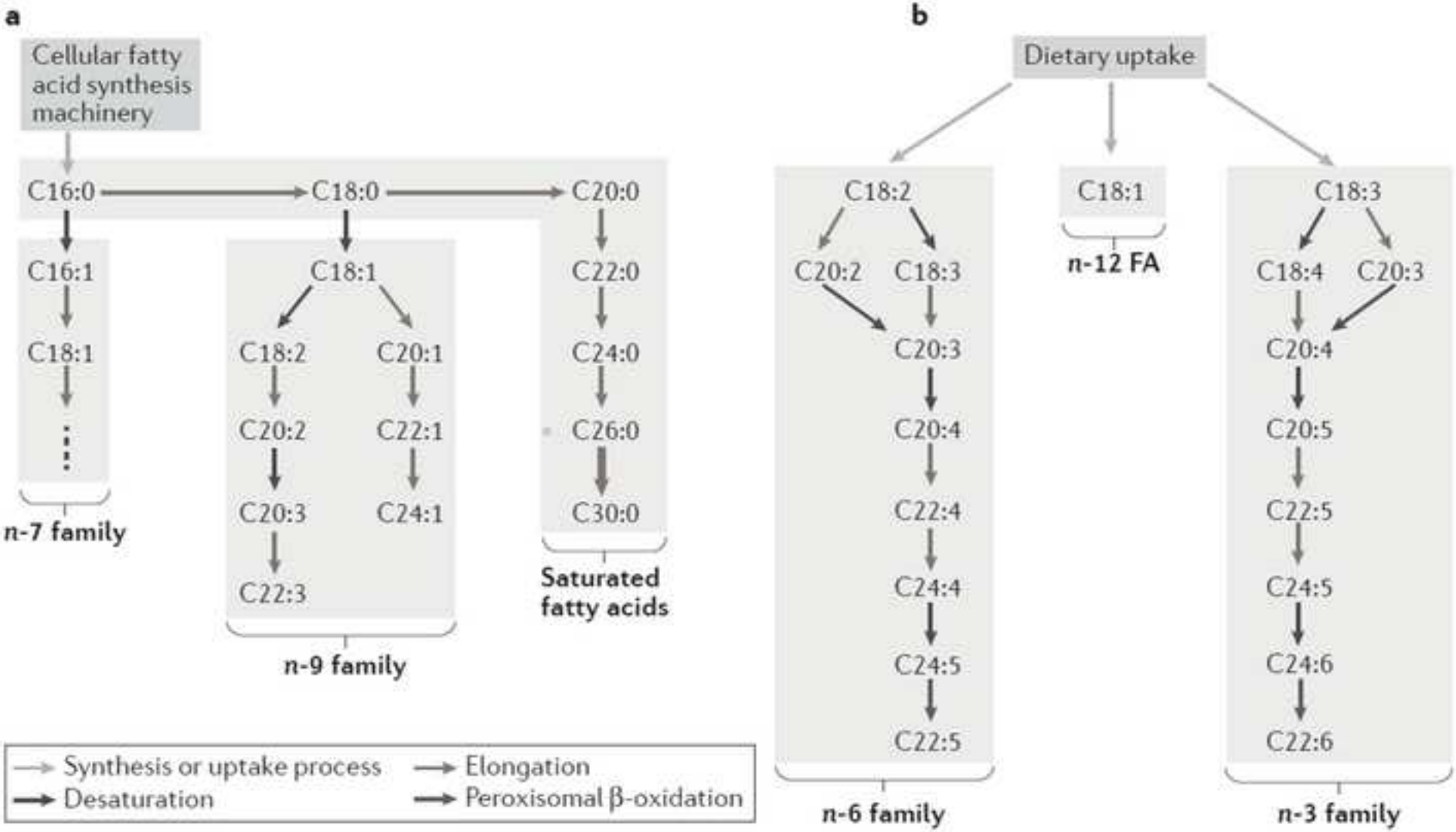

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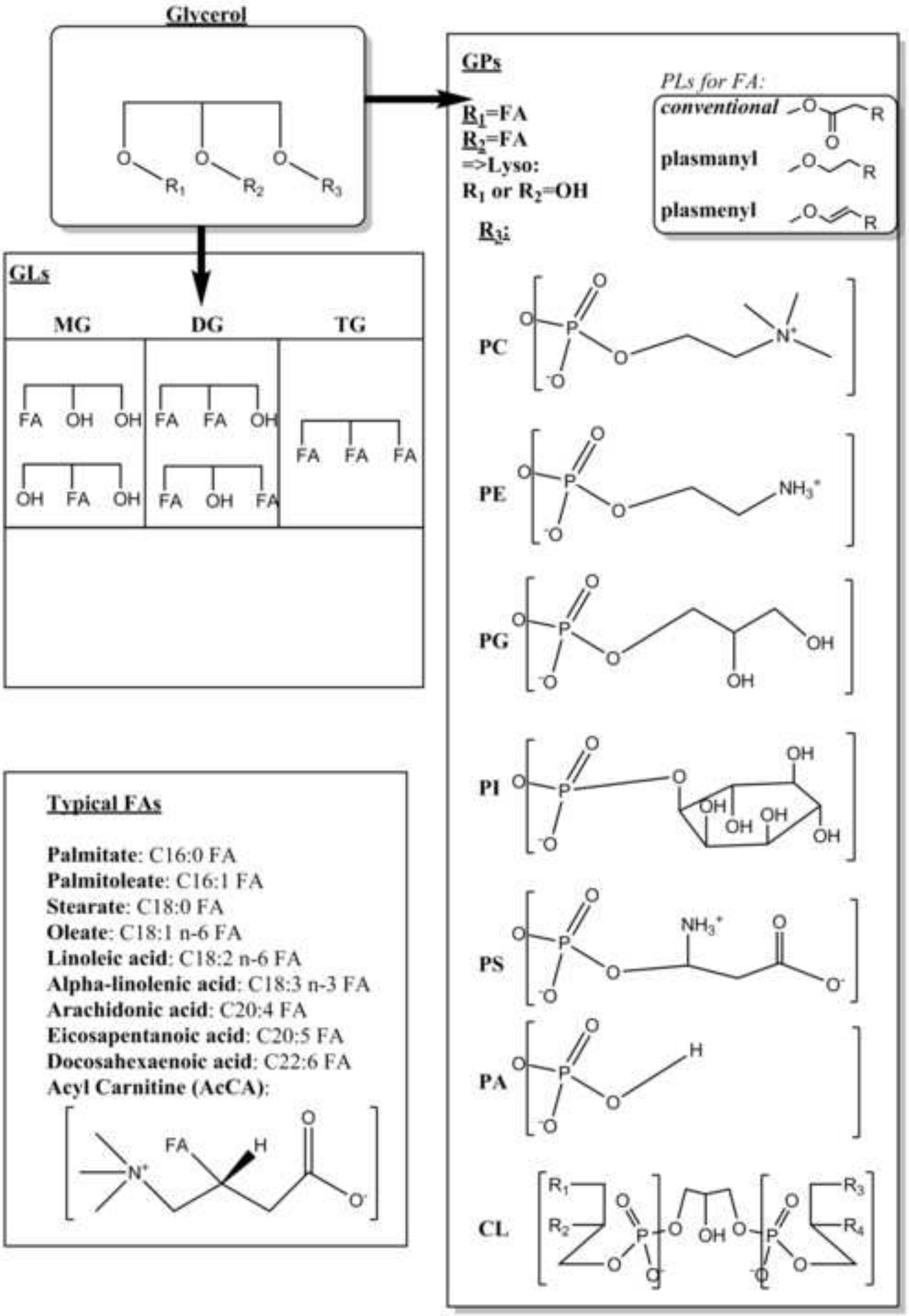

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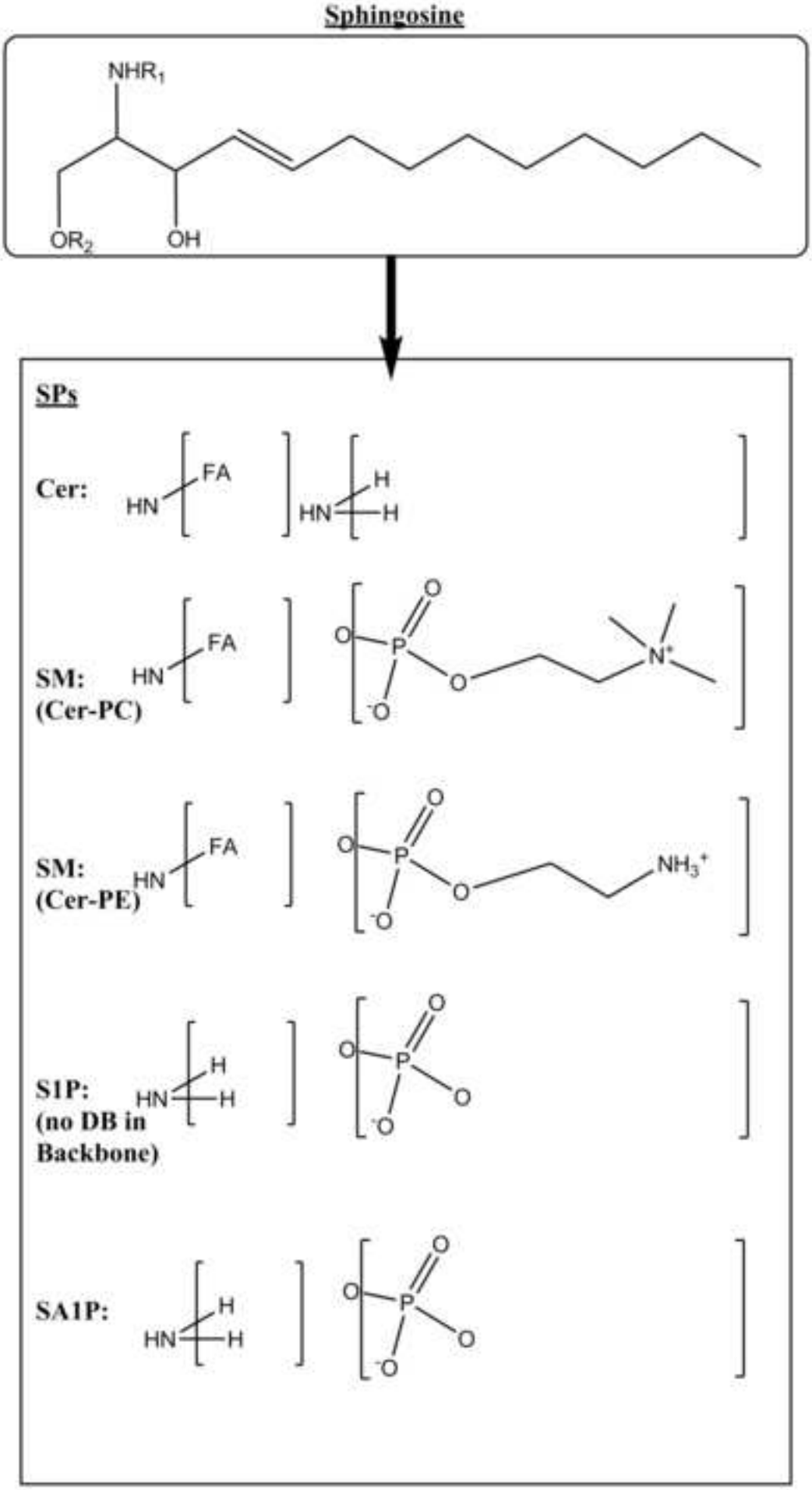

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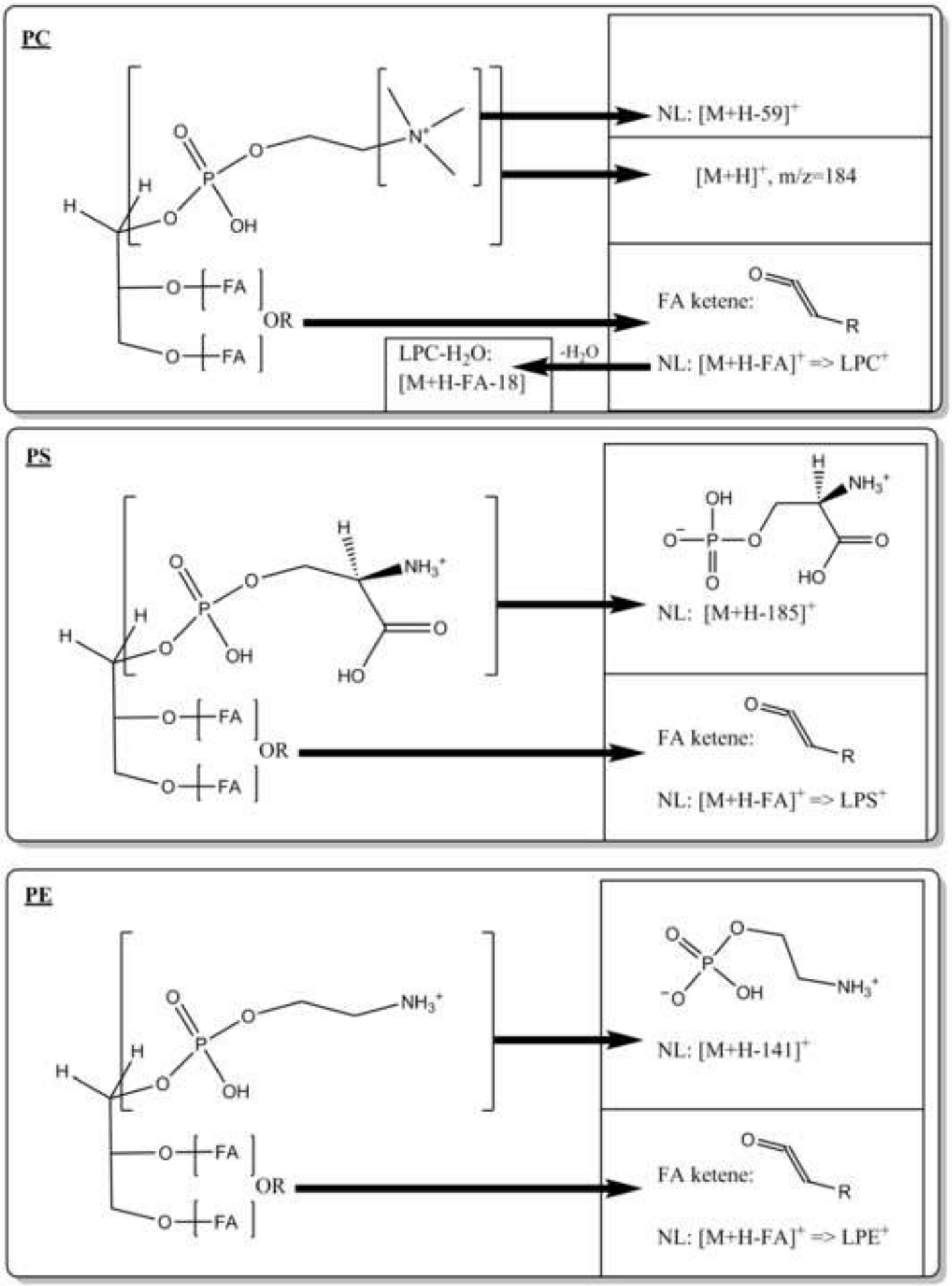

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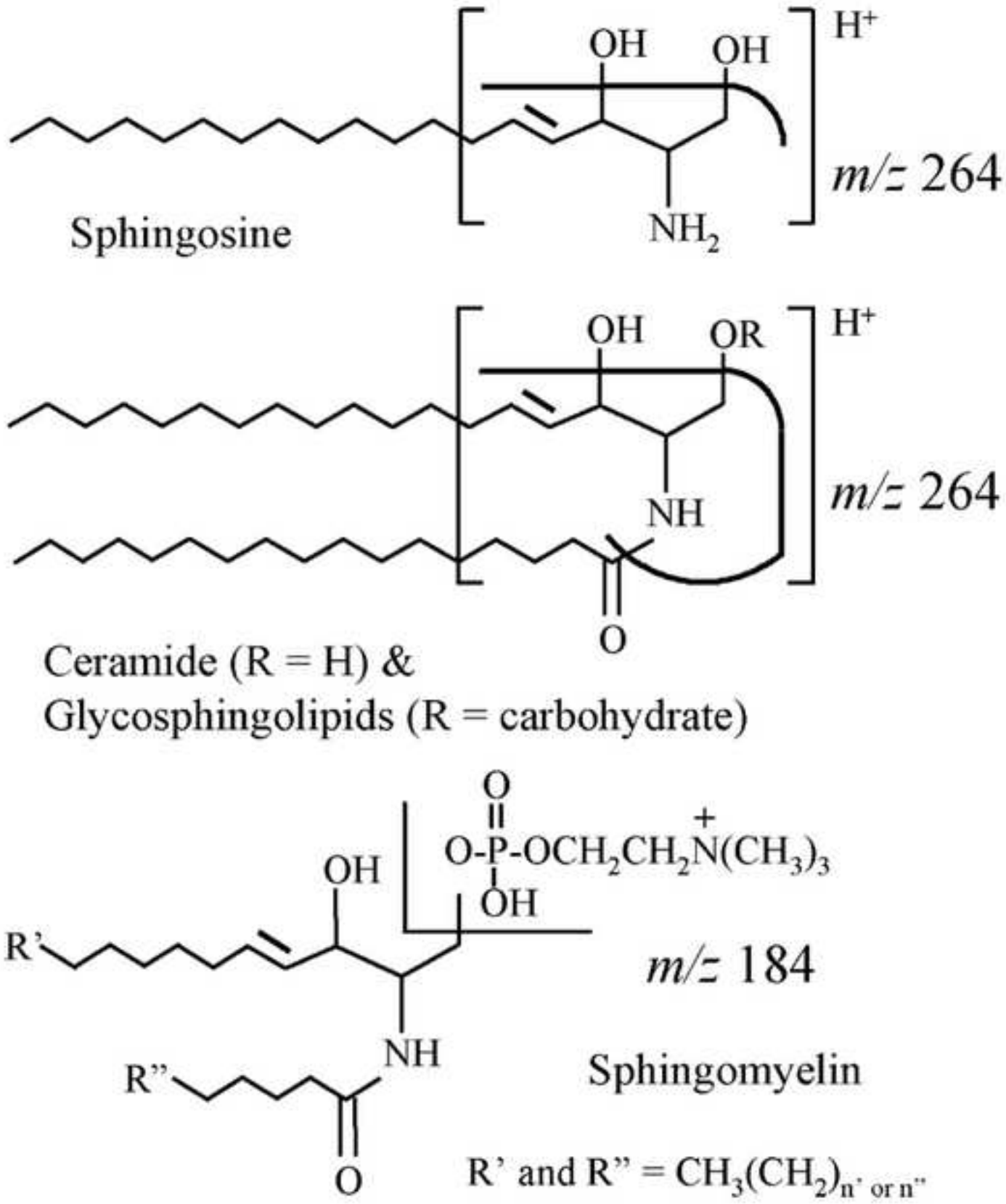

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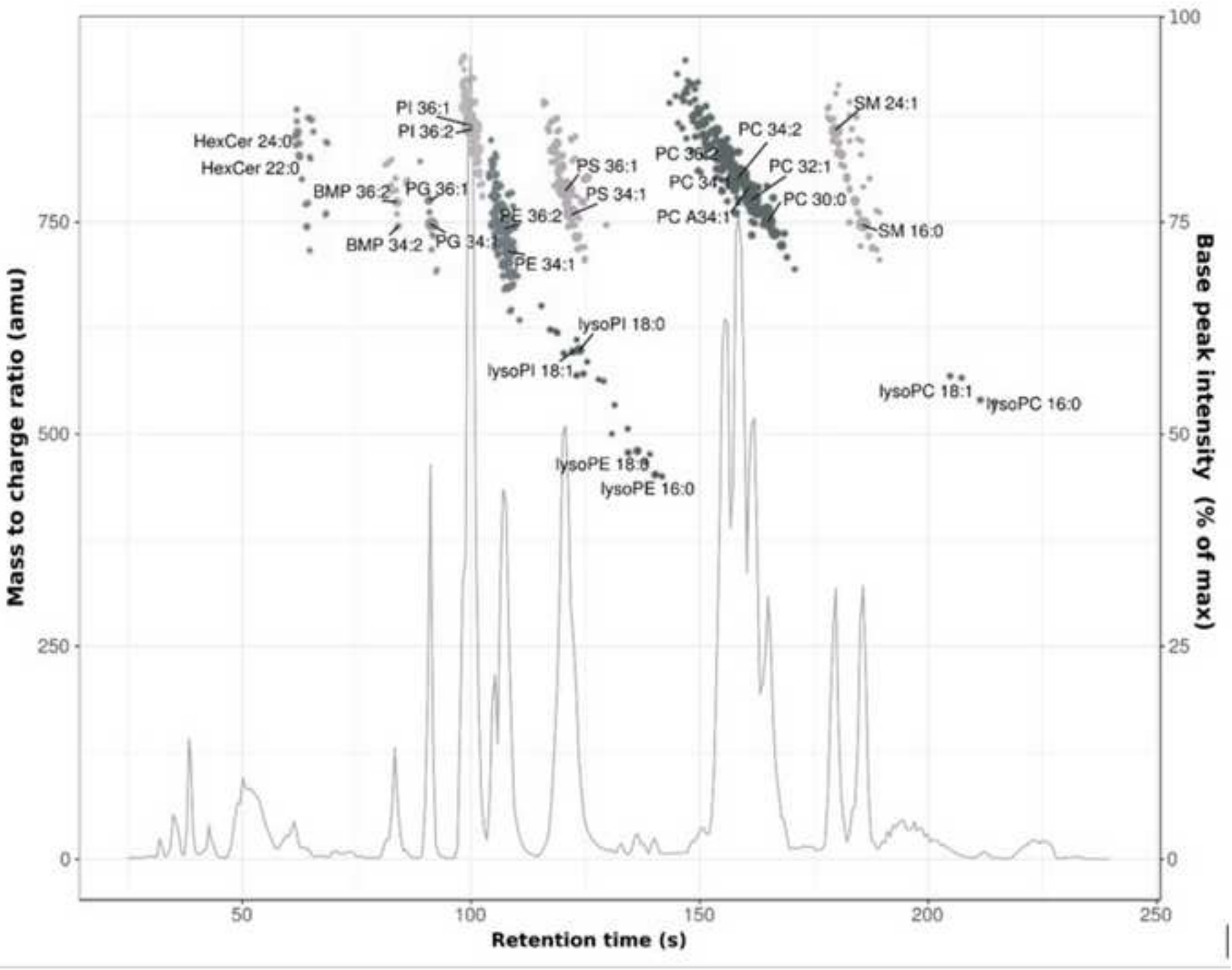

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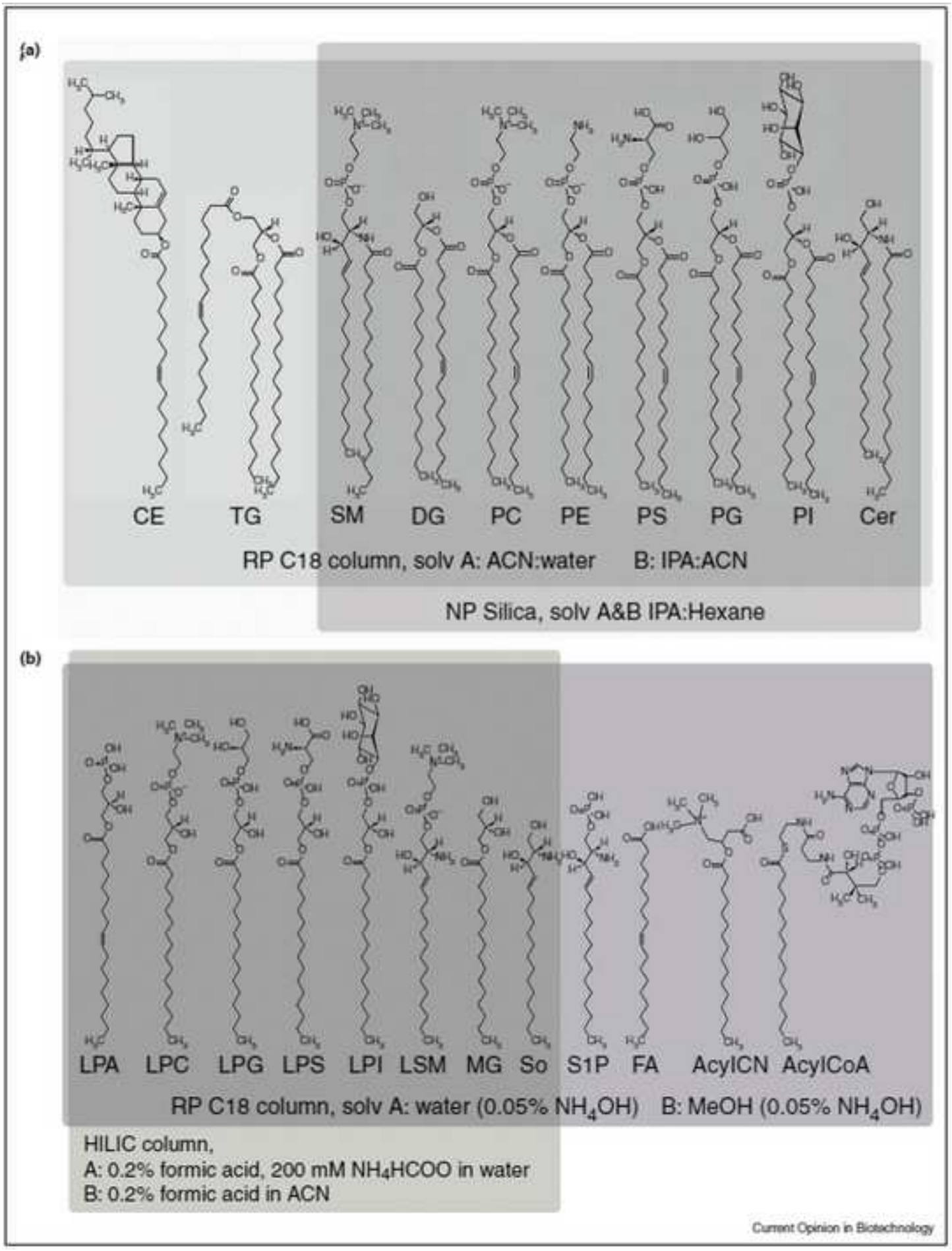

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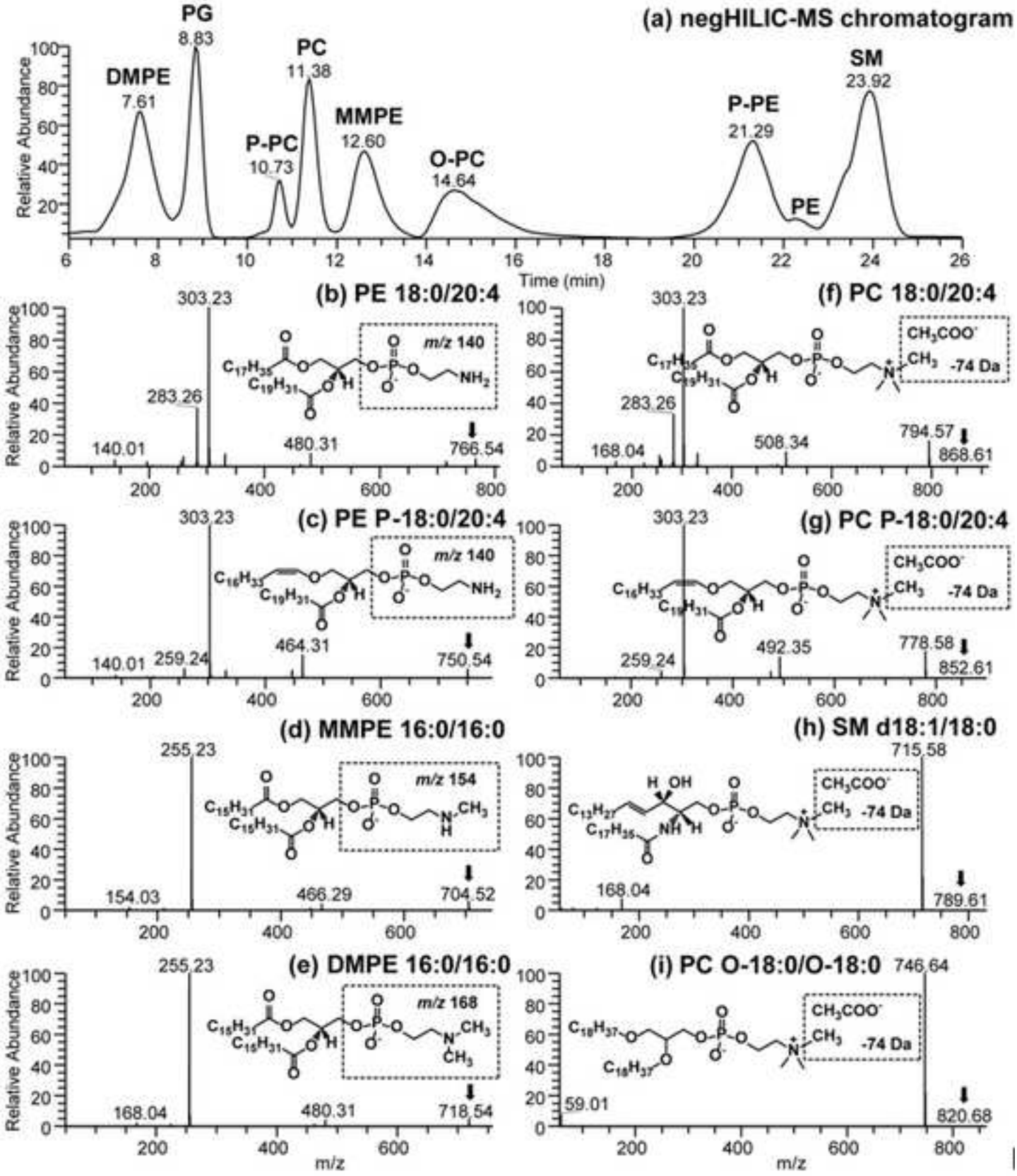

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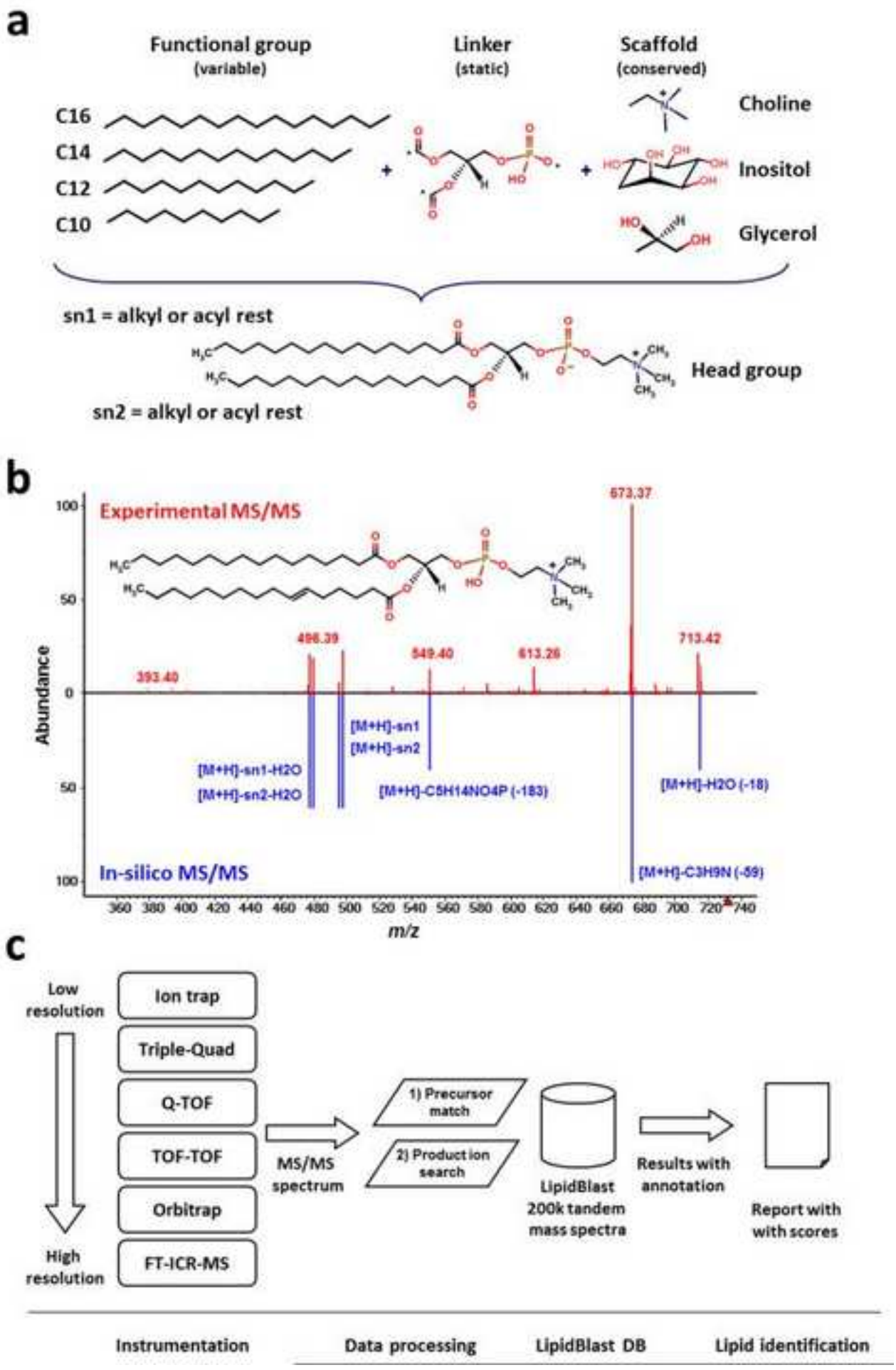

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Manuscript Draft

Manuscript Number:

Title: Lipidomics and recent applications in clinical and medical research with ultrahigh performance liquid chromatography-mass spectrometry

Article Type: Review Article

Keywords: Lipids, liquid chromatography, tandem mass spectrometry; clinical profiling, urine, blood.

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Abstract: Lipids are organic biomolecules, which contribute to energy storing, cellular and subcellular membrane assembly, functionality, signalling, gene expression regulation and surfactant formation. Lipidomics comprises of identification and quantitation of organic lipids, their derivatives and variants.
The present review article combines the literature on glycero-, glycerophospho- and sphingolipids in lipidomics from the years 2017-2019. In addition, a few papers which have promoted advancement in lipidomics are discussed. The primary focus is lipid profiling of biological lipidomic systems with ultrahigh performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS, tandem MS) detection, data handling, and calculations with analytical tools and current statistical methods. Research on in vivo samples includes matrix categories, such as primary blood derivatives (plasma, serum, erythrocytes, and blood platelets), faecal matter, urine, and liver tissues.

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Cover Letter

To
The Reviews Editor

Helsinki 11.11.2019

Dear Editor,

The paper was informed to be sent to the journal on 16th September, 2019 and asked about considering our review article for publication. The contacted person was Dr. Gregory S Makowski, Reviews Editor, CCA.

Now the review paper is ready and its title is

Lipidomics and recent applications in clinical and medical research with ultrahigh performance liquid chromatography-mass spectrometry – A review

written by Henri F. Avela and Heli Sirén
is sent to Clinica Chimica Acta.

For this paper we have compiled the newest literature between 2017 - 2019. The articles reviewed primarily were focused on applications of clinical and medical research made with ultrahigh-performance liquid chromatographic methods with mass spectrometric (MS, tandem MS) detection. The lipid articles were considered to be sufficient enough for making the current statement of the state from glycero-, glycerophospho- and sphingolipids in lipidomics and the identification with on-line coupled liquid chromatography with mass spectrometry research. Additionally, the data handling with computational methods alongside chemometric and statistical methods are discussed. That kind of evaluation was shown to have increased importance and usage for cross-validation and data-analysis. Please, find the abstract on a separate page.

The authors declare that they do not have competing financial interest concerning the project. They do not have any conflicts, either.
The manuscript has not published in any other journal. It has ten (10) figures and eleven (11) tables.

Thank you for considering the submission. We look forward to your response.

Sincerely
Heli Sirén,
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Lipidomics and recent applications in clinical and medical research with ultrahigh performance liquid chromatography-mass spectrometry – A review

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Abstract

Lipids are organic biomolecules, which contribute to energy storing, cellular and subcellular membrane assembly, functionality, signalling, gene expression regulation and surfactant formation. Lipidomics comprises of identification and quantitation of organic lipids, their derivatives and variants.

The present review article combines the literature on glycero-, glycerophospho- and sphingolipids in lipidomics from the years 2017-2019. In addition, a few papers which have promoted advancement in lipidomics are discussed. The primary focus is lipid profiling of biological lipidomic systems with ultrahigh performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS, tandem MS) detection, data handling, and calculations with analytical tools and current statistical methods. Research on *in vivo* samples includes matrix categories, such as primary blood derivatives (plasma, serum, erythrocytes, and blood platelets), faecal matter, urine, and liver tissues.

Keywords: Lipids, liquid chromatography, tandem mass spectrometry; clinical profiling, urine, blood.

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