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To International Journal of Mass Spectrometry (IJMS)

Helsinki, March 27th 2020

Dear Editor,

Enclosed, please, find an electronic submission of a review manuscript entitled

Advances in analytical tools and current statistical methods used in ultra-high-performance liquid chromatography-mass spectrometry of glycero-, glycerophospho- and sphingolipids written by Henri Avela and Heli Sirén for publication in International Journal of Mass Spectrometry.

We have broadly reviewed the status of current RPLC-MS analytics in the light of UHPLC-MS studies between the time frame of 2017-2019 to elucidate both standardized and variable aspects practiced by researchers for both identification and quantitation in lipidomics.

We focus to the importance of the topic and to orient current method validation towards factors (e.g. gradient, adducts) yet undecided in the lipidomic community and to clarify the choices made in lipidomic analyses. Moreover, complex lipidomic data demands high-throughput data processing as well as the appropriate chemometric and statistical tools, which are also represented in the review.

The manuscript contains 4 figures and 5 tables. Among them, Figure 4 needs permission for reprinting.

Looking forward to receive your decision about the review paper.

Sincerely,

Henri Avela and Heli Sirén

Cover letter

The response letter to the reviewer's comments on the manuscript

IJMS_2020_121

Advances in analytical tools and current statistical methods used in ultra-high-performance liquid chromatography-mass spectrometry of glycero-, glycerophospho- and sphingolipids

by Avela and Sirén

To the Editor of International Journal of Mass Spectrometry

Professor Zheng Ouyang

Please, find below our responses to the comments of the reviewers about the first version of the manuscript IJMS_2020_121.

1. Response to the reviewers' comments

Comment 1: This review contains a very wide coverage of literature of LC-MS methods for lipids analysis, this makes it too lengthy to read. I suggest the authors to remove basic knowledges of lipid nomenclature, liquid chromatography and mass spectrometry.

Response 1: The exposition on basic knowledge concerning the topics lipid analysis, LC and MS were minimized. However, some paragraphs were kept to preserve the text's flow and readability.

Comment 2: Besides, discussion of challenges specific to the analysis of each type of biological sample should be added.

Response 2: A brief introduction to biofluids, tissue and cell culture sample preparation was added to the Chapter 'Instrumentation'. Biological samples and their sample preparation is discussed elsewhere, since the main text was asked to be minimized.

Reviewer #1:

Comment 1: Although LC-MS are widely used for lipid analysis, shotgun lipidomics methods were also developed and employed into various biological samples.

Response 1: We have included our comments and compare shortly direct infusion techniques with HPLC-MS in the Chapter on 'Quantification of lipids'. We think that an extra paragraph on the mehod comparison will increase the basic information, which was suggested to be removed or decreased in the main paper

Comment 2: I understand that emphasizing UPLC can make this review more focusing, however, since many MS methods are developed based on HPLC, some very important MS methods for lipid analysis may be omitted.

Response 2: We have acknowledged this limitation in the 'Discussion' section and addressed some techniques mentioned by reviews on lipidomic MS-analysis.

Comment 3: During past few years, more attentions were paid onto lipid isomer analysis by developing new MS methods, e.g. C=C isomer and sn-isomer analysis. Related references should be included.

Response 3: The topic was extended upon up-to-date methods (e.g. Paterno-Büchi acetone derivatization) in the end of the Chapter 'Identification of lipids'. Furthermore, some methods were already mentioned in the text (e.g. SWATH, SONAR, PRM in the 'Introduction' and SWARM in the 'Instrumentation' chapter)

Comment 4: Reference [15] is a review paper, it cannot be used a source of experimental conditions in Table S1. Acetone has been reported as a major component of the mobile phase both for HILIC (2019) and RPLC (Anal. Chem. 2020) separation phospholipids.

Response 4: The study on buttermilk by Castro-Gómez et al. [15] includes an experimental section and results discussing them. Anyhow, acetone is now mentioned and cited as a organic phase in the limitations-paragraph of the Discussion-section (side note: UHPLC articles barely noted this solvent, though it must be used for the Posternó-Büchi photochemical derivatization)

Comment 5: Quantitation of lipids in biological samples should be discussed.

Response 5: A chapter ('Quantitation of lipids') was included to address the topic and its challenges. Furthermore, additional information had to be added for the normalization (i.e. standard usage) of lipidome data.

Advances in analytical tools and current statistical methods used in ultra-high-performance liquid chromatography-mass spectrometry of glycero-, glycerophospho- and sphingolipids

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Abstract

The review concentrates on the properties of analytical and statistical ultrahigh-performance liquid chromatographic (UHPLC) – mass spectrometric (MS) methods suitable for glycero-, glycerophospho- and sphingolipids in lipidomics published between the years 2017-2019. Trends and fluctuations of conventional and nano-UHPLC methods with MS and tandem MS detection were observed in context of analysis conditions and tools used for data-analysis. Whereas general workflow characteristics are agreed upon, more details related to the chromatographic methodology (i.e. stationary and mobile phase conditions) need evidently agreements. Lipid quantitation relies upon isotope-labelled standards in targeted analyses and fully standardless algorithm-based untargeted analyses. Furthermore, a wide spectrum of setups have shown potential for the elucidation of complex and large datasets by minimizing the risks of systematic misinterpretation like false positives. This kind of evaluation was shown to have increased importance and usage for cross-validation and data-analysis.

Keywords

Lipidomics, mass spectrometry, ultrahigh performance liquid chromatography, nano-liquid chromatography, chemometrics, statistical methods, multicomponent analysis

Highlights

- Method development and application enhancements in lipidomics
- The review sums up chemometric and statistical methods for current lipidomics
- State of the art data collection and evaluation is discussed
- Identification/quantitation of biological lipids
- Tandem MS data-independent and data-dependent analysis

Abbreviations

ACP acyl carrier protein, AF4 asymmetric flow field flow fractionation, ANOVA analysis of variance, AUC area under curve, CARS coherent anti-Stokes Raman scattering, CCS collision cross-section, CN total carbon, CRS coherent Raman scattering, DB double bond, DDA data dependent acquisition, DESI desorption electrospray ionization, DG diacylglycerol, DIA data independent acquisition, DIMS direct infusion MS, FDR false discovery rate, FA fatty acyl, FFA free fatty acid, GC gas chromatography, GL glycerolipid, GP glycerophospholipid, HCA hierarchical cluster analysis, i.d. internal diameter, IMS ion mobility spectrometry, HPLC liquid chromatography, LESA liquid extraction surface analysis, m/z mass-to-charge [ratio], LSI Lipidomic Standards Initiative, HR/LRMS high/low resolution mass spectrometry, MS^E all ions scans, MS/MS tandem mass spectrometry, MS/MS/MS second-generation fragmentation mass spectrometry MS^n on-line coupled mass spectrometry system, MSI mass spectrometric imaging, m/z mass-to-charge ratio, NP normal phase, NSI nano-electrospray ionization, nESI negative ion mode in electrospray ionization, OPLS-DA orthogonal projections to latent structures discriminant analysis, (L)PA (lyso-)phosphatidic acid, (L)PC (lyso-)phosphatidylcholine, (L)PE lyso-phosphatidylethanolamine, pESI positive ion mode in electrospray ionization, (L)PG (lyso-) phospatidylglycerol, (L)PI (lyso-)phosphatidylinositol, NP-HPLC normal phase liquid chromatography, PIS precursor ion scan, PLS-DA partial least squares discriminant analysis, PRM parallel reaction monitoring, (L)PS (lyso-

)phosphatidylserine, QqQ triple quadrupole mass analyzer, QTOF quadrupole - time of flight, ROC receiver operating characteristics curve, RP-HPLC reversed phase liquid chromatography, RT retention time, SP sphingolipid, SFC supercritical fluid chromatography, SWATH sequential window acquisition of all theoretical fragment ion mass spectra, SWARM sliding window adduct removal method, TG triacylglycerol, TOF time of flight mass analyzer, UHPLC ultra-high performance liquid chromatography, VIP variable importance projection

1. Introduction

Recent lipid research has emerged due to improved multidimensional computer algorithms and highly efficient commercial, open-source and in-house software platforms. Furthermore, data libraries for automated routine searching of mass spectra is adopted for lipid identification. International organizations have started together to harmonize knowledge about lipidomics. Especially, the Lipidomics Standard Initiative (LSI, https://lipidomics-standards-initiative.org/) found under the International Lipidomic Society (https://lipidomicssociety.org/about/) and Lipid Home (https://www.lipidhome.co.uk/) strive to standardize lipidomic information with the globally acknowledged platform Lipid MAPS (https://www.lipidmaps.org/). Furthermore, Sumner et al. [1] have stated minimal requirements for retraceable lipid analyses, **[1]** which are important for integrated research in lipidomics.

This review on lipid investigatios encompasses research conducted with ultra-high performance mass spectrometry (UHPLC-MS) during 2017-2019 **[2-79].** To clarify, this review uses the term high performance liquid chromatography (HPLC) to describe both conventional HPLC and UHPLC. However, blindspots of exclusive UHPLC-research are attempted to be covered in the Discussion-section.

Particularly, half of the reviewed studies deal with computerized platforms to identify lipid species of several classes. These computerized platforms have successfully enabled data handling with *in silico* analyses, commonly generalized as machine learning algorithms. After

all, the most popular means of comprehensive identification in -omics research is on-line coupled mass spectrometry systems (MS^n, e.g. tandem mass spectrometry [MS/MS]), which demand extensive and thorough data-handling. In the field of lipidomics, MS^n is often divided into data-dependent acquisition (DDA) and data-independent acquisition (DIA). Here, DDA is typically defined as fragmentation of only targeted precursors, as is the case in parallel reaction monitoring (PRM) **[15], [44], [45].** It is a technique, in which all fragments of the chosen lipid precursors are measured. In DIA, all available lipid precursors are fragmented and measured in a full scan with a set of collision energy (CE) or energies, detecting a substantial amount of lipid adducts fragmentation patterns, e.g. in an all ions scan (MS^E) **[9-15], [32-34], [55-61], [68], [74].**

Recent DIA research were applied in a novel MS technique called sequential window acquisition of all theoretical fragment ions spectra (SWATH MS) **[6], [44], [73-76]** and similarly, with QTOF-specific SONAR technology **[69].** The consecutive fragmentation or scan of precursors (i.e. PRM for DDA and SWATH or SONAR for DIA) and their product ions provide cleaner MS spectra in favour of library searches, an improved detection rate, broadened range, and expanded specificity in any given fragmentation frame compared to other methods **[34], [44], [73], [69].** Different MS and MS/MS techniques are compiled in **Table 1**. When significant, instrumental and experimental conditions for glycero-, glycerophospho- and sphingolipids are focused on and referred to **[80-88].**

2 Fatty acids, lipids and metabolites of the survey

Fatty acids are synthetized in cells and their cell membranes, endoplasmatic reticulum, Golgi apparatus, and mitochondria **[89], [90]**. Most lipids are products of free fatty acids in presence of coenzyme A and NADPH **[91].** This literature survey deals with a short area of lipidomics and contains commonly studied lipids, e.g. glycerolipids (GLs) like mono- (MG), di- (diacyl-, DG), and triacylsubstituted (triacyl-, TG) glycerols. **[91]** To look the structural challenges of

lipids in analytics, **Figure 1** illustrates the functionalities of TG lipids. Glycerophospholipids (GPs) from GLs are also included, since they are frequently detected like the most abundant lipids in eukaryotic cells, i.e. phosphatidylcholine (PC) and phosphatidylethanolamine (PE) **[92]**. Other GPs dealt in this review include phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerols (PGs), and PG-derived cardiolipins (CLs). **[92]** To observe the challenges of separation and identification issues some glycerolipids and glycerophospholipids are illustrated in **Figure 2**.

GPs may appear with fatty acids which both are bound with ester or ether groups, the latter being defined as plasmalogens (PLs) **[92]**. PLs are detected and identified either by an alkenyl linker with an oxygen (e.g. PI[O-18:0/17:0]) or with a phosphorous (e.g. PI[P-18:0/17:0]) **[92]**. Those structural hetero atoms help mass spectrometric detection, although there may not be improvements in chromatographic separation.

An interesting group of surveyed lipids is sphingolipids (SPs, **Figure 3**, [**28**]). They have a basic backbone with various kinds of lipids. The backbone is formed from serine amino acid and a long-chain fatty acyl catalyzed by coenzyme A in mammals **[92]**. The analytics is focused to ceramides (Cer), which have amine-bonds with fatty acids. To sum up, interest is focused on esterified fatty acids with glycerol head groups (glycerolipids, glycerophospholipids) or a sphingosine headgroup (sphingolipids), but not a sugar backbone (saccharolipids, a topic of worth its own review). Other groups left out are defined by characteristic hydrocarbon structures, i.e. fatty acyls, prenol lipids, sterols and polyketides **[94].** More analysis on lipid classes and metabolism is discussed elsewhere [101]

3 Instrumentation

In lipidomics, a wide range of articles introduce new or improved methods which are validated with UHPLC-MS, capillary-UHPLC-MS (nUHPLC-MS or nano-UHPLC-MS) instruments (**Table 2**). Most of these studies concentrate on sample preparation **[14], [20], [46], [66],**

development of chromatographic methods **[9], [22], [27], [45]**, MS/MS **identification [7], [16], [23], [27],** computational methods for improvement of measurement performance **[17],** and data analysis **[21], [25], [96].** Recent challenges in HPLC-MS seem to be fluctuation (repeatability) of analysis **[97], [98]** results, which hinder reliable identification and inter-laboratory tests of lipids. Since in chromatographic environments elution of different lipid types and their species may differ **[98]**, deconvolution of data via manual or computational analysis may be the only reasonable compromise.

The reviewed papers show that all lipid analyses have fluctuation of lipid composition and intensity based on the fingerprint profiles of different biological matrices. These observations can be explained by distinctive matrix effects, which in turn inform that there is a specific need of sample preparation before analyses. Usually, lipidome studies are done with simplified sample preparation to avoid recovery losses in clean-up steps. **[99]** Mostly, the clean-up steps are protein precipitation and extraction of solid matrix compounds (e.g. biological tissues) and fluids (e.g. plasma, serum, lavages, cell suspensions and supernatants).**[99]** Furthermore, solid materials may trap internal standards and analytes, which increases variation of results in quantification and leads to less accuracy and precision of the methods**. [100]** Sample preparation of biological and clinical samples is discussed more elsewhere **[101]**.

Though reconstitution with the most used polar acetonitrile - water mixture (60:40, v/v) and organic isopropanol - acetonitrile (90:10, v/v) eluents is practical, none of these dissolve lipids comprehensively. For instance, acetonitrile - water at 60:40 (v/v) recovers STs and TGs incompletely. [**45**] Furthermore, Danne-Rasche et al. [**45**] observed a distortion or even loss of lipids with i-propanol - acetonitrile mixture (9:1, v/v), when the eluent was used in nano-UHPLC. Thus, lipids need to be reconstituted into a standardized mixture such as butanol - i-propanol - water (8:23:69, v/v/v) [**45**] prior to injection. The solvent composition is important for lipid solubility. Due to high solvent volumes from pretreatment processes at the end of

sample preparation, all lipid samples need to be dried for getting concentrates of HPLC-MS research. Furthermore, concentrates are needed to measure multidimensional MS spectra to obtain accurate identification and algorithm-based data handling of features.

To reduce systematic noise and other disadvantages (e.g. adsorption, peak broadening, adduct formation) in chromatographic data has been attempted via computational methods, i.e. with the sliding window adduct removal method (SWARM). **[102]** SWARM is based on the systematic interference caused by multiple adduct ions of the same analyte. The interference induces systematic noise patterns that may be excluded to enhance accurate mass acquisition for metabolites. Then, the adduct signal overlap correction for low-to-moderate resolution mass spectra could be utilized.

Modern instrumental separation and detection methodologies are still on-going needed to avoid frequent appearance of false negative lipid identifications and to establish sufficiently reliable and label-free (i.e. standard-free) methods. Identification of lipids showed to be improved by multiply usability of analytical variables and by using more independent properties, such as chromatographic retention times (RT) and mass spectrometric mass-to-charge ratios *(m/z)* of lipid ions/adducts, fragmentation/isotope patterns, and collision reactions. Reliability for targeted lipid species identified by internal standards and calibrants have made lipid analyses possible in quite many case, but especially non-targeted lipid analyses demand the super power and speed of computers with algorithm-based libraries.**[96], [103], [104]** Thus, the analyses can be independently conducted without potent analytes. Evidently, the computerized methods have utilized automated systems when internal standards for normalization are neglected. **[2], [3], [10], [11], [13], [14], [16], [17], [20], [21], [22], [24], [26], [30], [32-36], [38], [44], [4 6], [47], [59], [63], [66], [73].**

3.1 UHPLC

HPLC techniques are preferred for their ability to enhance identification by predictable retention time patterns **[98], [105]** and reduced mass ambiguity **[106]**. In lipid research, the sub-method UHPLC is preferred over HPLC due to its lower dispersion, substantial decrease in solvent usage, shorter analysis times without compromising resolution, higher resolution performance due to smaller (sub-2 µm) fully porous particles or (sub-3 µm) core-shell material **[73]** in columns (enabled by higher pressure capabilities) and enhanced retention precision. **[107]**

Our dataset **[2-79], [108]** informs current lipidomic UHPLC-MS separations to be primarily done with additive-assisted reversed phase liquid chromatography (RP-HPLC) hyphenated with separate experiments on positive electrospray ionization (pESI) and negative electrospray ionization (nESI). Though ESI was predominantly used for UHPLC-MS, some atmospheric pressure chemical ionization (APCI) studies were also conducted**. [23], [44], [47].** ESI as a 'soft' and APCI as a 'hard' ionization technique, respectively, are suitable for supporting lipid identification. **[23]** In respect of that, Beccaria et al. **[23]** developed a method suitable for both detection approaches with no need to change HPLC parameters.

Elution of lipids in HPLC is done in many elution models (e.g. isocratic elution, curved gradient following an exponential or logarithmic function, stepped gradient, linear gradient, etc.). The suitable stationary and mobile phases with methods are also found in **Table 1**. More detailed information on stationary and mobile phases as well as lipid adduct concentrations is in **Supplementary (Table S1)**.

3.1.1 Reversed phase separation in HPLC

In lipidomics the commonly used RP-HPLC separation methods are based on the interaction of a nonpolar stationary phase with nonpolar lipid analytes. Nonlinear or second-degree curves between the retention times and total carbon (CN) double bonds (DB) are used for quantitative calculations. **[9], [16], [98], [105]** For example, they help to differentiate possible sodium

adducts from similar exact masses (e.g. [PC(38:4)+Na]$^+$ and [PC(40:7)+H]$^+$ **[9]).** However, retention time prediction can be complicated due to pH dependent speciation **[109],** experimentally made validation and multi-step liquid gradients **[23].** Separation of polar components (i.e. early retaining lipids) can be affected by the mobile phase gradient. When elution is started with high percentage of organic mixture, polar compounds are longer adsorbed and retained on RP-HPLC phases. **[110]** However, changes of eluent polarity are interlinked with the ionization rate of polar lipids, which in turn can have an impact on elution. **[110]** RP-HPLC with polar solvents (i.e. non-aqueous RP-HPLC) is enabled by polymer-based columns which are end-capped by a methyl or hydrogen group. **[110]** Some column materials are based on modified silica, which when not shielded are short-periodically used with water modified organic solvents due to free silanol groups having the attractive functions. **[110]**

In contrast to phenomena in reversed phases, normal phases in HPLC (NP-HPLC) typically separates analytes containing polar functionalities having silanol, amino- or diol groups. **[111]** NP-HPLC is excluded from the review, since only a single article on NP-HPLC with amide column was observed during 2017-2019 on lipid separation **[31].**

Since even the variation among lipid class species can be substantial, it is not surprising that the polarity of their classes varies a lot. Tumanov and Kamphorst **[85]** (**Figure 4**) demonstrated the lipid-subclass range of four distinct chromatographic approaches, which are divided to two groups (one a RP, one a NP separation strategy in each group). The subclasses in (**A**) include lipids of the nonpolar kind, whereas the separations of (**B**) are modelled to the polar lipid subclasses. Particularly, polar (lyso-forms, MGs, sphingosine-related compounds, FAs, acyl carnitines and acetyl coenzyme A) and mid-polar (PS, PG, PI, Cer) lipids seem to be species often analysed with the negative ionization mode, though more species are primarily found with positive ionization. From the GLs only MGs have FA chains with 16 counts, i.e. they are small enough to be rather polar. As a thumb rule, SPs are on the mid-polar lipid spectrum, when

they have amide-bound FA chains. **[85]** Thus for reliable research data, accurate UHPLC-MS methods are needed to identify the individual lipid class species.

3.1.2 HILIC

Essentially, hydrophilic interaction liquid chromatography (HILIC) columns are micro-bore columns with stationary phases of mixed hydrophilic interactions for nonpolar compounds and hydrophobic interactions for polar compounds. However, nonpolar lipids (e.g. CEs, TGs) and lipids with only one hydroxyl group (e.g. Cer, DG, MG, and cholesterol) are often barely retained. **[16]** Nevertheless, HILIC is a well-established subclass of NP chromatography that allows usage of water as the eluent (5-40%, >2% needed) as in contrast to conventional NP. **[112]** HILIC platforms are ideal for quantitation, since lipid class species co-elute with their respective calibrants. **[109]**

PAs and PSs species are known to have broad or barely detectable peaks in RP-HPLC, thus, HILIC is used. **[112]** Furthermore, as PAs and LPAs co-elute among major lipid components (e.g. PCs, PEs, SMs) in RP-HPLC. Because of that the comparatively lower abundance of lipids in this class, PAs and LPAs have no selective fragment to differentiate from other lipid classes. Thus, proper methods for PA/LPA separation have been specifically approached with HILIC. **[113], [109]**

Though being a well-established technique for HPLC-MS quantitation, not many UHPLC analyses have used HILIC-columns for separation. **[16], [47]** Thus, HILIC applications are also excluded from the scope of this review.

3.1.3 Nano-UHPLC

In nano-HPLC **[112]** columns of sub-millimetre internal diameter (i.d.) are filled with conventional column packing materials. Published papers **[41], [71], [24], [45], [42]** inform only about using a loaded capillary (i.d.: 1-0.001 mm **[114]**) or a nanobore column (i.d.: 0.1-0.025 mm **[114]**). For comparison, a large majority of the lipid studies are done with the

smallest possible narrow bore columns (2.1-4 mm). Surprisingly, along with nano-HPLC a low-resolution triple-quadrupole mass spectrometer was unequivocally preferred.

In contrast to a faster UHPLC separation, in nano-HPLC the "loading time" followed by the sample elution was extended due to the low flow rate restrictions (0.3-0.35 µl/min). **[71]** Nano-HPLC methods show a great capacity for lipid identification with a low-resolution mass analyser, since then both an extremely broad identification range and high sensitivity at the low fmol-scale are gained using a low throughput (analysis time: 40-60 min). **[41], [45]** The result was that the low fmol range for almost every GLs, GPs, SPs and their lipid derivatives was reached in the positive ionization electrospray mode (pESI). For example, the calibration curve for a specific PE (17:0/14:1) demonstrated a linear relationship between 16-10000 fmol by pESI-MS detection and 0.64-2000 fmol by nESI-MS detection **[41]** By contrast, with HPLC-ESI-MS the linear dynamic range for the same analyte reached around 80-10000 fmol by pESI-MS and 16-2000 fmol by nESI-MS. [45] Similarly, Kim et al. **[115]** achieved a LOD-range from 59 fmol (LPC(17:0)) to 507 fmol (LPG(14:0)) with untargeted nano-UHPLC-ESI-MS/MS of lipoprotein by pESI and nESI ionization, respectively. **[115]**

3.1.4 Mobile phases in hyphenation of HPLC with MS

Mobile phases in HPLC are usually modified to help in lipid detection and their separation with HPLC. **[112]** As the sensitivity issues are concerned, composition of eluents is important to obtain stabile adduct ions in MS. **[23]** Volatile buffers (e.g. formic acid, acetic acid, or their ammonium salts **[116]**) are used in lipidomics methods. **[113]** However, earlier studies report lipid results with 5 µM phosphate buffer (nanoelectrospray ionization) **[9]** and 5 mM phosphoric acid **[45]**

In some cases, additives in HPLC eluents may cause analytes to become undetectable due to signal suppression. Cajka and Fiehn **[117]** studied the effects of five different modifiers in both pESI and nESI mode with two different HPLC columns filled with ethylene bridged hybrid

(BEH) particles and the slightly better charged surface hybrid (CSH) particles. **[117]** Whereas the experiments could be concluded by choosing the optimal mobile phase modifier to be formic acid or formic acid/ammonium acetate for pESI and ammonium acetate for nESI. Using a CSH C18 column with HPLC-pESI-MS studies Monnin et al. **[118]** reported about a further enhanced ionization efficiency by choosing 0.02% acetic acid as the additive in eluent. Whereas the signal for LPLs (except LSM) and GPs certainly increased by manifold, Cer and PCs experienced a decrease in peak area when compared with analyses data with 10 mM ammonium acetate. **[118]** However, specific to PCs their carbonate ion adducts showed 20-fold stronger signals compared to their ammonium adducts with CID-MS/MS. **[119]**

3.2 Importance of mass spectrometry detection in lipid research

Formation of adducts is dependent on the molecular structure and functional groups of a lipid (e.g. deprotonation of carboxyl groups in fatty acid with nESI). Lists of exact masses in MS and MS/MS, non-protonated adducts, possible collision energies and lipid classes/topics are listed in **Table 3**. Furthermore, sources like Lipid maps providing a free MS/MS prediction tool (http://lipidmaps.org/resources/tools/index.php) and a structure database library (http://lipidmaps.org/data/classification/LM_classification_exp.php) allow data handling in lipidomics.

MS advancements, such as a quadrupole Orbitrap mass analyzer (Q-Orbitrap) **[9], [17], [20], [22], [27], [31], [46], [49], [51], [55], [56], [62-65], [77], [79],** and NSI **[24], [45], [71]** have extremely increased resolution capabilities (theoretical plate number in HPLC >35 000 /m) for lipid identification. However, compromises need to be done before the analyses, since either mass accuracy or ion resolution is emphasized depending on whether identification or quantitation is preferred. Furthermore, identification is improved with the help of orthogonal measurements, such as collisional cross-section (CCS) values with ion mobility spectrometry (IMS), achieved with a drift tube coupled with MS instruments, such as a quadrupole time of

flight (QTOF) **[6], [16], [25], [68].** Furthermore, the inclusion of trapped IMS (TIMS) combined with parallel accumulation serial fragmentation (PASEF) has emerged to enhance complementary MS/MS and IMS data. **[126]**

For high-resolution MS, an Orbitrap **[2], [5], [34], QTOF [4], [6], [10-16], [21], [25], [28], [30], [32], [33], [36-40], [43], [44], [53], [54], [59-61], [66], [68-71], [71], AA073, [74], [75], AA076, [78]** and QOrbitrap **[3], [5], [9], [17-20], [22], [26], [27], [31], [34], [35], [46], [47], [49-51], [55-58], [60], [62-65], [72], [77], [79]** were used. However, some studies settled for low-resolution MS, mostly with a triple quadrupole instrument **[7], [8], [23], [24], [30], [41-43]**. Most rarely, pseudotargeted methods **[21], [30], [54]** (i.e. lipid identification with an *in silico* library), polarity switching **[5], [17], [23], [41], [49],** standalone nESI mode in MS **[29], [43], [68], NSI [45], [71]** and atmospheric pressure chemical ionization (APCI) **[23], [44], [47]** were used.

3.3 Data acquisition and processing

Identification and determination of lipids and their metabolites need commercial, open-source, and sophisticated tailor-made tools **[127].** The statistical tools, analysis software, and algorithms enable visualization and perceiving of patterns from large datasets and raw data **[103]**. Metabolic profiling of MS data is done with multi-variant programs, such as Metaboanalyst, **[10], [11], [17], [27], [32], [34], [37], [40], [46], [59], [63], [77] or MeV [3], [30], [65], [61], [79], [103]**,. All in all, reported software environments for data processing constitute mostly of R and SPSS languages.

Manual programming from the ground up or with borrowed code demands more computational expertise. Koelmel et al. **[96]** compared identification algorithms with the commercial R-based LipidMatch-tool, which is tailored for lipid identification softwares. [96] For a deeper understanding of differences in program functionalities, the article on “LipidMatch: an

automated workflow for rule-based lipid identification using untargeted high-resolution tandem mass spectrometry data“ gives excellent information **[96]**.

4 Analysis

4.1 Identification of lipids

Lipid identification can be enhanced by using information about the individual lipid itself, e.g. by increasing orthogonal information via "polarity switching" **[5], [17], [23], [41], [49]** instead of only using one ionization mode in the measurements or by the introduction of supporting measurements or (lipid class) expanding methods. Identification demands are partly already mentioned in *Introduction* and chapters, where chromatographic parameters are discussed referenced to resolution and sensitivity.

In lipid studies, absolute retention time (RT) variation during sample analyses is easily increased by small differences in experimental conditions (preparatory, chromatographic, and instrumental parameters). However, more repeatable separation techniques along with feature alignment enables more reliable identification with RTs in inter-laboratory studies in the future. **[128]** Since measurements of m/z ratio are significantly less deviant (parameters affecting m/z: ionization efficiency and mass spectrometric setup), single m/z, feature, and low-resolution spectra matching are the most used means for quick identification. **[7]** If needed, high resolution precursor and product ion spectra are usually obtained to achieve accurate identification between highly similar species. For further optimization of mass spectrometric identification, adduct formation and control of collision energy should also be studied and used. It is commonly agreed that adduct ions are formed mostly during ionization **[9], [73],** which is why lipid species appear at the same retention time in both pESI-MS and nESI-MS. Apart from protonated and deprotonated species, adding of millimolar concentrations of salts can be harnessed for signal enhancement **[129]**. Though at too high concentrations lipid signals can drastically be inhibited due to background noise caused by ion clusters **[129]**. Common ligand

ions are acetate (CH_3COO^-) [56], [68], formate ($HCOO^-$) [48], [50], [57], [58], [60], [75-77], and ammonium (NH_4^+) [48], [50], [56-58], [60], [74-76]. Sodium (Na+) and potassium (K+) adducts are also commonly observed, though no controlled addition of such salts was noticed [92].

High resolution RP-HPLC separates structural isomers of lipid classes, [9] up to the point of carbon chain regioisomers (i.e. sn-positions of lipids). However, comprehensive identification of isobars and isomers could not be achieved by solely using MS/MS. For example, Lisa et al. [16] and Blaženović et al. [25] reported unresolved isomers and isobars with identical CNs and DBs but different fatty acyl composition. Adducts with same nominal mass can be separated with MS/MS [23], but specific information like sn-position of DBs needs other methods, like derivatizing of free fatty acids to form 4-iodobenzyl esters [130]. The reaction is made to esterified fatty acids into distinguishable epoxides via ketone dioxide catalyst and oxidant [131] or to oxetane-adducts via the Paternó-Büchi photochemical reaction. [132], [133], [134] Mass spectrometric solutions include second-generation fragmentation (MS/MS/MS) by dual stage collision-induced dissociation (CID) [135] or CID and ozone-induced dissociation combined, or silver-ion chromatography (currently achieved only with TGs) [81], [112], [136]. Recently, Zhao et al. [119] also mentioned electron impact excitation of ions from organics (EIEIO) and photo-ultraviolet dissociation (PUVD, photodissociation [130]) in the list of non-CID methods for sn and DB position. Since lyso-forms of GPs, i.e. sn-2 acyl lipids retain less in a HPLC column than their sn-1 isomers, they can be identified as separated double peaks. [9]

4.2 Quantification of lipids

Burla et al. [99] (a statement of the global lipidome community) compiled guideline recommendations for quantitative analysis in clinical sample matrices, specifically absolute quantification of lipids in plasma and serum. For the quick comparison of absolute

quantification results, lipid concentrations should be reported in the SI-units mol/L or molar percentages whenever possible. **[99]**

In comparison to lipid identification, quantitative analysis is increasingly dependent on control samples as well as the quality and concentration of included standards, i.e. internal standards and calibrants. Common control samples include blanks and quality control (QC): Blanks are included for standard impurity monitoring and validation purposes, whereas QC samples (a pooled sample of all study samples in a batch) serves to monitor and correct batch-related uncertainty, instrumental errors and evaluate the performance of lipid analytes for validation purposes. **[99]**

Absolute quantitative calibration of lipids is either conducted via spiked QC samples (surrogate calibration) or by direct introduction of standards in the study sample as is often done in single-point and standard addition calibration. [75] However, single-point calibrations are reported to overestimate target lipid concentrations due to inherent flaws in the regression. [75] An ideal calibrant for complex matrices has an identical response factor, ionization efficiency and experiences comparable matrix effects as the analyte. [137]

For better understanding quantitative UHPLC-MS, some differences between HPLC-MS and DIMS should be pointed out. Though DIMS is more robust at the high-concentration lipids (µmol/L-mmol/L) of a sample, HPLC-MS is advantageous for the identification and quantification of low- and very low-abundance lipids (<nmol/L-mmol/L). **[137], [99]** Anyway, lipid class coverage and sensitivity are considerably improved in HPLC-MS experiments. **[99]** As an inherent disadvantage in **HPLC**-MS, the risk of lipid-lipid interaction increases due to the enrichment of same lipid species occurring in the column, though hetero-interaction between different lipid species decreases. **[137]** HPLC-MS uses peak areas in contrast to DIMS, where more stable ionization conditions enable quantification via peak intensity. **[82]**

Moreover, the fluctuating quality and quantity of molecular species in the ion source is a recurring problem for lipid quantifications. These issues are amplified by the increasing complexity of experimental setups, such as chromatographic gradients affecting electrical properties (i.e. ionization efficiency) of the eluent. This has implications not only for the ionization efficiency, but also for the MS instrument's response factor: alongside solvent composition, Drotleff et al. **[75]** reports post-acquisition MS^n procedures like SWATH or multiple reaction monitoring (MRM) to further deviate the detector's response values from one unit per molecule. Drotleff et al. **[75]** proposed "post-acquisition recalibration" as a reasonable compromise, if not enough sample is available for using the standard addition method. Then, sensitivities of analytes can be determined via a certified reference material. For lipids, only the NIST CRM 1950 plasma reference is currently acknowledged. **[75]** Standard mixtures, such as the Splash® Lipidomix® mixture (Avanti Polar Lipids Inc., Alabaster, AL, USA) have been used for mimicking corresponding lipid concentrations in experiments as in biological fluids, e.g. human plasma, and for normalization of the analyses **[14], [28], [66].** Since lipidome isotope labelling of yeast (LILY, complete lipidome carbon labelling with C13 isotope **[138]**) technology is discovered very recently, studies on using it in practice could not be considered in this review.

Furthermore, solvent-system dependent lipid concentrations of >10-100 mmol/L are reported to form significant amounts of poorly ionic lipid aggregates (results found in a DIMS experiment). **[137]** The formation of aggregates like dimer, oligomer and micellar structures is further increased by the hydrophobicity of the lipid analyte and polarity of the mobile phase. **[137]**

Biological matrices include inherent variation in concentrations. UHPLC-MS experiments mostly revolve relative quantification and study changes in the lipidome of biological systems. However, in general HPLC-MS lipidomics has grown enough to advance further in absolute

quantification of targeted lipids. [99] Main limitations of absolute quantification in HPLC-MS concern ambiguities preceding calibration, i.e. the structural versatility of lipid species, lipid concentrations at the lower (or upper) border of their linearity range, and various matrix effects such as ion suppression and enhancement. [99] Whereas polar lipid species quantification reaches a ~5% accuracy (due to the polar headgroup predominantly explains their MS sensitivity) [137]. Mid- to nonpolar lipids like TGs and cholesteric esters are more effected by their specific structure in ESI-MS. [104] Thus, these lipids need more attention in standard representation. More details on quantitative and validation in lipid analyses are informed in Refs. [99] and [104].

5 Data analysis of lipids

According to the lipidome community, experimentally acquired raw data should be available for result-validation purposes and study re-analysis, whereas analytical details and results should be included in a file with acknowledged "XML[-based] or structured tabular format". [99] Corresponding raw data information can be taken from the proteomic field, which has practical formats for efficient data sharing. [99]

5.1 Normalization

Normalization of raw data is used to reduce systematic fluctuation concerning an accurate measurement. For example, Boysen et al. [139] developed the best matched internal standard normalization (B-MIS), an algorithm-driven solution for correcting non-biological variation of raw data (i.e. ion suppression by matrix effects, chromatographic quality and analytical drift). Obscuring variation, i.e. changes of peak area as a function of concentration, is compared between an unrestricted set of internal standards and analyte peaks in a QC sample. As a result, the algorithm chooses a suitable standard for normalization if the relative standard deviation of the peak area is improved by 40%. [139] Furthermore, Drotleff et al. [76] considered B-MIS to be the best normalization model for reducing intragroup variation between the coefficient of

variation, median absolute deviation and variance. His team observed a small difference in real positives favouring this normalization, but also noted that normalization models may work in a complementary fashion with their respective strengths and weaknesses. **[76]** Though not yet applied in absolute quantification experiments, B-MIS appears as a good alternative for model-based peak area normalization in relative quantification methods. **[139]**, [76]

Normalization is done with single standards representing the whole range of lipid classes. Suitable internal calibration standards with the same head groups and similar, but not identical fatty acids (e.g. odd-numbered <1% abundant in higher organisms **[104]**), or isotope-labeled fatty acids are preferred for normalization **[104]**. The most used internal standards are deuterated at the carbon chain of their FA end to have either 7 or 9 deuterium atoms. [75] Internal standards included in QC samples are used for post-correction of systematic errors (i.e. drift and other batch-biases) **[104], [99]** like changes in peak shapes influencing the peak areas and for monitoring carry over **[112]**.

5.2 Data libraries

Typically, lipid metabolite features in untargeted analyses are compared to available library spectra. **[66], [118]** Therefore, acquired data is represented by a “closest-match” for the m/z or total ion spectrum that is compared, typically accompanied by a score from 1-100%. Evidently, this approach may lead to false positive results, when the database used is incomplete or when the reference data is noisy **[128]**.

Along with home-made databases **[30], [32], [33], [36], [51], [54], [68], [78],** commercial and open-source libraries have gained popularity and variety both for monitoring the lipid range with MS by characterizing fragmentation patterns for selective identity searching of lipids. Information about individual libraries and their respective types are compiled in **Table 4**. Specific tools like open-source softwares are mentioned in **Table 5**, whereas chemometric and statistical concepts are introduced in the next chapters. Additional information can be found

from the LSI document (https://lipidomics-standards-initiative.org/links) and LIPID MAPS pages (http://lipidmaps.org/resources/tools/index.php).

5.3 Chemometric tools

Typically, metabolic correlation and significance analyses of lipids is conducted with a set of visual chemometric analyses and statistical methods. Chemometric methods provide an overview on analyte trends and outliers in relationship to their observations (features, spectra, etc.) with principal component analysis (PCA) **[5], [7], [13], [16], [17-19], [27], [30], [31], [33-36], [38-40], [43], [47-49], [51], [52], [54], [56-60], [62-64], [66], [68], [69], [72], [73], [75-79], [124],** systematic but hidden/uncorrelated variation between lipidome states with partial least-squares regression – discriminant analysis (PLS-DA) **[3], [7], [10], [11], [26], [32], [34], [35], [48], [59], [60], [64], [76], [79],** or orthogonal projection of latent structures – discriminant analysis (OPLS-DA) **[4], [6], [9], [19], [27], [33], [36], [37], [49], [50], [52], [54], [57], [60], [69], [78], [79],** (relative or exact) concentration evaluation with boxplots **[10], [18], [19], [32], [34], [37], [40], [44], [45], [48], [58], [60], [61], [67], [69], [73], [76]** or a heatmap **[3], [5], [7], [10], [11], [17], [26-28], [30-32], [34-38], [40-42], [45], [47], [48], [52], [56-58], [62], [68], [69], [73], [77], [79]** visualizations for significant outlier detection such as the Bland-Altman plot **[16], [40], [43-45], [55],** analyte interaction or metabolic interlinkage of lipid species via hierarchical cluster analysis (HCA, often included with heatmap analysis) **[3], [5], [7], [10], [17], [26], [27], [34], [37], [39], [40], [47-49], [68], [69], [73], [77], [79],** interactive network **[3], [26], [47]** or pathway analysis **[3], [7], [11], [26], [27], [37], [60],** and diagnostic tools such as importance testing of PLS-DA variables with variable importance projection (VIP) **[3], [10], [26], [33-37], [48], [50], [52], [57], [64], [79],** OPLS-DA variable reliability and importance evaluation via S-plots **[6], [27], [50], [57], [69], [79]** or volcano plots **[11], [31], [41], [42], [48], [60], [63], [67], [74], [77], [78],** and method or sample comparisons with Venn diagrams **[11], [17], [27], [30], [45], [49], [60], [66], [76].**

PCA [140] pursues to flatten a large dataset with multiple variables to find the most important two sources of variation, i.e. principal components. This is done by comparing the transitions between all variables and flattening them into two dimensions with matrix operations. Thus, the most important information about multivariate data is found. This reduction process may help to divide the studied compounds into different groups and characterize them. [141] Similarly, PLS-DA seeks to flatten multivariate data to find the most fitting parallel two-dimensional plane representing the whole dataset, [140] whereas OPLS-DA reduces the dataset to an orthogonal plane found with the partial least square plateau of the dataset. [142] Briefly, HCA clusters and categorizes sample components according to their apparent covariant relationship. [143]

Before actual multivariate data-analysis, the data needs often to be transformed, i.e. cleaned, scaled and re-centered, as is often done with e.g. statistical noise corrections or discarding of noisy data/spectra/outliers, unit variance or Pareto scaling and mean centering for PCA. [144] Chemometric methods, such as PCA, PLS-DA and OPLS-DA have kept their positions as the most common tools for visualizing analyte groupings (PCA) and metabolic changes (PLS-DA, OPLS-DA). Furthermore, VIP has emerged as an emphasis estimator of PLS-DA variables [3], [8], [26], [32], [34], [37], [64], [79] emphasizing the importance (i.e. magnitude) of each variable. In this context, lipids in the projection plot with VIP scores >1 are most accountable to predict changes in metabolism. More extensive analyses can be found in *Ref.* [88]

5.4 Statistical tools

In lipidomic research, statistical methods have developed into a broad variety of numerical tests and visualization techniques. Targeted and untargeted methods are two distinctively different approaches, as well as the means of data processing. Gorrochategui et al. [103] divided the targeted processing of metabolites into 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. **[103]** In contrast to targeted approaches, untargeted approaches need more careful experimental MS data processing, data modification, and evaluation for pre-processing to attain sufficient data quality. By this way, relevant biomarkers can be identified from the totality of detected features/peaks. The data quality is directly affected by the sensitivity and resolving power of the applied instrumentation. The non-targeted steps include the same pathway elucidation as in the targeted analyses, however, with respective appropriate tools. **[103]**

Statistical methods include a null hypothesis to test similarities by using one dataset. However, the *p*-value parameter in the Student's *t*-test **[2], [10], [14], [15], [18], [19], [26], [27], [32], [37-39], [42], [50], [52], [54], [56], [57], [60], [61], [63], [67], [72], [79],** the non-parametric Mann-Whitney *U*-test, **[15], [31], [35], [41], [50], [57], [58], [74], [77],** and the analysis of variance test (ANOVA) **[2], [7], [10], [17], [22], [27], [32], [33], [46], [64], [69], [79]** are for comparison of two or more datasets. These methods are often used for validation of analytical data and for detection of changes (e.g. analyte concentrations) between a control sample and authentic samples. Together with statistical tests, correction methods such as the Benjamini-Hochberg test **[145]** are used to calculate the false discovery rate (FDR), i.e. minimizing false positive data in the dataset **[56-58], [60], [74], [76].**

The *t*-test designed for comparing two independent variables (e.g. patient versus control group lipidome data and that before and after drug intake) may be unreliable, when the sample size number is small (n<30) or when other kinds than normal distributions arise through the data processing **[63].** This is the case for most sample number described in the articles reviewed for this paper. The average number of real samples studied was around 10-20 with the excluded outliers of 283 **[36]** and 10115 **[94]** test subjects. As to analyse control samples, there was a

clear split of 7-16 and ~30 in the number of controls used in the individual studies **[30], [34].** Some cases of statistical testing of the datasets may be questionable, since e.g. *t*-tests were not reliable, when variances between populations were different. **[146]** For these kinds of cases, an unequal variance test like the **Storey [74], [115]** or Welch **[48]** *t*-test may be used instead **[48], [74].**

The Mann-Whitney U-test **[147]** (also known as the Wilcoxon rank-sum test) is used in mathematical data handling, when there are not any fixed parameters, which can be used 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 one. The ranking integer is divided, if two values are identical: for instance, when two datapoints have the value 2, they will be both noted as 1.5 **[147]**. Then, the separately ranked sample value sets are individually summed. When any of the sums reaches the critical value range of the *U*-test, the null hypothesis can be rejected. Akin to the Student´s *t*-test [63], the *U*-test is evaluated by determining the z-score ($z = \frac{x_i - \mu}{SD}$, where x_i is a value of a single datapoint, μ the population mean and SD the population standard deviation) from a normal distribution, suitable for a large number of studied samples (>30) **[147]**. In essence, the z-score is a distance-measurement of a single datapoint in relation to a normal distribution's standard deviation, determining the distance between its mean and single measurement.

A Mann-Whitney *U*-test **[147]** can be fitted for non-Gaussian distribution data. It is typically combined with a Benjamini-Hochberg (or Bonferroni-Holm **[63])** test to exclude false positive values, thus providing the FDR. These methods were applied in multiple studies to limit uncertainty in the results of lipids. Furthermore, Paepe et al. **[27]** and Gong et al. **[60]** used cross-validated ANOVA test (CV-ANOVA) to improve reliability of the identified analytes. The reviewed papers also gave information that Mann-Whitney *U*-tests and FDR corrections

with the Benjamini-Hochberg test **[145]** were used to discover the real positive results from analysed MS datasets.

In the Benjamini-Hochberg method **[145]** *p*-values of two sample sets (between the control and the test groups) are inflated mathematically to reveal possible false positive results. Since affected and unaffected lipids represent two distinct normal distributions, false positive values can be ideally seen to differentiate a distribution from the normal when using a big sample number in the study. In the data analysis of lipid species, the original *p*-values are inflated and excluded by their significance desired for the *p*-values (e.g. p=0.1, 10% significance). The *p*-values are then ranked from the smallest to the largest, after which the individual *p*-values are converted to a largest-to-smallest sequence. The largest *p*-value is kept, but the second largest *p*-value is determined as the smaller value of the two options. Therefore, either it is the same value preceding it, or it is the value calculated with a separate equation **[145]**.

Lately, Tietz-Bogert et al. **[31]** calculated the FDR value in a lipido-metabolomic study by searching significant biomarkers of primary sclerosing cholangitis **[31]**, 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 p=0.01 **[31].** Simpler classical methods like ANOVA for multiple variables **[2], [32], [46]** were only occasionally used.

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 within our scope **[3], [18], [21], [25], [33], [67**]. Due to machine learning techniques and their discovered use in omics, automated lipidomic analyses, receiver operating characteristic curve/area under curve (ROC/AUC) cross-validation analyses **[3], [60], [67], [76]** random forest studies **[18], [32], [59], [67],** neural network applications **[67], [96],** *in silico* spectra evaluation **[21],** and CCS value generation algorithms **[25]** have become in use.

6 Discussion

UHPLC-MS lipidomics is divided into isotope-labelled standard methods and fully standardless algorithm-based analyses. Furthermore, the separation and acquisition of highly abundant lipid classes (mmol/L) from mid- (µmol/L) and low-abundance (≤nmol/L) classes and their species is a challenge. **[113]** This is especially the case when (semi-)quantitative analysis is conducted for all quantifiable lipids in the sample, which can be the case for in-depth studies on biological materials.

When comparing matrix effects in pharmacokinetic analysis made with HPLC and supercritical fluid chromatography (SFC) coupled with ESI-MS/MS, Svan et al. **[148]** observed a higher amount of ion suppression in SFC than in HPLC due to more ion enhancement. Is this general notion also applicable to **HPLC**-MS in lipidomics? Further information may be obtained from matrix effects via post-column infusion **[148]** as demonstrated by Drotleff et al. **[75]** or observations from more laborious (though more informative) post-extraction process. **[149]** Both of these tools may be important for improving the understanding of lipidome analysis in the future, as sightings of such matrix effect analysis is rare in recent lipid papers.

For practical reasons, a limitation of the comprehensive lipidome analysis is caused by the lipids without reasonable sensitivity. **[9]** Thus, the solution in that case is the Pareto principle, i.e. a limited number of biomolecules (lipid species) can be explained and measured using the main part of the studied biomass. **[9]** This is especially understandable in studies, where all the components are not relevant to the scientific question proposed. For example, metabolic lipid profiling with uncorrelated variation analysis (PLS-DA, OPLS-DA) in combination with metabolic knowledge highlight certain lipids from others to determine the most viable biomarkers. These biomarkers may form a conjoint array, which can already explain the changes in metabolism.

The studies reviewed revealed a constant change, which affect retention time and peak focusing in a HPLC method: flow rate, gradient and oven temperature. Apart from an isocratic **[43]** and three-solvent eluents **[10], [32],** using aqueous - organic solvent gradients by increasing organic solvent percentage appeared in stepped (two **[21], [40], [48] to three [25], [29], [38], [39]** plateau steps or two **[6], [69]** to three **[51]** inclined steps), curved (exponential, **[8], [37], [45], [67]** logarithmic, **[2], [19], [24], [41], [42], [50], [71]** or s-curve **[20], [27], [39], [49], [59], [63], [66])** and most popularly in linear **[3], [9], [12], [13], [16], [17], [18], [22], [26], [28], [33], [34], [47], [52-54], [56], [67], [78]** form. Modifications and combinations such as an inverted (i.e. increase in polar solvent percentage) linear **[60], [79]** or logarithmic [**11]** gradient, pyramidic **[57], [77],** pit-like **[15],** modified *S*-curve **[5], [7], [45], [64]** linear/isocratic **[19], [23],** exponential/linear **[62], [68], [75],** exponential/exponential [65], *S*-curve/exponential **[64]** and *S*-curve/isocratic **[5]** gradient were also used.

In particular, it was observed that the lipid studies were done under various column heating temperatures, from room temperature to 65 ˚C. **[65]** Instrumentation for keeping the oven temperature stabile is very important in lipids analysis standardization. Furthermore, the research was done under various mobile phase temperatures, i.e. lipid analytes experienced significant temperature changes (up to 10-20 ˚C) from column inlet to outlet. **[107], [150]** In addition, as heat is propagated from the column walls to the centre, temperature gradients are evident. **[107]** Though this is partly considered by narrow bore packed columns and the pre-equilibration of the analyses, the eluents flown from solvent units in room-temperature may cool down the column unevenly resulting in separation of lipids with low resolution.

The choose of column properties in lipid analyses should be considered by sorbent chemistry **[109]**, particle size, pore size [86], and particle technology **[117]**. The unanimously preferred column (i.d. 2.1 mm) is not necessarily optimal, since narrower columns are more prone to wall effects. **[107], [144]** This is due to a relatively more heterogeneous packing in comparison to

bigger columns (internal diameter larger, i.d.). Moreover, a bigger i.d. (3 mm) is argued to enhance column efficiency and allow higher flow rates. **[107]** However, this may be an insignificant issue compared to the current disparity in lipidome method standardization and validation. **[97]**

Avanti Polar Lipids (https://avantilipids.com/tech-support/physical-properties/ionization-constants) provides pKa-values of phospholipids to be closest to buffer pKa at 2.6 (PS phosphate group), 3 (PAs), 2.9-3.5 (PGs) and 5.5 (carboxyl group of PS) when compared to the pKa values of formic acid (3.74 **[151]**) and acetic acid (4.75 **[151]**). This means that when those organic acids are used PSs (and PAs/PGs at low pH) are never once charged ions. Further problems may arise for amines such as in sphingosine (pKa 9.1 **[152]**), PE (9.6 **[153]**) or PS (9.8 **[153]**) when ammonia (pKa 9.25 **[151]**) is used. Modifiers used as eluent additives may also lead to fluctuations of pH in non-buffered systems in on-line coupled HPLC-MS. **[154]** The phenomena are possible either in the eluent during electrochemical ionization due to charge-balancing redox reactions, and during droplet shrinkage, since it may cause structural perturbations and discrepancy in the ratios of ionization species. **[154]**

In lipidome analytics, interdisciplinary analyses on multifunctional and computational methods will be crucial to effective and improved data processing. Furthermore, it is important to use the most practical parameters such as precursors, fragments, and CCS values in evaluation of data to get fast identification and determination of lipids. Multifunctional methods used are statistic and chemometric analyses, whereas computational methods include algorithms, data-processing interfaces, specific software, and machine learning strategies. Both method categories were attempted to be refined with tailored software platforms for necessary data pre-processing, pattern recognition, and for using large datasets with an immense amount of data-processing interfaces.

Concerning common terminology, Aristizabal et al. [155] suggest names depending on the acquired structural information: brutto (e.g. PC 36:1), medio (e.g. PC(18:0/18:1), sn-position not known), genio (e.g. PC(18:0/18:1), sn-position known) and infinio (e.g. PC(18:0/18:1-9Z), sn-position not known). The same research group identified two distinct trends in lipidomics, which are the global analysis of lipids (coined macrolipidomics) and the specialized analyses for low-abundant lipids (microlipidomics).

Finally, the recognition correlation of HCA analyses is derived from information often visualized in the Heatmaps. Since HCA sorts out (i.e. clusters) groups of similar analyte species with each other, similar tools can potentially be developed by means of identifying and distinguishing matrices from each other.

As the scope of this review focuses mainly on UHPLC applications, it may limit some aspects of progress in HPLC-MS lipidomics as a whole. However, this was deemed necessary as publications on lipidomics have drastically increased. [156], [157] In our opinion -as may be representative for lipid studies in general- the most neglected topic mainly left out in the dataset was nutritional lipidomics [133] (mainly personalized nutrition [158] and nutritional intervention [159] studies). This is in tune with the main tendency of lipidomics towards life-sciences and bioanalytical questions concerning physiology and pathology. Furthermore, UHPLC-IMS-MS^n studies were rarer than might be expected from current trends in HPLC-IMS-MS^n lipidomics. Our dataset between the years 2017-2019 (topics discussed elsewhere [101]) included research on lipid method development, physiological profiling and metabolic changes via multicomponent analysis including drug development and biomarker studies. Sample matrices included plants, microbiota, mammals, fish and human patients. [101]

As UHPLC instruments are less frequent in the total scheme of lipidomics compared to the commonly used HPLC instruments, limitations concerning this review's scope on lipidome applications must be noted. For instance, the use of acetone in an eluent mixture was only used

by Castro-Gómez et al. [15] though some studies use a HPLC-MS ternary gradient separation in the fashion of Graeve et al. **[160]** ; acetone/ethyl acetate (v/v 2:1) and alkane:ethyl acetate (alkane: e.g. isooctane or n-heptane, v/v 99.8:0.2) is applied. **[161]** The lipidome community has also gone out of its way to develop a comprehensive and high-throughput method for lipid analysis, which puts emphasis on simple and quick sample preparation, high resolution/sensitivity MS^n analysis and data handling via extensively automated data processing. Consequently, a derivatization step for UHPLC-MS was used only once in our dataset **[8]** where TMSD methylation was applied for separate acidic lipid analysis; neutral and basic lipids were directly analysed after sample preparation. **[8]** Though derivatization may be necessary for very low-abundance lipids or to increase lipid class specificity, another reason for the reduction of sample preparation steps is the minimization of experimental errors. **[137]** A particularly important outlier in lipidome applications was the research on double bond position elucidation; for the absolute determination of double bond positions, other options than multiple collision MS^n-experiments or spectral library matching have been developed.

7 Conclusions

Current challenges in lipidomics are closely related to the lack of uniform methods across the field. Compared with the lipid research generally made with RP-HPLC-MS, UHPLC-MS needs more focus on the chromatographic method parameters, such as stationary (e.g. column choice, oven temperature) and mobile phase properties (flow rate, pH, solvents, gradients, eluent modifiers).

Normalization with representative or single internal standards has been widely adopted in the field. Since computational methods alongside chemometric and statistical methods have increased in both importance and usage in HPLC-MS technology for cross-validation and data-analysis, lipidomics needs interdisciplinary studies to reach full potential with big and complex datasets. The process of typical data-analyses in lipidomics is suggested to be reduced into four

parts: Pre-processing, identification/quantitation, chemometric, and statistical analyses. A substantial amount of specific processing tools are provided, i.e. commercial and open-source ones, including self-made algorithms. Since ever, more lipid libraries have emerged as well for untargeted, relatively targeted and *in silico* methods. Currently, chemometric analyses appear to be popular in lipidomics, i.e. for developing methods, profiling lipids in samples, and evaluating metabolic relationships between lipid species. For accurate identification, ion mobility mass spectrometry has been applied with liquid chromatography, alongside chromatographic separation modes with mass-spectrometric ionization, or/and MS^n systems. When chemometric methods shed light to the inter- and intra-analyte properties of typically biological medium, statistical analyses were used for data-validation (e.g. significance testing and filtering out of false positives) and detection of abonormal changes (e.g. metabolic profiling of diseases).

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Supplementary Table S1. Experimental conditions for the solid and mobile phase in chromatography

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
Yes	T3 C18	1:1 MeOH:H_2O (pH 7.5) (PG;PS)	6:4 MeOH:ACN (GP;PS)		AmFo, FoA	2017	[15]
	C18 HSS	19:19:2 ACN:MeOH:H_2O	IPA		AmFo, FoA	2017	[8]
	C18 HSS T3	3:4:3 ACN:acetone:IPA (DG;TG)	3:7 ACN:IPA (DG;TG)		AmOH	2017	[15]
	C18 BEH	40:60 ACN:H_2O	90:10 IPA:ACN		AmAc, AcA	2017	[19]
	N/A	40:60 ACN:H_2O	90:10 IPA:ACN		AmAc	2017	[18]
	C18	50:50 ACN:H_2O	95:5 IPA:ACN		AmFo, FoA	2017	[20]
	C18 CSH	H_2O	50:50 IPA:ACN		AmFo, FoA	2017	[11]
	C18	60:40 MeOH/H_2O	60:40 MeOH:IPA		AmAc, AcA	2017	[7]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2017	[22]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2017	[21]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		-	2017	[4]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc, FoA	2017	[6]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2017	[22]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2017	[14]
	C18 HSS	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2017	[3]
	C18 BEH	90:10 ACN:H_2O	90:80:1 IPA:ACN:H_2O		AmFo, FoA	2017	[17]
	C18 BEH*	90:10 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, FoA	2017	[24]*
	C18 HSS T3	90:10 IPA:ACN	30:70 H_2O:ACN		AmFo	2017	[12]
	HILIC, BEH	H_2O	96:4 ACN:H_2O		AmAc	2017	[16]
	C8 BEH	H_2O	2:5 IPA:ACN		AmFo, FoA	2017	[9]
	C18 BEH	H_2O	50:50 IPA:ACN		-	2017	[19]
	C18**	H_2O	60:36:4 IPA:ACN:H_2O		AmFo	2017	[23]**
	C8	H_2O	70:30 ACN:IPA		AmAc, FoA	2017	[2]
	C18 BEH	H_2O	ACN	90:10 IPA:ACN	FoA	2017	[10]
	C18 HSS T3	H_2O	ACN		FoA	2017	[13]
	C18 BEH	H_2O	MeOH		AmAc	2017	[5]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
	C18 CSH	40:60 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[35]
	C18	45:55 ACN:H_2O			AcA	2018	[43]
	C18 BEH	60:40 ACN:H_2O	81:10:9 IPA:ACN:H_2O		AmFo, FoA	2018	[40]
	C18 CSH	60:40 ACN:H_2O	90:10 ACN:IPA		AmFo, FoA	2018	[39]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2018	[33]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2018	[38]
Yes	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[25]
	C18 HSS	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2018	[26]
Yes	C18*	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[45]*
Yes	C18***	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[45]***
	C8 BEH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2018	[30]
	C8 BEH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2018	[30]
	C18 BEH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2018	[28]
	C18 BEH	60:40 ACN:H_2O	90:8:2 IPA:ACN:H_2O		AmFo, FoA	2018	[46]
Yes	C18 BEH*	90:10 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, FoA	2018	[41]*
Yes	C18 BEH*	90:10 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, FoA	2018	[42]*
	C18 SB	H_2O	90:10 ACN:H_2O		AmAc, AcA	2018	[29]
	C18**	H_2O	ACN		FoA	2018	[44]**
	C18 BEH	H_2O	ACN	90:10 IPA:ACN	FoA	2018	[32]
	C18 BEH	H_2O	ACN		AcA	2018	[37]
	C18 HSS	H_2O	ACN		FoA	2018	[27]
	Amide	H_2O	MeOH		AmHCO3	2018	[31]
	Amide	H_2O	MeOH		AmFo	2018	[31]
	C18 BEH	H_2O	MeOH		PFPA, FoA	2018	[31]
	C18 BEH	H_2O	MeOH		PFPA, FoA	2018	[31]
	C18 CSH	H_2O	MeOH		FoA	2018	[34]
	C18 HSS	H_2O	MeOH		FoA	2018	[39]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
	C18 BEH*	10:90 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, NH3	2019	[71]
	C18 CSH	40:60 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2019	[77]
	C18	50:50 ACN:H_2O	20:80 IPA:MeOH		AmAc	2019	[52]
	C18	50:50 H2O:ACN	5:95 ACN:IPA		AmFo, FoA	2019	[50]
	C18	50:50 H2O:ACN	5:95 ACN:IPA		AmFo, FoA	2019	[57]
Yes	C18 CSH	60:40 ACN:H_2O	90:10 ACN:H_2O		AmFo, FoA	2019	[55]
	C18 HSS T3	60:40 ACN:H2O	90:10 ACN:IPA		AmFo, FoA	2019	[60]
	C18 CSH	60:40 ACN:H2O	90:10 ACN:IPA		AmFo, FoA	2019	[60]
Yes	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2019	[66]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo, FoA	2019	[74]
	C8	60:40 ACN:H2O	90:10 IPA:ACN		AmAc	2019	[56]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo, FoA	2019	[65]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo	2019	[79]
	C18	60:40 ACN:H2O	90:10 IPA:ACN		AmFo	2019	[78]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo, FoA	2019	[69]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo	2019	[62]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmAc	2020	[64]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmAc	2020	[64]
Yes	C18 BEH	60:40 ACN:H_2O	90:8:2 IPA:ACN:H_2O		AmFo, FoA	2019	[63]
Yes	C18 CSH	60:40 ACN:H2O	90:9:1 IPA:ACN:H2O		AmFo, FoA	2019	[75]
Yes	C18 CSH	60:40 ACN:H2O	90:9:1 IPA:ACN:H2O		AmFo, FoA	2019	[76]
	C18 CSH	60:40 H2O:ACN	90:10 IPA:ACN		AmFo, FoA	2019	[58]
	HILIC	70:30 ACN:H_2O	90:10 ACN:acetone		AmFo, FoA	2019	[47]
	HILIC**	70:30 ACN:H_2O	90:10 ACN:acetone		AmFo, FoA	2019	[47]**
	C18 BEH	ACN:H2O	IPA:ACN		AmFo	2019	[67]
	C18 CSH	H2O	16:3 ACN:MeOH		AcA	2019	[68]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
Yes	C8	H_2O	55:40:5 ACN:IPA:H_2O		AmAc	2019	[68]
	C18	H_2O	75:25 IPA:ACN		AmFo	2019	[59]
	C18	H_2O	75:25 IPA:ACN		AmAc	2019	[59]
	C18 BEH	H2O	ACN		FoA	2019	[67]
	F5	H2O	IPA		AmFo, FoA	2019	[51]
	C18	H_2O	MeOH		AmAc, FoA	2019	[53]
	C18	H_2O	MeOH		AmAc, FoA	2019	[53]
Yes	Phenyl	H_2O	MeOH		AmAc	2019	[49]
	C8 BEH	H2O	MeOH		AmFo, FoA	2019	[48]
	C18 BEH	MeOH	2:5 ACN:IPA		AmAc, FoA	2019	[54]
	C18 HSS	N/A	N/A		N/A	2019	[61]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

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Highlights

- Method development and application enhancements in lipidomics
- The review sums up chemometric and statistical methods for current lipidomics
- State of the art data collection and evaluation is discussed
- Identification/quantitation of biological lipids
- Tandem MS data-independent and data-dependent analysis

Advances in analytical tools and current statistical methods used in ultra-high-performance liquid chromatography-mass spectrometry of glycero-, glycerophospho- and sphingolipids

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Abstract

The review concentrates on the properties of analytical and statistical ultrahigh-performance liquid chromatographic (UHPLC) – mass spectrometric (MS) methods suitable for glycero-, glycerophospho- and sphingolipids in lipidomics published between the years 2017-2019. Trends and fluctuations of conventional and nano-UHPLC methods with MS and tandem MS detection were observed in context of analysis conditions and tools used for data-analysis. Whereas general workflow characteristics are agreed upon, more details related to the chromatographic methodology (i.e. stationary and mobile phase conditions) need evidently agreements. Lipid quantitation relies upon isotope-labelled standards in targeted analyses and fully standardless algorithm-based untargeted analyses. Furthermore, a wide spectrum of setups have shown potential for the elucidation of complex and large datasets by minimizing the risks of systematic misinterpretation like false positives. This kind of evaluation was shown to have increased importance and usage for cross-validation and data-analysis.

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Lipidomics, mass spectrometry, ultrahigh performance liquid chromatography, nano-liquid chromatography, chemometrics, statistical methods, multicomponent analysis

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Figures included: 4 **Tables included:** 5

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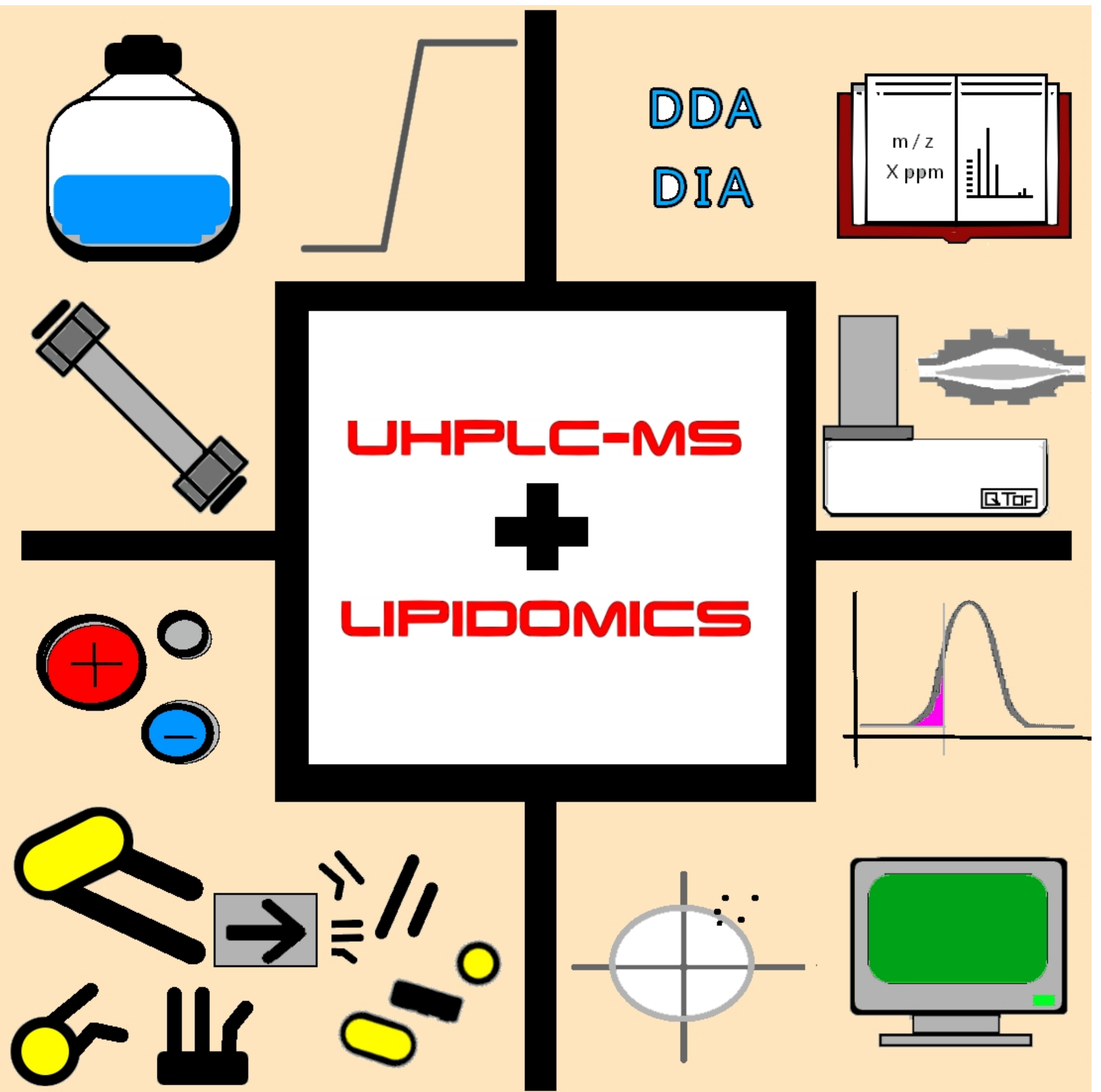

DDA
DIA
m / z
X ppm
UHPLC-MS
LIPIDOMICS
QTOF

Advances in analytical tools and current statistical methods used in ultra-high-performance liquid chromatography-mass spectrometry of glycero-, glycerophospho- and sphingolipids

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Abbreviations

ACP acyl carrier protein, AF4 asymmetric flow field flow fractionation, ANOVA analysis of variance, AUC area under curve, CARS coherent anti-Stokes Raman scattering, CCS collision cross-section, CN total carbon, CRS coherent Raman scattering, DB double bond, DDA data dependent acquisition, DESI desorption electrospray ionization, DG diacylglycerol, DIA data independent acquisition, DIMS direct infusion MS, FDR false discovery rate, FA fatty acyl, FFA free fatty acid, GC gas chromatography, GL glycerolipid, GP glycerophospholipid, HCA hierarchical cluster analysis, i.d. internal diameter, IMS ion mobility spectrometry, HPLC liquid chromatography, LESA liquid extraction surface analysis, m/z mass-to-charge [ratio], LSI Lipidomic Standards Initiative, HR/LRMS high/low resolution mass spectrometry, MS^E all ions scans, MS/MS tandem mass spectrometry, MS/MS/MS second-generation fragmentation mass spectrometry MS^n on-line coupled mass spectrometry system, MSI mass spectrometric imaging, m/z mass-to-charge ratio, NP normal phase, NSI nano-electrospray ionization, nESI negative ion mode in electrospray ionization, OPLS-DA orthogonal projections to latent structures discriminant analysis, (L)PA (lyso-)phosphatidic acid, (L)PC (lyso-)phosphatidylcholine, (L)PE lyso-phosphatidylethanolamine, pESI positive ion mode in electrospray ionization, (L)PG (lyso-) phospatidylglycerol, (L)PI (lyso-)phosphatidylinositol, NP-HPLC normal phase liquid chromatography, PIS precursor ion scan, PLS-DA partial least squares discriminant analysis, PRM parallel reaction monitoring, (L)PS (lyso-

)phosphatidylserine, QqQ triple quadrupole mass analyzer, QTOF quadrupole - time of flight, ROC receiver operating characteristics curve, RP-HPLC reversed phase liquid chromatography, RT retention time, SP sphingolipid, SFC supercritical fluid chromatography, SWATH sequential window acquisition of all theoretical fragment ion mass spectra, SWARM sliding window adduct removal method, TG triacylglycerol, TOF time of flight mass analyzer, UHPLC ultra-high performance liquid chromatography, VIP variable importance projection

1. Introduction

Recent lipid research has emerged due to improved multidimensional computer algorithms and highly efficient commercial, open-source and in-house software platforms. Furthermore, data libraries for automated routine searching of mass spectra is adopted for lipid identification. International organizations have started together to harmonize knowledge about lipidomics. Especially, the Lipidomics Standard Initiative (LSI, https://lipidomics-standards-initiative.org/) found under the International Lipidomic Society (https://lipidomicssociety.org/about/) and Lipid Home (https://www.lipidhome.co.uk/) strive to standardize lipidomic information with the globally acknowledged platform Lipid MAPS (https://www.lipidmaps.org/). Furthermore, Sumner et al. [1] have stated minimal requirements for retraceable lipid analyses, [1] which are important for integrated research in lipidomics.

This review on lipid investigatios encompasses research conducted with ultra-high performance mass spectrometry (UHPLC-MS) during 2017-2019 [2-79]. To clarify, this review uses the term high performance liquid chromatography (HPLC) to describe both conventional HPLC and UHPLC. However, blindspots of exclusive UHPLC-research are attempted to be covered in the Discussion-section.

Particularly, half of the reviewed studies deal with computerized platforms to identify lipid species of several classes. These computerized platforms have successfully enabled data handling with *in silico* analyses, commonly generalized as machine learning algorithms. After

all, the most popular means of comprehensive identification in -omics research is on-line coupled mass spectrometry systems (MS^n, e.g. tandem mass spectrometry [MS/MS]), which demand extensive and thorough data-handling. In the field of lipidomics, MS^n is often divided into data-dependent acquisition (DDA) and data-independent acquisition (DIA). Here, DDA is typically defined as fragmentation of only targeted precursors, as is the case in parallel reaction monitoring (PRM) [15], [44], [45]. It is a technique, in which all fragments of the chosen lipid precursors are measured. In DIA, all available lipid precursors are fragmented and measured in a full scan with a set of collision energy (CE) or energies, detecting a substantial amount of lipid adducts fragmentation patterns, e.g. in an all ions scan (MS^E) [9-15], [32-34], [55-61], [68], [74].

Recent DIA research were applied in a novel MS technique called sequential window acquisition of all theoretical fragment ions spectra (SWATH MS) [6], [44], [73-76] and similarly, with QTOF-specific SONAR technology [69]. The consecutive fragmentation or scan of precursors (i.e. PRM for DDA and SWATH or SONAR for DIA) and their product ions provide cleaner MS spectra in favour of library searches, an improved detection rate, broadened range, and expanded specificity in any given fragmentation frame compared to other methods [34], [44], [73], [69]. Different MS and MS/MS techniques are compiled in **Table 1**. When significant, instrumental and experimental conditions for glycero-, glycerophospho- and sphingolipids are focused on and referred to [80-88].

2 Fatty acids, lipids and metabolites of the survey

Fatty acids are synthetized in cells and their cell membranes, endoplasmatic reticulum, Golgi apparatus, and mitochondria [89], [90]. Most lipids are products of free fatty acids in presence of coenzyme A and NADPH [91]. This literature survey deals with a short area of lipidomics and contains commonly studied lipids, e.g. glycerolipids (GLs) like mono- (MG), di- (diacyl-, DG), and triacylsubstituted (triacyl-, TG) glycerols. [91] To look the structural challenges of

lipids in analytics, **Figure 1** illustrates the functionalities of TG lipids. Glycerophospholipids (GPs) from GLs are also included, since they are frequently detected like the most abundant lipids in eukaryotic cells, i.e. phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [92]. Other GPs dealt in this review include phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerols (PGs), and PG-derived cardiolipins (CLs). [92] To observe the challenges of separation and identification issues some glycerolipids and glycerophospholipids are illustrated in **Figure 2**.

GPs may appear with fatty acids which both are bound with ester or ether groups, the latter being defined as plasmalogens (PLs) [92]. PLs are detected and identified either by an alkenyl linker with an oxygen (e.g. PI[O-18:0/17:0]) or with a phosphorous (e.g. PI[P-18:0/17:0]) [92]. Those structural hetero atoms help mass spectrometric detection, although there may not be improvements in chromatographic separation.

An interesting group of surveyed lipids is sphingolipids (SPs, **Figure 3**, [28]). They have a basic backbone with various kinds of lipids. The backbone is formed from serine amino acid and a long-chain fatty acyl catalyzed by coenzyme A in mammals [92]. The analytics is focused to ceramides (Cer), which have amine-bonds with fatty acids. To sum up, interest is focused on esterified fatty acids with glycerol head groups (glycerolipids, glycerophospholipids) or a sphingosine headgroup (sphingolipids), but not a sugar backbone (saccharolipids, a topic of worth its own review). Other groups left out are defined by characteristic hydrocarbon structures, i.e. fatty acyls, prenol lipids, sterols and polyketides [94]. More analysis on lipid classes and metabolism is discussed elsewhere [101]

3 Instrumentation

In lipidomics, a wide range of articles introduce new or improved methods which are validated with UHPLC-MS, capillary-UHPLC-MS (nUHPLC-MS or nano-UHPLC-MS) instruments (**Table 2**). Most of these studies concentrate on sample preparation [14], [20], [46], [66],

development of chromatographic methods [9], [22], [27], [45], MS/MS identification [7], [16], [23], [27], computational methods for improvement of measurement performance [17], and data analysis [21], [25], [96]. Recent challenges in HPLC-MS seem to be fluctuation (repeatability) of analysis [97], [98] results, which hinder reliable identification and inter-laboratory tests of lipids. Since in chromatographic environments elution of different lipid types and their species may differ [98], deconvolution of data via manual or computational analysis may be the only reasonable compromise.

The reviewed papers show that all lipid analyses have fluctuation of lipid composition and intensity based on the fingerprint profiles of different biological matrices. These observations can be explained by distinctive matrix effects, which in turn inform that there is a specific need of sample preparation before analyses. Usually, lipidome studies are done with simplified sample preparation to avoid recovery losses in clean-up steps. [99] Mostly, the clean-up steps are protein precipitation and extraction of solid matrix compounds (e.g. biological tissues) and fluids (e.g. plasma, serum, lavages, cell suspensions and supernatants).[99] Furthermore, solid materials may trap internal standards and analytes, which increases variation of results in quantification and leads to less accuracy and precision of the methods. [100] Sample preparation of biological and clinical samples is discussed more elsewhere [101].

Though reconstitution with the most used polar acetonitrile - water mixture (60:40, v/v) and organic isopropanol - acetonitrile (90:10, v/v) eluents is practical, none of these dissolve lipids comprehensively. For instance, acetonitrile - water at 60:40 (v/v) recovers STs and TGs incompletely. [45] Furthermore, Danne-Rasche et al. [45] observed a distortion or even loss of lipids with i-propanol - acetonitrile mixture (9:1, v/v), when the eluent was used in nano-UHPLC. Thus, lipids need to be reconstituted into a standardized mixture such as butanol - i-propanol - water (8:23:69, v/v/v) [45] prior to injection. The solvent composition is important for lipid solubility. Due to high solvent volumes from pretreatment processes at the end of

sample preparation, all lipid samples need to be dried for getting concentrates of HPLC-MS research. Furthermore, concentrates are needed to measure multidimensional MS spectra to obtain accurate identification and algorithm-based data handling of features.

To reduce systematic noise and other disadvantages (e.g. adsorption, peak broadening, adduct formation) in chromatographic data has been attempted via computational methods, i.e. with the sliding window adduct removal method (SWARM). [102] SWARM is based on the systematic interference caused by multiple adduct ions of the same analyte. The interference induces systematic noise patterns that may be excluded to enhance accurate mass acquisition for metabolites. Then, the adduct signal overlap correction for low-to-moderate resolution mass spectra could be utilized.

Modern instrumental separation and detection methodologies are still on-going needed to avoid frequent appearance of false negative lipid identifications and to establish sufficiently reliable and label-free (i.e. standard-free) methods. Identification of lipids showed to be improved by multiply usability of analytical variables and by using more independent properties, such as chromatographic retention times (RT) and mass spectrometric mass-to-charge ratios *(m/z)* of lipid ions/adducts, fragmentation/isotope patterns, and collision reactions. Reliability for targeted lipid species identified by internal standards and calibrants have made lipid analyses possible in quite many case, but especially non-targeted lipid analyses demand the super power and speed of computers with algorithm-based libraries.[96], [103], [104] Thus, the analyses can be independently conducted without potent analytes. Evidently, the computerized methods have utilized automated systems when internal standards for normalization are neglected. [2], [3], [10], [11], [13], [14], [16], [17], [20], [21], [22], [24], [26], [30], [32-36], [38], [44], [4 6], [47], [59], [63], [66], [73].

3.1 UHPLC

HPLC techniques are preferred for their ability to enhance identification by predictable retention time patterns [98], [105] and reduced mass ambiguity [106]. In lipid research, the sub-method UHPLC is preferred over HPLC due to its lower dispersion, substantial decrease in solvent usage, shorter analysis times without compromising resolution, higher resolution performance due to smaller (sub-2 µm) fully porous particles or (sub-3 µm) core-shell material [73] in columns (enabled by higher pressure capabilities) and enhanced retention precision. [107]

Our dataset [2-79], [108] informs current lipidomic UHPLC-MS separations to be primarily done with additive-assisted reversed phase liquid chromatography (RP-HPLC) hyphenated with separate experiments on positive electrospray ionization (pESI) and negative electrospray ionization (nESI). Though ESI was predominantly used for UHPLC-MS, some atmospheric pressure chemical ionization (APCI) studies were also conducted. [23], [44], [47]. ESI as a 'soft' and APCI as a 'hard' ionization technique, respectively, are suitable for supporting lipid identification. [23] In respect of that, Beccaria et al. [23] developed a method suitable for both detection approaches with no need to change HPLC parameters.

Elution of lipids in HPLC is done in many elution models (e.g. isocratic elution, curved gradient following an exponential or logarithmic function, stepped gradient, linear gradient, etc.). The suitable stationary and mobile phases with methods are also found in **Table 1**. More detailed information on stationary and mobile phases as well as lipid adduct concentrations is in Supplementary (**Table S1**).

3.1.1 Reversed phase separation in HPLC

In lipidomics the commonly used RP-HPLC separation methods are based on the interaction of a nonpolar stationary phase with nonpolar lipid analytes. Nonlinear or second-degree curves between the retention times and total carbon (CN) double bonds (DB) are used for quantitative calculations. [9], [16], [98], [105] For example, they help to differentiate possible sodium

adducts from similar exact masses (e.g. $[PC(38:4)+Na]^+$ and $[PC(40:7)+H]^+$ [9]). However, retention time prediction can be complicated due to pH dependent speciation [109], experimentally made validation and multi-step liquid gradients [23]. Separation of polar components (i.e. early retaining lipids) can be affected by the mobile phase gradient. When elution is started with high percentage of organic mixture, polar compounds are longer adsorbed and retained on RP-HPLC phases. [110] However, changes of eluent polarity are interlinked with the ionization rate of polar lipids, which in turn can have an impact on elution. [110] RP-HPLC with polar solvents (i.e. non-aqueous RP-HPLC) is enabled by polymer-based columns which are end-capped by a methyl or hydrogen group. [110] Some column materials are based on modified silica, which when not shielded are short-periodically used with water modified organic solvents due to free silanol groups having the attractive functions. [110]

In contrast to phenomena in reversed phases, normal phases in HPLC (NP-HPLC) typically separates analytes containing polar functionalities having silanol, amino- or diol groups. [111] NP-HPLC is excluded from the review, since only a single article on NP-HPLC with amide column was observed during 2017-2019 on lipid separation [31].

Since even the variation among lipid class species can be substantial, it is not surprising that the polarity of their classes varies a lot. Tumanov and Kamphorst [85] (**Figure 4**) demonstrated the lipid-subclass range of four distinct chromatographic approaches, which are divided to two groups (one a RP, one a NP separation strategy in each group). The subclasses in (A) include lipids of the nonpolar kind, whereas the separations of (B) are modelled to the polar lipid subclasses. Particularly, polar (lyso-forms, MGs, sphingosine-related compounds, FAs, acyl carnitines and acetyl coenzyme A) and mid-polar (PS, PG, PI, Cer) lipids seem to be species often analysed with the negative ionization mode, though more species are primarily found with positive ionization. From the GLs only MGs have FA chains with 16 counts, i.e. they are small enough to be rather polar. As a thumb rule, SPs are on the mid-polar lipid spectrum, when

they have amide-bound FA chains. [85] Thus for reliable research data, accurate UHPLC-MS methods are needed to identify the individual lipid class species.

3.1.2 HILIC

Essentially, hydrophilic interaction liquid chromatography (HILIC) columns are micro-bore columns with stationary phases of mixed hydrophilic interactions for nonpolar compounds and hydrophobic interactions for polar compounds. However, nonpolar lipids (e.g. CEs, TGs) and lipids with only one hydroxyl group (e.g. Cer, DG, MG, and cholesterol) are often barely retained. [16] Nevertheless, HILIC is a well-established subclass of NP chromatography that allows usage of water as the eluent (5-40%, >2% needed) as in contrast to conventional NP. [112] HILIC platforms are ideal for quantitation, since lipid class species co-elute with their respective calibrants. [109]

PAs and PSs species are known to have broad or barely detectable peaks in RP-HPLC, thus, HILIC is used. [112] Furthermore, as PAs and LPAs co-elute among major lipid components (e.g. PCs, PEs, SMs) in RP-HPLC. Because of that the comparatively lower abundance of lipids in this class, PAs and LPAs have no selective fragment to differentiate from other lipid classes. Thus, proper methods for PA/LPA separation have been specifically approached with HILIC. [113], [109]

Though being a well-established technique for HPLC-MS quantitation, not many UHPLC analyses have used HILIC-columns for separation. [16], [47] Thus, HILIC applications are also excluded from the scope of this review.

3.1.3 Nano-UHPLC

In nano-HPLC [112] columns of sub-millimetre internal diameter (i.d.) are filled with conventional column packing materials. Published papers [41], [71], [24], [45], [42] inform only about using a loaded capillary (i.d.: 1-0.001 mm [114]) or a nanobore column (i.d.: 0.1-0.025 mm [114]). For comparison, a large majority of the lipid studies are done with the

smallest possible narrow bore columns (2.1-4 mm). Surprisingly, along with nano-HPLC a low-resolution triple-quadrupole mass spectrometer was unequivocally preferred.

In contrast to a faster UHPLC separation, in nano-HPLC the "loading time" followed by the sample elution was extended due to the low flow rate restrictions (0.3-0.35 µl/min). [71]

Nano-HPLC methods show a great capacity for lipid identification with a low-resolution mass analyser, since then both an extremely broad identification range and high sensitivity at the low fmol-scale are gained using a low throughput (analysis time: 40-60 min). [41], [45] The result was that the low fmol range for almost every GLs, GPs, SPs and their lipid derivatives was reached in the positive ionization electrospray mode (pESI). For example, the calibration curve for a specific PE (17:0/14:1) demonstrated a linear relationship between 16-10000 fmol by pESI-MS detection and 0.64-2000 fmol by nESI-MS detection [41] By contrast, with HPLC-ESI-MS the linear dynamic range for the same analyte reached around 80-10000 fmol by pESI-MS and 16-2000 fmol by nESI-MS. [45] Similarly, Kim et al. [115] achieved a LOD-range from 59 fmol (LPC(17:0)) to 507 fmol (LPG(14:0)) with untargeted nano-UHPLC-ESI-MS/MS of lipoprotein by pESI and nESI ionization, respectively. [115]

3.1.4 Mobile phases in hyphenation of HPLC with MS

Mobile phases in HPLC are usually modified to help in lipid detection and their separation with HPLC. [112] As the sensitivity issues are concerned, composition of eluents is important to obtain stabile adduct ions in MS. [23] Volatile buffers (e.g. formic acid, acetic acid, or their ammonium salts [116]) are used in lipidomics methods. [113] However, earlier studies report lipid results with 5 µM phosphate buffer (nanoelectrospray ionization) [9] and 5 mM phosphoric acid [45]

In some cases, additives in HPLC eluents may cause analytes to become undetectable due to signal suppression. Cajka and Fiehn [117] studied the effects of five different modifiers in both pESI and nESI mode with two different HPLC columns filled with ethylene bridged hybrid

(BEH) particles and the slightly better charged surface hybrid (CSH) particles. [117] Whereas the experiments could be concluded by choosing the optimal mobile phase modifier to be formic acid or formic acid/ammonium acetate for pESI and ammonium acetate for nESI. Using a CSH C18 column with HPLC-pESI-MS studies Monnin et al. [118] reported about a further enhanced ionization efficiency by choosing 0.02% acetic acid as the additive in eluent. Whereas the signal for LPLs (except LSM) and GPs certainly increased by manifold, Cer and PCs experienced a decrease in peak area when compared with analyses data with 10 mM ammonium acetate. [118] However, specific to PCs their carbonate ion adducts showed 20-fold stronger signals compared to their ammonium adducts with CID-MS/MS. [119]

3.2 Importance of mass spectrometry detection in lipid research

Formation of adducts is dependent on the molecular structure and functional groups of a lipid (e.g. deprotonation of carboxyl groups in fatty acid with nESI). Lists of exact masses in MS and MS/MS, non-protonated adducts, possible collision energies and lipid classes/topics are listed in **Table 3**. Furthermore, sources like Lipid maps providing a free MS/MS prediction tool (http://lipidmaps.org/resources/tools/index.php) and a structure database library (http://lipidmaps.org/data/classification/LM_classification_exp.php) allow data handling in lipidomics.

MS advancements, such as a quadrupole Orbitrap mass analyzer (Q-Orbitrap) [9], [17], [20], [22], [27], [31], [46], [49], [51], [55], [56], [62-65], [77], [79], and NSI [24], [45], [71] have extremely increased resolution capabilities (theoretical plate number in HPLC >35 000 /m) for lipid identification. However, compromises need to be done before the analyses, since either mass accuracy or ion resolution is emphasized depending on whether identification or quantitation is preferred. Furthermore, identification is improved with the help of orthogonal measurements, such as collisional cross-section (CCS) values with ion mobility spectrometry (IMS), achieved with a drift tube coupled with MS instruments, such as a quadrupole time of

flight (QTOF) [6], [16], [25], [68]. Furthermore, the inclusion of trapped IMS (TIMS) combined with parallel accumulation serial fragmentation (PASEF) has emerged to enhance complementary MS/MS and IMS data. [126]

For high-resolution MS, an Orbitrap [2], [5], [34], QTOF [4], [6], [10-16], [21], [25], [28], [30], [32], [33], [36-40], [43], [44], [53], [54], [59-61], [66], [68-71], [71], AA073, [74], [75], AA076, [78] and QOrbitrap [3], [5], [9], [17-20], [22], [26], [27], [31], [34], [35], [46], [47], [49-51], [55-58], [60], [62-65], [72], [77], [79] were used. However, some studies settled for low-resolution MS, mostly with a triple quadrupole instrument [7], [8], [23], [24], [30], [41-43]. Most rarely, pseudotargeted methods [21], [30], [54] (i.e. lipid identification with an *in silico* library), polarity switching [5], [17], [23], [41], [49], standalone nESI mode in MS [29], [43], [68], NSI [45], [71] and atmospheric pressure chemical ionization (APCI) [23], [44], [47] were used.

3.3 Data acquisition and processing

Identification and determination of lipids and their metabolites need commercial, open-source, and sophisticated tailor-made tools [127]. The statistical tools, analysis software, and algorithms enable visualization and perceiving of patterns from large datasets and raw data [103]. Metabolic profiling of MS data is done with multi-variant programs, such as Metaboanalyst, [10], [11], [17], [27], [32], [34], [37], [40], [46], [59], [63], [77] or MeV [3], [30], [65], [61], [79], [103],. All in all, reported software environments for data processing constitute mostly of R and SPSS languages.

Manual programming from the ground up or with borrowed code demands more computational expertise. Koelmel et al. [96] compared identification algorithms with the commercial R-based LipidMatch-tool, which is tailored for lipid identification softwares. [96] For a deeper understanding of differences in program functionalities, the article on “LipidMatch: an

automated workflow for rule-based lipid identification using untargeted high-resolution tandem mass spectrometry data“ gives excellent information [96].

4 Analysis

4.1 Identification of lipids

Lipid identification can be enhanced by using information about the individual lipid itself, e.g. by increasing orthogonal information via “polarity switching” [5], [17], [23], [41], [49] instead of only using one ionization mode in the measurements or by the introduction of supporting measurements or (lipid class) expanding methods. Identification demands are partly already mentioned in *Introduction* and chapters, where chromatographic parameters are discussed referenced to resolution and sensitivity.

In lipid studies, absolute retention time (RT) variation during sample analyses is easily increased by small differences in experimental conditions (preparatory, chromatographic, and instrumental parameters). However, more repeatable separation techniques along with feature alignment enables more reliable identification with RTs in inter-laboratory studies in the future. [128] Since measurements of m/z ratio are significantly less deviant (parameters affecting m/z: ionization efficiency and mass spectrometric setup), single m/z, feature, and low-resolution spectra matching are the most used means for quick identification. [7] If needed, high resolution precursor and product ion spectra are usually obtained to achieve accurate identification between highly similar species. For further optimization of mass spectrometric identification, adduct formation and control of collision energy should also be studied and used. It is commonly agreed that adduct ions are formed mostly during ionization [9], [73], which is why lipid species appear at the same retention time in both pESI-MS and nESI-MS. Apart from protonated and deprotonated species, adding of millimolar concentrations of salts can be harnessed for signal enhancement [129]. Though at too high concentrations lipid signals can drastically be inhibited due to background noise caused by ion clusters [129]. Common ligand

ions are acetate (CH_3COO^-) [56], [68], formate ($HCOO^-$) [48], [50], [57], [58], [60], [75-77], and ammonium (NH_4^+) [48], [50], [56-58], [60], [74-76]. Sodium (Na+) and potassium (K+) adducts are also commonly observed, though no controlled addition of such salts was noticed [92].

High resolution RP-HPLC separates structural isomers of lipid classes, [9] up to the point of carbon chain regioisomers (i.e. sn-positions of lipids). However, comprehensive identification of isobars and isomers could not be achieved by solely using MS/MS. For example, Lisa et al. [16] and Blaženović et al. [25] reported unresolved isomers and isobars with identical CNs and DBs but different fatty acyl composition. Adducts with same nominal mass can be separated with MS/MS [23], but specific information like sn-position of DBs needs other methods, like derivatizing of free fatty acids to form 4-iodobenzyl esters [130]. The reaction is made to esterified fatty acids into distinguishable epoxides via ketone dioxide catalyst and oxidant [131] or to oxetane-adducts via the Paternó-Büchi photochemical reaction. [132], [133], [134] Mass spectrometric solutions include second-generation fragmentation (MS/MS/MS) by dual stage collision-induced dissociation (CID) [135] or CID and ozone-induced dissociation combined, or silver-ion chromatography (currently achieved only with TGs) [81], [112], [136]. Recently, Zhao et al. [119] also mentioned electron impact excitation of ions from organics (EIEIO) and photo-ultraviolet dissociation (PUVD, photodissociation [130]) in the list of non-CID methods for sn and DB position. Since lyso-forms of GPs, i.e. sn-2 acyl lipids retain less in a HPLC column than their sn-1 isomers, they can be identified as separated double peaks. [9]

4.2 Quantification of lipids

Burla et al. [99] (a statement of the global lipidome community) compiled guideline recommendations for quantitative analysis in clinical sample matrices, specifically absolute quantification of lipids in plasma and serum. For the quick comparison of absolute

quantification results, lipid concentrations should be reported in the SI-units mol/L or molar percentages whenever possible. [99]

In comparison to lipid identification, quantitative analysis is increasingly dependent on control samples as well as the quality and concentration of included standards, i.e. internal standards and calibrants. Common control samples include blanks and quality control (QC): Blanks are included for standard impurity monitoring and validation purposes, whereas QC samples (a pooled sample of all study samples in a batch) serves to monitor and correct batch-related uncertainty, instrumental errors and evaluate the performance of lipid analytes for validation purposes. [99]

Absolute quantitative calibration of lipids is either conducted via spiked QC samples (surrogate calibration) or by direct introduction of standards in the study sample as is often done in single-point and standard addition calibration. [75] However, single-point calibrations are reported to overestimate target lipid concentrations due to inherent flaws in the regression. [75] An ideal calibrant for complex matrices has an identical response factor, ionization efficiency and experiences comparable matrix effects as the analyte. [137]

For better understanding quantitative UHPLC-MS, some differences between HPLC-MS and DIMS should be pointed out. Though DIMS is more robust at the high-concentration lipids (µmol/L-mmol/L) of a sample, HPLC-MS is advantageous for the identification and quantification of low- and very low-abundance lipids (<nmol/L-mmol/L). [137], [99] Anyway, lipid class coverage and sensitivity are considerably improved in HPLC-MS experiments. [99] As an inherent disadvantage in HPLC-MS, the risk of lipid-lipid interaction increases due to the enrichment of same lipid species occurring in the column, though hetero-interaction between different lipid species decreases. [137] HPLC-MS uses peak areas in contrast to DIMS, where more stable ionization conditions enable quantification via peak intensity. [82]

Moreover, the fluctuating quality and quantity of molecular species in the ion source is a recurring problem for lipid quantifications. These issues are amplified by the increasing complexity of experimental setups, such as chromatographic gradients affecting electrical properties (i.e. ionization efficiency) of the eluent. This has implications not only for the ionization efficiency, but also for the MS instrument's response factor: alongside solvent composition, Drotleff et al. [75] reports post-acquisition MS^n procedures like SWATH or multiple reaction monitoring (MRM) to further deviate the detector's response values from one unit per molecule. Drotleff et al. [75] proposed "post-acquisition recalibration" as a reasonable compromise, if not enough sample is available for using the standard addition method. Then, sensitivities of analytes can be determined via a certified reference material. For lipids, only the NIST CRM 1950 plasma reference is currently acknowledged. [75] Standard mixtures, such as the Splash® Lipidomix® mixture (Avanti Polar Lipids Inc., Alabaster, AL, USA) have been used for mimicking corresponding lipid concentrations in experiments as in biological fluids, e.g. human plasma, and for normalization of the analyses [14], [28], [66]. Since lipidome isotope labelling of yeast (LILY, complete lipidome carbon labelling with C13 isotope [138]) technology is discovered very recently, studies on using it in practice could not be considered in this review.

Furthermore, solvent-system dependent lipid concentrations of >10-100 mmol/L are reported to form significant amounts of poorly ionic lipid aggregates (results found in a DIMS experiment). [137] The formation of aggregates like dimer, oligomer and micellar structures is further increased by the hydrophobicity of the lipid analyte and polarity of the mobile phase. [137]

Biological matrices include inherent variation in concentrations. UHPLC-MS experiments mostly revolve relative quantification and study changes in the lipidome of biological systems. However, in general HPLC-MS lipidomics has grown enough to advance further in absolute

quantification of targeted lipids. [99] Main limitations of absolute quantification in HPLC-MS concern ambiguities preceding calibration, i.e. the structural versatility of lipid species, lipid concentrations at the lower (or upper) border of their linearity range, and various matrix effects such as ion suppression and enhancement. [99] Whereas polar lipid species quantification reaches a ~5% accuracy (due to the polar headgroup predominantly explains their MS sensitivity) [137]. Mid- to nonpolar lipids like TGs and cholesteric esters are more effected by their specific structure in ESI-MS. [104] Thus, these lipids need more attention in standard representation. More details on quantitative and validation in lipid analyses are informed in Refs. [99] and [104].

5 Data analysis of lipids

According to the lipidome community, experimentally acquired raw data should be available for result-validation purposes and study re-analysis, whereas analytical details and results should be included in a file with acknowledged "XML[-based] or structured tabular format". **[99]** Corresponding raw data information can be taken from the proteomic field, which has practical formats for efficient data sharing. **[99]**

5.1 Normalization

Normalization of raw data is used to reduce systematic fluctuation concerning an accurate measurement. For example, Boysen et al. [139] developed the best matched internal standard normalization (B-MIS), an algorithm-driven solution for correcting non-biological variation of raw data (i.e. ion suppression by matrix effects, chromatographic quality and analytical drift). Obscuring variation, i.e. changes of peak area as a function of concentration, is compared between an unrestricted set of internal standards and analyte peaks in a QC sample. As a result, the algorithm chooses a suitable standard for normalization if the relative standard deviation of the peak area is improved by 40%. [139] Furthermore, Drotleff et al. [76] considered B-MIS to be the best normalization model for reducing intragroup variation between the coefficient of

variation, median absolute deviation and variance. His team observed a small difference in real positives favouring this normalization, but also noted that normalization models may work in a complementary fashion with their respective strengths and weaknesses. [76] Though not yet applied in absolute quantification experiments, B-MIS appears as a good alternative for model-based peak area normalization in relative quantification methods. [139], [76]

Normalization is done with single standards representing the whole range of lipid classes. Suitable internal calibration standards with the same head groups and similar, but not identical fatty acids (e.g. odd-numbered <1% abundant in higher organisms [104]), or isotope-labeled fatty acids are preferred for normalization [104]. The most used internal standards are deuterated at the carbon chain of their FA end to have either 7 or 9 deuterium atoms. [75] Internal standards included in QC samples are used for post-correction of systematic errors (i.e. drift and other batch-biases) [104], [99] like changes in peak shapes influencing the peak areas and for monitoring carry over [112].

5.2 Data libraries

Typically, lipid metabolite features in untargeted analyses are compared to available library spectra. [66], [118] Therefore, acquired data is represented by a "closest-match" for the m/z or total ion spectrum that is compared, typically accompanied by a score from 1-100%. Evidently, this approach may lead to false positive results, when the database used is incomplete or when the reference data is noisy [128].

Along with home-made databases [30], [32], [33], [36], [51], [54], [68], [78], commercial and open-source libraries have gained popularity and variety both for monitoring the lipid range with MS by characterizing fragmentation patterns for selective identity searching of lipids. Information about individual libraries and their respective types are compiled in **Table 4**. Specific tools like open-source softwares are mentioned in **Table 5**, whereas chemometric and statistical concepts are introduced in the next chapters. Additional information can be found

from the LSI document (https://lipidomics-standards-initiative.org/links) and LIPID MAPS pages (http://lipidmaps.org/resources/tools/index.php).

5.3 Chemometric tools

Typically, metabolic correlation and significance analyses of lipids is conducted with a set of visual chemometric analyses and statistical methods. Chemometric methods provide an overview on analyte trends and outliers in relationship to their observations (features, spectra, etc.) with principal component analysis (PCA) [5], [7], [13], [16], [17-19], [27], [30], [31], [33-36], [38-40], [43], [47-49], [51], [52], [54], [56-60], [62-64], [66], [68], [69], [72], [73], [75-79], [124], systematic but hidden/uncorrelated variation between lipidome states with partial least-squares regression – discriminant analysis (PLS-DA) [3], [7], [10], [11], [26], [32], [34], [35], [48], [59], [60], [64], [76], [79], or orthogonal projection of latent structures – discriminant analysis (OPLS-DA) [4], [6], [9], [19], [27], [33], [36], [37], [49], [50], [52], [54], [57], [60], [69], [78], [79], (relative or exact) concentration evaluation with boxplots [10], [18], [19], [32], [34], [37], [40], [44], [45], [48], [58], [60], [61], [67], [69], [73], [76] or a heatmap [3], [5], [7], [10], [11], [17], [26-28], [30-32], [34-38], [40-42], [45], [47], [48], [52], [56-58], [62], [68], [69], [73], [77], [79] visualizations for significant outlier detection such as the Bland-Altman plot [16], [40], [43-45], [55], analyte interaction or metabolic interlinkage of lipid species via hierarchical cluster analysis (HCA, often included with heatmap analysis) [3], [5], [7], [10], [17], [26], [27], [34], [37], [39], [40], [47-49], [68], [69], [73], [77], [79], interactive network [3], [26], [47] or pathway analysis [3], [7], [11], [26], [27], [37], [60], and diagnostic tools such as importance testing of PLS-DA variables with variable importance projection (VIP) [3], [10], [26], [33-37], [48], [50], [52], [57], [64], [79], OPLS-DA variable reliability and importance evaluation via S-plots [6], [27], [50], [57], [69], [79] or volcano plots [11], [31], [41], [42], [48], [60], [63], [67], [74], [77], [78], and method or sample comparisons with Venn diagrams [11], [17], [27], [30], [45], [49], [60], [66], [76].

PCA [140] pursues to flatten a large dataset with multiple variables to find the most important two sources of variation, i.e. principal components. This is done by comparing the transitions between all variables and flattening them into two dimensions with matrix operations. Thus, the most important information about multivariate data is found. This reduction process may help to divide the studied compounds into different groups and characterize them. [141] Similarly, PLS-DA seeks to flatten multivariate data to find the most fitting parallel two-dimensional plane representing the whole dataset, [140] whereas OPLS-DA reduces the dataset to an orthogonal plane found with the partial least square plateau of the dataset. [142] Briefly, HCA clusters and categorizes sample components according to their apparent covariant relationship. [143]

Before actual multivariate data-analysis, the data needs often to be transformed, i.e. cleaned, scaled and re-centered, as is often done with e.g. statistical noise corrections or discarding of noisy data/spectra/outliers, unit variance or Pareto scaling and mean centering for PCA. [144] Chemometric methods, such as PCA, PLS-DA and OPLS-DA have kept their positions as the most common tools for visualizing analyte groupings (PCA) and metabolic changes (PLS-DA, OPLS-DA). Furthermore, VIP has emerged as an emphasis estimator of PLS-DA variables [3], [8], [26], [32], [34], [37], [64], [79] emphasizing the importance (i.e. magnitude) of each variable. In this context, lipids in the projection plot with VIP scores >1 are most accountable to predict changes in metabolism. More extensive analyses can be found in *Ref.* [88]

5.4 Statistical tools

In lipidomic research, statistical methods have developed into a broad variety of numerical tests and visualization techniques. Targeted and untargeted methods are two distinctively different approaches, as well as the means of data processing. Gorrochategui et al. [103] divided the targeted processing of metabolites into 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. [103] In contrast to targeted approaches, untargeted approaches need more careful experimental MS data processing, data modification, and evaluation for pre-processing to attain sufficient data quality. By this way, relevant biomarkers can be identified from the totality of detected features/peaks. The data quality is directly affected by the sensitivity and resolving power of the applied instrumentation. The non-targeted steps include the same pathway elucidation as in the targeted analyses, however, with respective appropriate tools. [103]

Statistical methods include a null hypothesis to test similarities by using one dataset. However, the *p*-value parameter in the Student's *t*-test [2], [10], [14], [15], [18], [19], [26], [27], [32], [37-39], [42], [50], [52], [54], [56], [57], [60], [61], [63], [67], [72], [79], the non-parametric Mann-Whitney *U*-test, [15], [31], [35], [41], [50], [57], [58], [74], [77], and the analysis of variance test (ANOVA) [2], [7], [10], [17], [22], [27], [32], [33], [46], [64], [69], [79] are for comparison of two or more datasets. These methods are often used for validation of analytical data and for detection of changes (e.g. analyte concentrations) between a control sample and authentic samples. Together with statistical tests, correction methods such as the Benjamini-Hochberg test [145] are used to calculate the false discovery rate (FDR), i.e. minimizing false positive data in the dataset [56-58], [60], [74], [76].

The *t*-test designed for comparing two independent variables (e.g. patient versus control group lipidome data and that before and after drug intake) may be unreliable, when the sample size number is small ($n<30$) or when other kinds than normal distributions arise through the data processing [63]. This is the case for most sample number described in the articles reviewed for this paper. The average number of real samples studied was around 10-20 with the excluded outliers of 283 [36] and 10115 [94] test subjects. As to analyse control samples, there was a

clear split of 7-16 and ~30 in the number of controls used in the individual studies [30], [34]. Some cases of statistical testing of the datasets may be questionable, since e.g. *t*-tests were not reliable, when variances between populations were different. [146] For these kinds of cases, an unequal variance test like the Storey [74], [115] or Welch [48] *t*-test may be used instead [48], [74].

The Mann-Whitney U-test [147] (also known as the Wilcoxon rank-sum test) is used in mathematical data handling, when there are not any fixed parameters, which can be used 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 one. The ranking integer is divided, if two values are identical: for instance, when two datapoints have the value 2, they will be both noted as 1.5 [147]. Then, the separately ranked sample value sets are individually summed. When any of the sums reaches the critical value range of the *U*-test, the null hypothesis can be rejected. Akin to the Student´s *t*-test [63], the *U*-test is evaluated by determining the z-score ($z = \frac{x_i - \mu}{SD}$, where x_i is a value of a single datapoint, μ the population mean and SD the population standard deviation) from a normal distribution, suitable for a large number of studied samples (>30) [147]. In essence, the z-score is a distance-measurement of a single datapoint in relation to a normal distribution's standard deviation, determining the distance between its mean and single measurement.

A Mann-Whitney *U*-test [147] can be fitted for non-Gaussian distribution data. It is typically combined with a Benjamini-Hochberg (or Bonferroni-Holm [63]) test to exclude false positive values, thus providing the FDR. These methods were applied in multiple studies to limit uncertainty in the results of lipids. Furthermore, Paepe et al. [27] and Gong et al. [60] used cross-validated ANOVA test (CV-ANOVA) to improve reliability of the identified analytes. The reviewed papers also gave information that Mann-Whitney *U*-tests and FDR corrections

with the Benjamini-Hochberg test [145] were used to discover the real positive results from analysed MS datasets.

In the Benjamini-Hochberg method [145] *p*-values of two sample sets (between the control and the test groups) are inflated mathematically to reveal possible false positive results. Since affected and unaffected lipids represent two distinct normal distributions, false positive values can be ideally seen to differentiate a distribution from the normal when using a big sample number in the study. In the data analysis of lipid species, the original *p*-values are inflated and excluded by their significance desired for the *p*-values (e.g. p=0.1, 10% significance). The *p*-values are then ranked from the smallest to the largest, after which the individual *p*-values are converted to a largest-to-smallest sequence. The largest *p*-value is kept, but the second largest *p*-value is determined as the smaller value of the two options. Therefore, either it is the same value preceding it, or it is the value calculated with a separate equation [145].

Lately, Tietz-Bogert et al. [31] calculated the FDR value in a lipido-metabolomic study by searching significant biomarkers of primary sclerosing cholangitis [31], 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 p=0.01 [31]. Simpler classical methods like ANOVA for multiple variables [2], [32], [46] were only occasionally used.

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 within our scope [3], [18], [21], [25], [33], [67]. Due to machine learning techniques and their discovered use in omics, automated lipidomic analyses, receiver operating characteristic curve/area under curve (ROC/AUC) cross-validation analyses [3], [60], [67], [76] random forest studies [18], [32], [59], [67], neural network applications [67], [96], *in silico* spectra evaluation [21], and CCS value generation algorithms [25] have become in use.

6 Discussion

UHPLC-MS lipidomics is divided into isotope-labelled standard methods and fully standardless algorithm-based analyses. Furthermore, the separation and acquisition of highly abundant lipid classes (mmol/L) from mid- (µmol/L) and low-abundance (≤nmol/L) classes and their species is a challenge. [113] This is especially the case when (semi-)quantitative analysis is conducted for all quantifiable lipids in the sample, which can be the case for in-depth studies on biological materials.

When comparing matrix effects in pharmacokinetic analysis made with HPLC and supercritical fluid chromatography (SFC) coupled with ESI-MS/MS, Svan et al. [148] observed a higher amount of ion suppression in SFC than in HPLC due to more ion enhancement. Is this general notion also applicable to HPLC-MS in lipidomics? Further information may be obtained from matrix effects via post-column infusion [148] as demonstrated by Drotleff et al. [75] or observations from more laborious (though more informative) post-extraction process. [149] Both of these tools may be important for improving the understanding of lipidome analysis in the future, as sightings of such matrix effect analysis is rare in recent lipid papers.

For practical reasons, a limitation of the comprehensive lipidome analysis is caused by the lipids without reasonable sensitivity. [9] Thus, the solution in that case is the Pareto principle, i.e. a limited number of biomolecules (lipid species) can be explained and measured using the main part of the studied biomass. [9] This is especially understandable in studies, where all the components are not relevant to the scientific question proposed. For example, metabolic lipid profiling with uncorrelated variation analysis (PLS-DA, OPLS-DA) in combination with metabolic knowledge highlight certain lipids from others to determine the most viable biomarkers. These biomarkers may form a conjoint array, which can already explain the changes in metabolism.

The studies reviewed revealed a constant change, which affect retention time and peak focusing in a HPLC method: flow rate, gradient and oven temperature. Apart from an isocratic [43] and three-solvent eluents [10], [32], using aqueous - organic solvent gradients by increasing organic solvent percentage appeared in stepped (two [21], [40], [48] to three [25], [29], [38], [39] plateau steps or two [6], [69] to three [51] inclined steps), curved (exponential, [8], [37], [45], [67] logarithmic, [2], [19], [24], [41], [42], [50], [71] or s-curve [20], [27], [39], [49], [59], [63], [66]) and most popularly in linear [3], [9], [12], [13], [16], [17], [18], [22], [26], [28], [33], [34], [47], [52-54], [56], [67], [78] form. Modifications and combinations such as an inverted (i.e. increase in polar solvent percentage) linear [60], [79] or logarithmic [11] gradient, pyramidic [57], [77], pit-like [15], modified *S*-curve [5], [7], [45], [64] linear/isocratic [19], [23], exponential/linear [62], [68], [75], exponential/exponential [65], *S*-curve/exponential [64] and *S*-curve/isocratic [5] gradient were also used.

In particular, it was observed that the lipid studies were done under various column heating temperatures, from room temperature to 65 ˚C. [65] Instrumentation for keeping the oven temperature stabile is very important in lipids analysis standardization. Furthermore, the research was done under various mobile phase temperatures, i.e. lipid analytes experienced significant temperature changes (up to 10-20 ˚C) from column inlet to outlet. [107], [150] In addition, as heat is propagated from the column walls to the centre, temperature gradients are evident. [107] Though this is partly considered by narrow bore packed columns and the pre-equilibration of the analyses, the eluents flown from solvent units in room-temperature may cool down the column unevenly resulting in separation of lipids with low resolution.

The choose of column properties in lipid analyses should be considered by sorbent chemistry [109], particle size, pore size [86], and particle technology [117]. The unanimously preferred column (i.d. 2.1 mm) is not necessarily optimal, since narrower columns are more prone to wall effects. [107], [144] This is due to a relatively more heterogeneous packing in comparison to

bigger columns (internal diameter larger, i.d.). Moreover, a bigger i.d. (3 mm) is argued to enhance column efficiency and allow higher flow rates. [107] However, this may be an insignificant issue compared to the current disparity in lipidome method standardization and validation. [97]

Avanti Polar Lipids (https://avantilipids.com/tech-support/physical-properties/ionization-constants) provides pKa-values of phospholipids to be closest to buffer pKa at 2.6 (PS phosphate group), 3 (PAs), 2.9-3.5 (PGs) and 5.5 (carboxyl group of PS) when compared to the pKa values of formic acid (3.74 [151]) and acetic acid (4.75 [151]). This means that when those organic acids are used PSs (and PAs/PGs at low pH) are never once charged ions. Further problems may arise for amines such as in sphingosine (pKa 9.1 [152]), PE (9.6 [153]) or PS (9.8 [153]) when ammonia (pKa 9.25 [151]) is used. Modifiers used as eluent additives may also lead to fluctuations of pH in non-buffered systems in on-line coupled HPLC-MS. [154] The phenomena are possible either in the eluent during electrochemical ionization due to charge-balancing redox reactions, and during droplet shrinkage, since it may cause structural perturbations and discrepancy in the ratios of ionization species. [154]

In lipidome analytics, interdisciplinary analyses on multifunctional and computational methods will be crucial to effective and improved data processing. Furthermore, it is important to use the most practical parameters such as precursors, fragments, and CCS values in evaluation of data to get fast identification and determination of lipids. Multifunctional methods used are statistic and chemometric analyses, whereas computational methods include algorithms, data-processing interfaces, specific software, and machine learning strategies. Both method categories were attempted to be refined with tailored software platforms for necessary data pre-processing, pattern recognition, and for using large datasets with an immense amount of data-processing interfaces.

Concerning common terminology, Aristizabal et al. [155] suggest names depending on the acquired structural information: brutto (e.g. PC 36:1), medio (e.g. PC(18:0/18:1), sn-position not known), genio (e.g. PC(18:0/18:1), sn-position known) and infinio (e.g. PC(18:0/18:1-9Z), sn-position not known). The same research group identified two distinct trends in lipidomics, which are the global analysis of lipids (coined macrolipidomics) and the specialized analyses for low-abundant lipids (microlipidomics).

Finally, the recognition correlation of HCA analyses is derived from information often visualized in the Heatmaps. Since HCA sorts out (i.e. clusters) groups of similar analyte species with each other, similar tools can potentially be developed by means of identifying and distinguishing matrices from each other.

As the scope of this review focuses mainly on UHPLC applications, it may limit some aspects of progress in HPLC-MS lipidomics as a whole. However, this was deemed necessary as publications on lipidomics have drastically increased. [156], [157] In our opinion -as may be representative for lipid studies in general- the most neglected topic mainly left out in the dataset was nutritional lipidomics [133] (mainly personalized nutrition [158] and nutritional intervention [159] studies). This is in tune with the main tendency of lipidomics towards life-sciences and bioanalytical questions concerning physiology and pathology. Furthermore, UHPLC-IMS-MS^n studies were rarer than might be expected from current trends in HPLC-IMS-MS^n lipidomics. Our dataset between the years 2017-2019 (topics discussed elsewhere [101]) included research on lipid method development, physiological profiling and metabolic changes via multicomponent analysis including drug development and biomarker studies. Sample matrices included plants, microbiota, mammals, fish and human patients. [101]

As UHPLC instruments are less frequent in the total scheme of lipidomics compared to the commonly used HPLC instruments, limitations concerning this review's scope on lipidome applications must be noted. For instance, the use of acetone in an eluent mixture was only used

by Castro-Gómez et al. [15] though some studies use a HPLC-MS ternary gradient separation in the fashion of Graeve et al. [160] ; acetone/ethyl acetate (v/v 2:1) and alkane:ethyl acetate (alkane: e.g. isooctane or n-heptane, v/v 99.8:0.2) is applied. [161] The lipidome community has also gone out of its way to develop a comprehensive and high-throughput method for lipid analysis, which puts emphasis on simple and quick sample preparation, high resolution/sensitivity MS^n analysis and data handling via extensively automated data processing. Consequently, a derivatization step for UHPLC-MS was used only once in our dataset [8] where TMSD methylation was applied for separate acidic lipid analysis; neutral and basic lipids were directly analysed after sample preparation. [8] Though derivatization may be necessary for very low-abundance lipids or to increase lipid class specificity, another reason for the reduction of sample preparation steps is the minimization of experimental errors. [137] A particularly important outlier in lipidome applications was the research on double bond position elucidation; for the absolute determination of double bond positions, other options than multiple collision MS^n-experiments or spectral library matching have been developed.

7 Conclusions

Current challenges in lipidomics are closely related to the lack of uniform methods across the field. Compared with the lipid research generally made with RP-HPLC-MS, UHPLC-MS needs more focus on the chromatographic method parameters, such as stationary (e.g. column choice, oven temperature) and mobile phase properties (flow rate, pH, solvents, gradients, eluent modifiers).

Normalization with representative or single internal standards has been widely adopted in the field. Since computational methods alongside chemometric and statistical methods have increased in both importance and usage in HPLC-MS technology for cross-validation and data-analysis, lipidomics needs interdisciplinary studies to reach full potential with big and complex datasets. The process of typical data-analyses in lipidomics is suggested to be reduced into four

parts: Pre-processing, identification/quantitation, chemometric, and statistical analyses. A substantial amount of specific processing tools are provided, i.e. commercial and open-source ones, including self-made algorithms. Since ever, more lipid libraries have emerged as well for untargeted, relatively targeted and *in silico* methods. Currently, chemometric analyses appear to be popular in lipidomics, i.e. for developing methods, profiling lipids in samples, and evaluating metabolic relationships between lipid species. For accurate identification, ion mobility mass spectrometry has been applied with liquid chromatography, alongside chromatographic separation modes with mass-spectrometric ionization, or/and MS^n systems. When chemometric methods shed light to the inter- and intra-analyte properties of typically biological medium, statistical analyses were used for data-validation (e.g. significance testing and filtering out of false positives) and detection of abonormal changes (e.g. metabolic profiling of diseases).

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Supplementary Table S1. Experimental conditions for the solid and mobile phase in chromatography

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
Yes	T3 C18	1:1 MeOH:H_2O (pH 7.5) (PG;PS)	6:4 MeOH:ACN (GP;PS)		AmFo, FoA	2017	[15]
	C18 HSS	19:19:2 ACN:MeOH:H_2O	IPA		AmFo, FoA	2017	[8]
	C18 HSS T3	3:4:3 ACN:acetone:IPA (DG;TG)	3:7 ACN:IPA (DG;TG)		AmOH	2017	[15]
	C18 BEH	40:60 ACN:H_2O	90:10 IPA:ACN		AmAc, AcA	2017	[19]
	N/A	40:60 ACN:H_2O	90:10 IPA:ACN		AmAc	2017	[18]
	C18	50:50 ACN:H_2O	95:5 IPA:ACN		AmFo, FoA	2017	[20]
	C18 CSH	H_2O	50:50 IPA:ACN		AmFo, FoA	2017	[11]
	C18	60:40 MeOH/H_2O	60:40 MeOH:IPA		AmAc, AcA	2017	[7]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2017	[22]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2017	[21]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		-	2017	[4]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc, FoA	2017	[6]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2017	[22]
	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2017	[14]
	C18 HSS	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2017	[3]
	C18 BEH	90:10 ACN:H_2O	90:80:1 IPA:ACN:H_2O		AmFo, FoA	2017	[17]
	C18 BEH*	90:10 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, FoA	2017	[24]*
	C18 HSS T3	90:10 IPA:ACN	30:70 H_2O:ACN		AmFo	2017	[12]
	HILIC, BEH	H_2O	96:4 ACN:H_2O		AmAc	2017	[16]
	C8 BEH	H_2O	2:5 IPA:ACN		AmFo, FoA	2017	[9]
	C18 BEH	H_2O	50:50 IPA:ACN		-	2017	[19]
	C18**	H_2O	60:36:4 IPA:ACN:H_2O		AmFo	2017	[23]**
	C8	H_2O	70:30 ACN:IPA		AmAc, FoA	2017	[2]
	C18 BEH	H_2O	ACN	90:10 IPA:ACN	FoA	2017	[10]
	C18 HSS T3	H_2O	ACN		FoA	2017	[13]
	C18 BEH	H_2O	MeOH		AmAc	2017	[5]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
	C18 CSH	40:60 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[35]
	C18	45:55 ACN:H_2O			AcA	2018	[43]
	C18 BEH	60:40 ACN:H_2O	81:10:9 IPA:ACN:H_2O		AmFo, FoA	2018	[40]
	C18 CSH	60:40 ACN:H_2O	90:10 ACN:IPA		AmFo, FoA	2018	[39]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2018	[33]
	C18	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2018	[38]
Yes	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[25]
	C18 HSS	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2018	[26]
Yes	C18*	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[45]*
Yes	C18***	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2018	[45]***
	C8 BEH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2018	[30]
	C8 BEH	60:40 ACN:H_2O	90:10 IPA:ACN		AmAc	2018	[30]
	C18 BEH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo	2018	[28]
	C18 BEH	60:40 ACN:H_2O	90:8:2 IPA:ACN:H_2O		AmFo, FoA	2018	[46]
Yes	C18 BEH*	90:10 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, FoA	2018	[41]*
Yes	C18 BEH*	90:10 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, FoA	2018	[42]*
	C18 SB	H_2O	90:10 ACN:H_2O		AmAc, AcA	2018	[29]
	C18**	H_2O	ACN		FoA	2018	[44]**
	C18 BEH	H_2O	ACN	90:10 IPA:ACN	FoA	2018	[32]
	C18 BEH	H_2O	ACN		AcA	2018	[37]
	C18 HSS	H_2O	ACN		FoA	2018	[27]
	Amide	H_2O	MeOH		AmHCO3	2018	[31]
	Amide	H_2O	MeOH		AmFo	2018	[31]
	C18 BEH	H_2O	MeOH		PFPA, FoA	2018	[31]
	C18 BEH	H_2O	MeOH		PFPA, FoA	2018	[31]
	C18 CSH	H_2O	MeOH		FoA	2018	[34]
	C18 HSS	H_2O	MeOH		FoA	2018	[39]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
	C18 BEH*	10:90 H_2O:ACN	20:20:60 MeOH:ACN:IPA		AmFo, NH3	2019	[71]
	C18 CSH	40:60 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2019	[77]
	C18	50:50 ACN:H_2O	20:80 IPA:MeOH		AmAc	2019	[52]
	C18	50:50 H2O:ACN	5:95 ACN:IPA		AmFo, FoA	2019	[50]
	C18	50:50 H2O:ACN	5:95 ACN:IPA		AmFo, FoA	2019	[57]
Yes	C18 CSH	60:40 ACN:H_2O	90:10 ACN:H_2O		AmFo, FoA	2019	[55]
	C18 HSS T3	60:40 ACN:H2O	90:10 ACN:IPA		AmFo, FoA	2019	[60]
	C18 CSH	60:40 ACN:H2O	90:10 ACN:IPA		AmFo, FoA	2019	[60]
Yes	C18 CSH	60:40 ACN:H_2O	90:10 IPA:ACN		AmFo, FoA	2019	[66]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo, FoA	2019	[74]
	C8	60:40 ACN:H2O	90:10 IPA:ACN		AmAc	2019	[56]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo, FoA	2019	[65]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo	2019	[79]
	C18	60:40 ACN:H2O	90:10 IPA:ACN		AmFo	2019	[78]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo, FoA	2019	[69]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmFo	2019	[62]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmAc	2020	[64]
	C18 CSH	60:40 ACN:H2O	90:10 IPA:ACN		AmAc	2020	[64]
Yes	C18 BEH	60:40 ACN:H_2O	90:8:2 IPA:ACN:H_2O		AmFo, FoA	2019	[63]
Yes	C18 CSH	60:40 ACN:H2O	90:9:1 IPA:ACN:H2O		AmFo, FoA	2019	[75]
Yes	C18 CSH	60:40 ACN:H2O	90:9:1 IPA:ACN:H2O		AmFo, FoA	2019	[76]
	C18 CSH	60:40 H2O:ACN	90:10 IPA:ACN		AmFo, FoA	2019	[58]
	HILIC	70:30 ACN:H_2O	90:10 ACN:acetone		AmFo, FoA	2019	[47]
	HILIC**	70:30 ACN:H_2O	90:10 ACN:acetone		AmFo, FoA	2019	[47]**
	C18 BEH	ACN:H2O	IPA:ACN		AmFo	2019	[67]
	C18 CSH	H2O	16:3 ACN:MeOH		AcA	2019	[68]

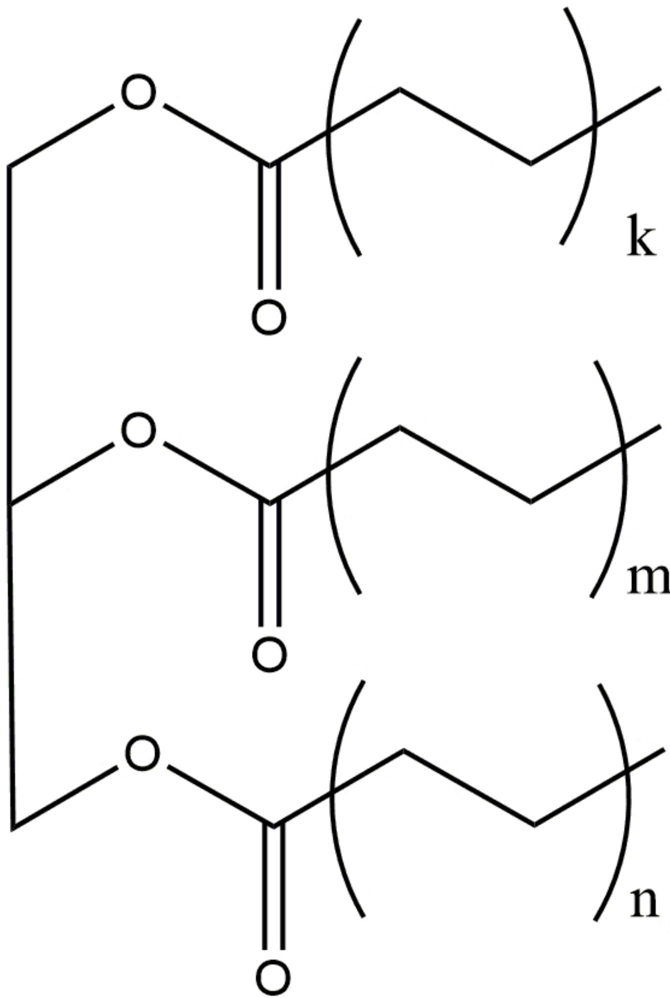
*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing

Precolumn	Column	Polar Solvent (v/v ratios)	Organic Solvent (v/v ratios)	Solvent C	Additives	Year	Citation
Yes	C8	H_2O	55:40:5 ACN:IPA:H_2O		AmAc	2019	[68]
	C18	H_2O	75:25 IPA:ACN		AmFo	2019	[59]
	C18	H_2O	75:25 IPA:ACN		AmAc	2019	[59]
	C18 BEH	H2O	ACN		FoA	2019	[67]
	F5	H2O	IPA		AmFo, FoA	2019	[51]
	C18	H_2O	MeOH		AmAc, FoA	2019	[53]
	C18	H_2O	MeOH		AmAc, FoA	2019	[53]
Yes	Phenyl	H_2O	MeOH		AmAc	2019	[49]
	C8 BEH	H2O	MeOH		AmFo, FoA	2019	[48]
	C18 BEH	MeOH	2:5 ACN:IPA		AmAc, FoA	2019	[54]
	C18 HSS	N/A	N/A		N/A	2019	[61]

*nano-LC, **APCI instead of ESI, ***narrow-bore UHPLC

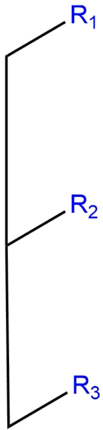
BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica, **SB**: stable bond packing



TG(N[k+2]:0,N[m+2]:0,N[n+2]:0)

k=m=n=16 => TG(18:0,18:0,18:0), TG(54:0)
k+m+n=54 => TG(54:0) (FAs unknown)

A



MG: R_1=FA,R_2=OH,R_3=OH *or* R_1=OH,R_2=FA,R_3=OH
DG: R_1=FA,R_2=FA,R_3=OH *or* R_1=FA,R_2=OH,R_3=FA
TG: R_1=FA,R_2=FA,R_3=FA

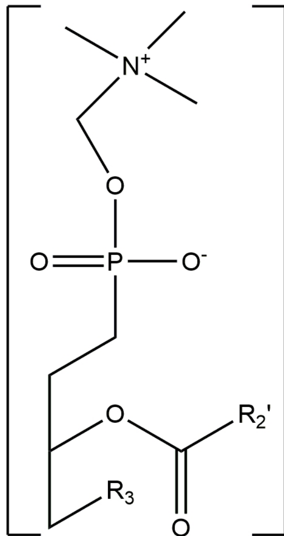
PC: e.g. $R_1=OPO_3^-CH_2CH_2N(CH_3)_3^+$,$R_2$=FA,$R_3$=FA
PE: e.g. $R_1=OPO_3^-CH_2CH_2NH_3^+$,$R_2$=FA,$R_3$=FA
PG: e.g. $R_1=OPO_3^-CH_2CH(OH)CH_2OH$,$R_2$=FA,$R_3$=FA
PI: e.g. $R_1=OPO_3^-CH_2CH_2NH_3^+$,$R_2$=FA,$R_3$=FA
PS: e.g. $R_1=OPO_3^-CH_2CH(COO^-)NH_2$,$R_2$=FA,$R_3$=FA

LPC: e.g. $R_1=OPO_3^-CH_2CH_2N(CH_3)_3^+$,$R_2$=FA,$R_3$=OH
LPE: e.g. $R_1=OPO_3CH_2CH_2NH_3^+$,R_2=FA,R_3=OH

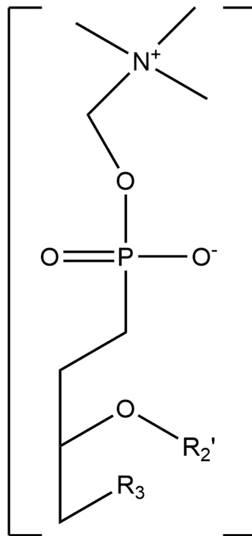
FA: fatty acid residue

B

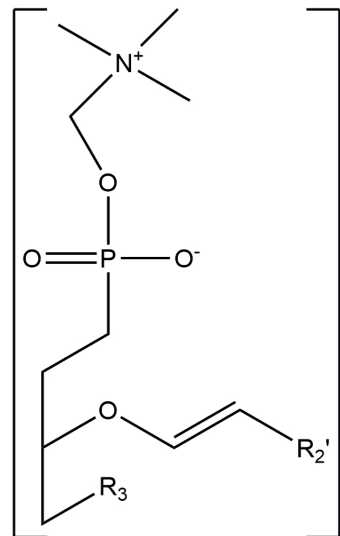
PC(...):

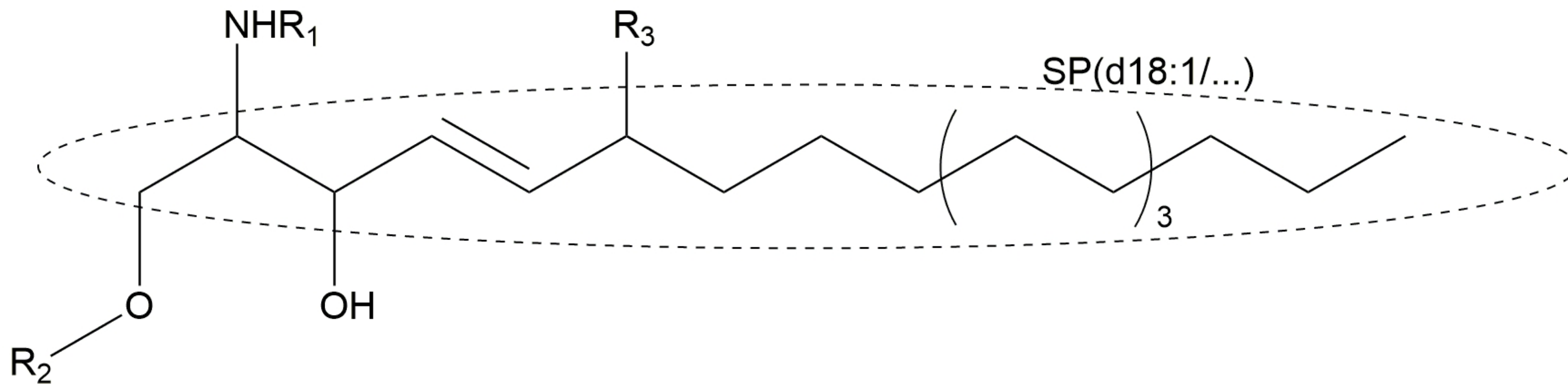


PC(O-...):



PC(P-...):



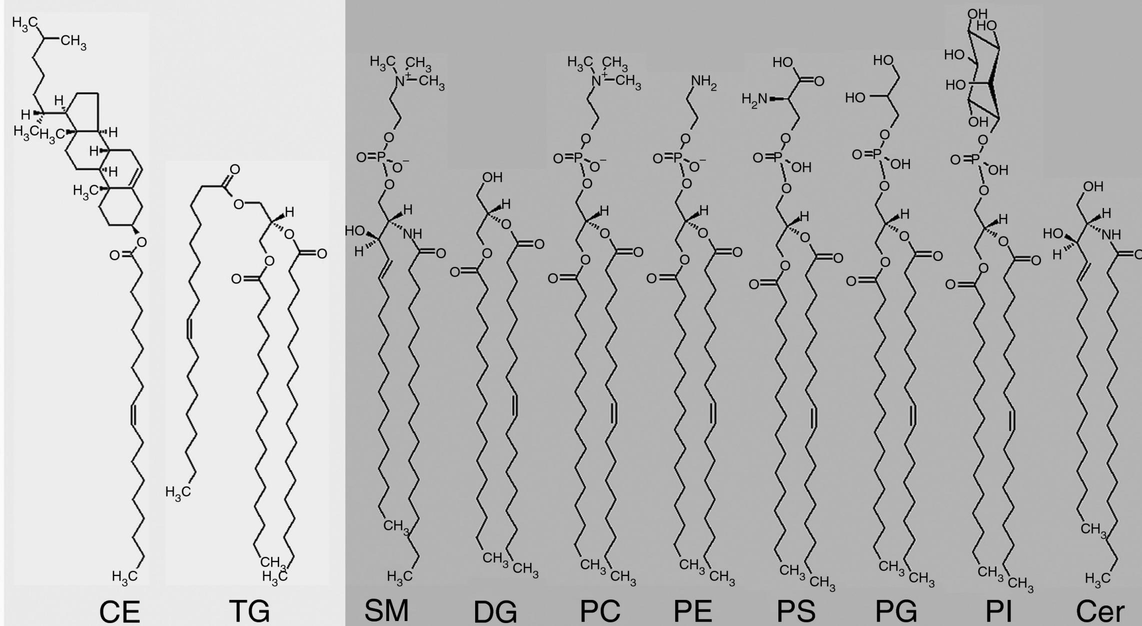


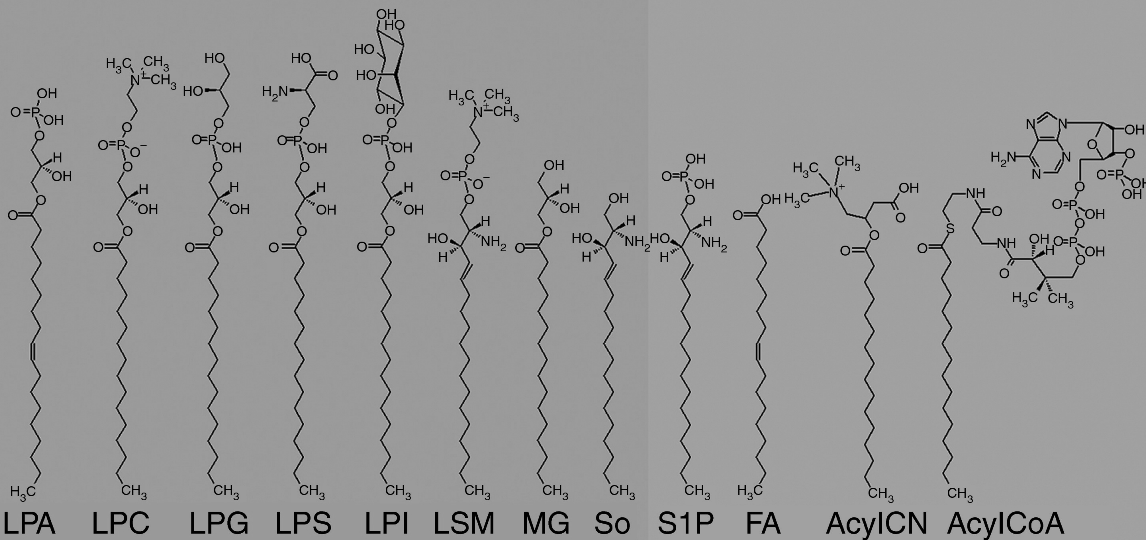
Cer:	R_1=CO-FA, R_2=H, R_3=H
S1P:	R_1=H, R_2=PO_3^-H, R_3=H
SA1P*:	R_1=H, R_2=PO_3^-H, R_3=H
SM(Cer-PC**):**	R_1=CO-FA, R_2=$PO_3^-CH_2CH_2NH_2$, R_3=H
SM(Cer-PE**):**	R_1=CO-FA, R_2=$PO_3^-CH_2CH_2N(CH_3)_3^+$, R_3=H
Phytosphingosine*:	R_1=H, R_2=H, R_3=OH
6-hydroxy-sphingosine:	R_1=H, R_2=H, R_3=OH

S1P: sphingosine phosphate

SA1P: sphinganine phosphate

***no double bond**


(a)
CE
TG
SM
DG
PC
PE
PS
PG
PI
Cer
RP C18 column, solv A: ACN:water B: IPA:ACN
NP Silica, solv A&B IPA:Hexane


(b)
LPA
LPC
LPG
LPS
LPI
LSM
MG
So
S1P
FA
AcylCN
AcylCoA
RP C18 column, solv A: water (0.05% NH4OH) B: MeOH (0.05% NH4OH)
HILIC column,
A: 0.2% formic acid, 200 mM NH4HCOO in water
B: 0.2% formic acid in ACN

Tables:

Table 1. Instrumental approaches and MS or MS/MS chromatographic methods. Ionization mode was done either in positive (pos), negative (neg) or polarity switch (Switch) ion mode.

Approach	Column	ESI mode	MS/MS	Tandem mode	Year	Citation
UHPLC-ESI-Orbitrap		Pos/Neg	No	-	2017	[2]
UHPLC-ESI-QOrbitrap	Silica	Pos/Neg	No	-	2017	[3]
UHPLC-ESI-QTOF	CSH	Pos	No	-	2017	[4]
UHPLC-HESI-Orbitrap	BEH	Switch	No	-	2017	[5]
UHPLC-Zspray-IMS-QTOF	CSH	Pos/Neg	No	-	2017	[6]
UHPLC-ESI-QqQ-MS/MS		Pos (SPs)/Neg (SM)	Yes	MRM	2017	[7]
UHPLC-ESI-QqQ-MS/MS	Silica	Pos/Neg	Yes	MRM	2017	[8]
UHPLC-ESI-QOrbitrap-MS/MS	C8 BEH	Pos/Neg	Yes	MSE	2017	[9]
UHPLC-ESI-QTOF-MS/MS	BEH	Pos/Neg	Yes	MSE	2017	[10]
UHPLC-ESI-QTOF-MS/MS	CSH	Pos/Neg	Yes	MSE	2017	[11]
UHPLC-ESI-QTOF-MS/MS	HSS	Pos/Neg	Yes	MSE	2017	[12]
UHPLC-ESI-QTOF-MS/MS		Pos	Yes	MSE	2017	[13]
UHPLC-ESI-QTOF-MSMS	CSH	Pos/Neg	Yes	MSE	2017	[14]
UHPLC-ESI-QTOF-MS/MS	HSS	Pos/Neg	Yes	MSE, PRM	2017	[15]
UHPLC-ESI-IMS-QTOF-MS/MS		Pos	Yes	N/A	2017	[16]
UHPLC-ESI-QOrbitrap-MS/MS	BEH	Switch	Yes	N/A	2017	[17]
UHPLC-ESI-QOrbitrap-MS/MS		Pos/Neg	Yes	N/A	2017	[18]
UHPLC-HESI-QOrbitrap-MS/MS	BEH	Pos/Neg	Yes	PIS	2017	[19]
UHPLC-HESI-QOrbitrap-MS/MS		Pos/Neg	Yes	PIS	2017	[20]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	PIS?	2017	[21]
UHPLC-HESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	product ion scan	2017	[22]
UHPLC-ESI(/APCI)-QqQ-MS/MS	**	Switch	Yes	SIM	2017	[23]
AF4, nUHPLC-ESI-QqQ-MS/MS	*	Pos/Neg	Yes	SRM, PIS	2017	[24]
UHPLC-ESI-IMS-QTOF	CSH	Pos	No	-	2018	[25]
UHPLC-ESI-QOrbitrap	Silica	Pos/Neg	No	-	2018	[26]
UHPLC-HESI-QOrbitrap	HSS	Switch	No	-	2018	[27]
UHPLC-ESI-QTOF	BEH	Pos	No?	-	2018	[28]
UHPLC-ESI-Qtrap-MS/MS	SB	Neg	Yes	enh. product ion scan, MRM	2018	[29]
UHPLC-ESI-QQQ-MS/MS	C8 BEH	Pos/Neg	Yes	MRM	2018	[30]
UHPLC-HESI-QOrbitrap-MS/MS	BEH, CSH, Amide	Pos/Neg	Yes	MRM	2018	[31]
UHPLC-ESI-QTOF-MS/MS	BEH	Pos/Neg	Yes	MSE	2018	[32]

Enh.: enhanced, **PIS**: precursor ion scan, *nano-LC, **APCI instead of ESI, ***narrow-bore LC
BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica,
SB: stable bond packing

Approach	Column	ESI mode	MS/MS	Tandem mode	Year	Citation
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	MSE	2018	[33]
UHPLC-ESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	MSE, MRM	2018	[34]
UHPLC-HESI-QOrbitrap		Pos/Neg	Yes	N/A	2018	[35]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	PIS	2018	[36]
UHPLC-QTOF-MS/MS	BEH	Pos/Neg	Yes	PIS	2018	[37]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	Targeted	2018	[38]
UHPLC-QTOF-MS/MS	CSH, HSS	Pos/Neg	Yes	Targeted	2018	[39]
UHPLC-ESI-QTOF-MS/MS	BEH	Pos/Neg	Yes	product ion scan	2018	[40]
AF4, nUHPLC-ESI-QqQ-MS/MS	*	Switch	Yes	SRM	2018	[41]
AF4, nUHPLC-ESI-QqQ-MS/MS	*	Pos/Neg	Yes	SRM	2018	[42]
UHPLC-ESI-QQQ		Neg	Yes	SRM	2018	[43]
UHPLC-ESI(neg: APCI)-QTOF-MS/MS	**	Pos/Neg	Yes	SWATH, PRM	2018	[44]
nUHPLC-NSI-QTOF-MS/MS	*/***	Pos/Neg	Yes	Top10 ddMS2, PRM	2018	[45]
UHPLC-HESI-QOrbitrap-MS/MS	BEH	Pos/Neg	Yes	top10-ddMS2	2018	[46]
UHPLC-APCI-QLIT-MS	HILIC**	Pos	No	-	2019	[47]
UHPLC-ESI-TOF	C8 BEH	Pos	No	-	2019	[48]
UHPLC-HESI-QOrbitrap	Phenyl	Switch	No	-	2019	[49]
UHPLC-ESI-Orbitrap-MS/MS		Pos/Neg	Yes	autoMS	2019	[50]
UHPLC-HESI-QOrbitrap-MS/MS	F5	Pos/Neg	Yes	autoMS	2019	[51]
UHPLC-ESI-Qtrap-MS/MS		Pos/Neg	Yes	MRM	2019	[52]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	MRM (GPs), product ion scan (FAs)	2019	[53]
UHPLC-ESI-QTOF-MS/MS	BEH	Pos	Yes	MRM, PIS	2019	[54]
UHPLC-ESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	MSE	2019	[55]
UHPLC-ESI-QOrbitrap-MS/MS	C8	Pos/Neg	Yes	MSE	2019	[56]
UHPLC-ESI-QOrbitrap-MS/MS		Pos/Neg	Yes	MSE	2019	[57]
UHPLC-ESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	MSE	2019	[58]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	MSE	2019	[59]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	MSE	2019	[60]
UHPLC-ESI-QTOF-MS/MS	HSS	Pos	Yes	MSE	2019	[61]
UHPLC-ESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	Untargeted	2019	[62]
UHPLC-ESI-QOrbitrap-MS/MS	BEH	Pos/Neg	Yes	N/A	2019	[63]
UHPLC-ESI-QOrbitrap-MS/MS	CSH**	Pos/Neg	Yes	N/A	2020	[64]
UHPLC-ESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	PIS	2019	[65]

Enh.: enhanced, **PIS**: precursor ion scan, *nano-LC, **APCI instead of ESI, ***narrow-bore LC
BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica,
SB: stable bond packing

Approach	Column	ESI mode	MS/MS	Tandem mode	Year	Citation
UHPLC-ESI-QTOF-MS/MS	CSH	Pos/Neg	Yes	PIS	2019	[66]
UHPLC-ESI-QOrbitrap-MS/MS	CSH, HSS	Pos	Yes	Targeted	2019	[60]
UHPLC-HESI-QOrbitrap-MS/MS	HILIC**	Neg	Yes	Targeted	2019	[47]
UHPLC-ESI-QTOF-MS/MS	BEH**	Pos/Neg	Yes	Product ion scan, NLS	2019	[67]
UHPLC-ESI-IMS-QTOF-MS/MS	CSH	Neg	Yes	Product ion scan, PIS	2019	[68]
UHPLC-ESI-QTOF-MS/MS	CSH	Pos/Neg	Yes	SONAR	2019	[69]
nUHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	SRM	2019	[70]
nUHPLC-NSI-QTOF-MS/MS	*	Pos/Neg	Yes	SRM	2019	[71]
AF4, nUHPLC-ESI-QOrbitrap-MS/MS		Pos/Neg	Yes	SRM, PIS	2019	[72]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	SWATH	2019	[73]
UHPLC-ESI-QTOF-MS/MS	CSH	Pos/Neg	Yes	SWATH	2019	[74]
UHPLC-ESI-QTOF-MS/MS	CSH	Pos/Neg	Yes	SWATH	2019	[75]
UHPLC-ESI-QTOF-MS/MS	CSH	Pos/Neg	Yes	SWATH	2019	[76]
UHPLC-HESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	Top10-ddMS2	2019	[77]
UHPLC-ESI-QTOF-MS/MS		Pos/Neg	Yes	N/A	2019	[78]
UHPLC-HESI-QOrbitrap-MS/MS	CSH	Pos/Neg	Yes	Targeted	2019	[79]

Enh.: enhanced, **PIS**: precursor ion scan, *nano-LC, **APCI instead of ESI, ***narrow-bore LC
BEH: ethylene bridged hybrid, **CSH**: charged surface hybrid, **HSS:** high strength silica,
SB: stable bond packing

Table 2. New analytic method developments and application enhancements in lipidomics

Year	Citation	Subtheme
2017	[21]	Improvement to in-silico fragmentation prediction
2017	[9]	Ultrahigh performance chromatography lipidomics
2017	[14]	Super absorbent polymer extraction chip testing
2017	[95]	Single-cell resolution, PDMS microfluid droplet chip Raman method
2017	[96]	LipidMatch comparison to other software
2017	[23]	Method development, low resolution MS identification
2017	[19]	*in vitro* computational data analysis method, excessive adipocyte lipolysis
2017	[16]	Comparison of LC/MS, SCF/MS and DIMS, kidney cancer patient analysis
2017	[22]	Bee pollen analysis, method validation
2017	[20]	Lipid extraction comparison with pancreatic cancer cell line
2017	[17]	Data processing improvements, nonalcoholic fatty liver disease analysis
2018	[25]	Machine learning algorithms for CCS values
2018	[45]	Reproducible nano-LC NSI method
2018	[27]	Multi-matrix platform validation
2018	[46]	Optimization of established extraction techniques
2018	[30]	Multimatrix method development, a mixture of untargeted/targeted
2019	[66]	Extraction comparison ("IPA-75", "IPA-90" vs "Bligh & Dyer"), MS-DIAL, SWATH
2019	[68]	Oxylipin, eicosanoid and FA identification method with IMS-MS/MS (DIA),
2019	[76]	Guide for choosing a suitable strategy for an ISTD-based untargeted approach
2019	[51]	Method for hepatotoxicity evaluation
2019	[69]	DIA method with rapid "SONAR" sequential ion feeding scan, sitaxentan (antihypertension drug) effects in animals

Table 3. Mass-to-charge values for several lipid classes found during research. **Ch**: cholesterol and its derivatives, **Hex**: SPs hexosyl-derivatives

Citation	Table	FA	FFA	GL	GP	LPL	PL	Ox	Sulfo-GP	SP	Hex	ST	Ch	SL	PK	PR	adduct	m/z	CE	more
[120]	S3	x	x	x	x	X	X			x	x		x				x	x		
[121]	A2	x	x	x	x	X				x							x	x		Metabolomic study
[5]	S1	x	x	x	x		X			x		x	x	X	x	x	x	x		Authentic standards
[49]	S7	x	x	x	x							x	x			x		x		Metabolomic study
[7]	S1, S2	x	x		x	X	X		x	x		x		X		x	x	x		Metabolomic study
[39]	S6	x	x		x	x								X			x	x		Protonated ions only
[122]	S1, S4	x	x		x	x											x	x		Daphnia Magna baseline
[123]	S1	x		x	x	x				x	x							x		FA-chains, lipoproteins
[63]	S2, S3	x			x	x	X	x									x	x		Metabolomic study
[43]	T2	x																x	x	Prostaglandines
[23]	S1, S2, S3		x	x	x	x	X		x	x			x	x			x	x		
[30]	T1, T2		x	x	x	x	X			x	x						x	x		part of the precursors
[86]	S1		x	x	x	x	X			x		x			x		x	x		Column comparison
[124]	S1		x	x	x	x	X			x			x				x	x		
[58]	S1-S4		x	x	x	x	X										x	x		
[75]	S1		x	x	x	x				x			x				x	x		Standards, SWATH
[22]	T2, T3		x	x	x	x				x							x	x		Gene expression manipulation
[76]	S1		x	x	x	x											x			
[66]	S1		x	x	x		X			x				x			x	x		
[73]	A1, A5, A10		x		x	x											x	x	x	SWATH, exclusion list MS-DIAL
[37]	T1		x			x	X											x		PAF C-16

Citation	Table	FA	FFA	GL	GP	LPL	PL	Ox	Sulfo-GP	SP	Hex	ST	Ch	SL	PK	PR	adduct	m/z	CE	more
[19]	S2		x															x		
[42]	S3			x	x	x	X		x	x	x							x		
[41]	S1, S2			x	x	x	X			x	x						x	x		List for possible acyl chains (S3)
[8]	T2			x	x	x	X			x	x							x		Abundances in 5 cancer types
[9]	S7			x	x	x	X			x	x						x	x		Adduct list
[14]	S1, S2			x	x	x	X			x			x	x			x	x		APCI
[16]	S2			x	x	x	X			x			x				x	x		Adduct list, UHPLC/SFC/DIMS & tissue comparison, Des-lipids
[60]	S1			x	x	x	X			x			x	x				x		
[48]	S3			x	x	x	X			x			x					x		
[11]	S2, S3			x	x	x	X			x							x	x		
[65]	S1, S2, S3			x	x	x	X			x							x	x		PE(16:0/18:1)+H m/z fragments
[96]	S1, S4			x	x	x		x					x	x			x	x		Adduct list, Software comparison
[34]	S1, S2			x	x	x				x			x				x	x	x	MRM validation, methylated species
[25]	S1, S2			x	x	x				x			x				x			CCS
[52]	A3			x	x	x				x			x							SWATH, standards only
[15]	T1			x	x	x				x				x				x		Saccharolipids
[45]	S4, S6, S8			x	x	x				x				x			x	x	x	PRM, sensitivity data
[72]	S1			x	x	x				x							x	x	x	
[79]	S2, S3, S4			x	x	x				x							x	x		
[83]	T2			x	x	x											x	x		
[24]	T2, S1, S2			x	x		x			x	x						x	x	x	nUHPLC, adduct list
[12]	S1, S2			x	x					x							x	x		

Citation	Table	FA	FFA	GL	GP	LPL	PL	Ox	Sulfo-GP	SP	Hex	ST	Ch	SL	PK	PR	adduct	m/z	CE	more
[69]	T1			x	x					x							x	x		
[59]	T2			x	x							x			x		x	x		Plant flavonoids included
[2]	S1			x	x									x	x		x			Plant flavonoids included
[125]	S1, S2, S3			x	x									x		x	x	x		archaeal isoprene-based and ether-linked lipid list (incl. fragment info)
[18]	S3			x	x												x	x		
[50]	T2, T5				x	x				x							x	x		
[47]	S1, S2				x					x								x		csv files (S2)
[4]	T1				x													x		
[61]	T1				x												x	x		PC only
[29]	S1							x										x	x	Oxylipins
[55]	T2, S1									x			x				x	x		SM and cholesterol
[40]	S2									x							x	x		protonated species only
[27]	S1											x					x	x		Metabolomic study
[44]	A.144, A.155											x								SWATH
[54]	S1												x							Math. model-assisted identification

Table 4. Databases used for lipid identification

Database	Content	Focus	Used in Citation
in-house	Compound lists	Lipids	[30], [32], [36], [51], [54], [68], [78]
ChemSpider	Structures, Library	Comprehensive	[13], [127]
Foodb	Library	Food constituents	[59]
HMDB	Various	Metabolites	[4], [12], [19], [32], [34], [39], [40], [59], [60], [63], [127]
KEGG	Pathway maps	Genes	[33], [34], [60], [79]
LipidBlast	Spectra	Lipids	[6], [21], [25], [35], [76]
LIPID MAPS	Library	Lipids	[4], [6], [9], [12], [16], [19], [26], [34], [39], [40], [54], [65], [69], [127]
MassBank	Mass spectra	Comprehensive	[21]
MetLin	MS/MS	Metabolites	[34], [39], [40], [63]
NIST 11	Library	Comprehensive	[60]
Nist 14	Library	Comprehensive	[25]
PubChem	Library	Comprehensive	[13], [125]
ChEBI*	Library	Metabolites	[125]
Lipidbank	Compound lists	Lipids	[125]
Reactome	Library	Pathways	[26]
Home-made	Various	Various	[30], [32], [36], [54], [68]

Table 5. Coding languages, possible packages and specific softwares for analysis of lipids typically under the m/z value of 1500.

Availability	Program	A/IQ/S/V	More	Used in Citation
MathWorks	**Matlab**	**n/y/y/y**	Coding language	[49]
DoubleClick	**Origin**	**n/n/y/y**	Coding language	[44], [75], [76]
Open-source	**Python**	**n/y/y/y**	Coding language	[25]
Open-source	**R**	**n/y/y/y**	Coding language, statistics	[2], [3], [6], [7], [10], [18], [21], [32], [33], [36], [37], [39], [40], [46], [47], [55], [65], [67], [68], [74], [76], [78]
	-Out of which CAMERA	**n/y/n/n**	Isotope screening	[6], [21], [37], [55]
	-Out of which MeV	**n/y/y/y**	**DB** search, visualization	[3], [30]
	-Out of which XCMS	**n/y/n/n**	Feature detection	[7], [10], [19], [32], [33], [36], [37], [39], [46], [47], [55], [61], [65], [76], [78]
IBM	**SPSS**	**n/y/y/y**	Coding language, statistics	[7], [26], [33], [42], [44], [50], [53], [54], [55], [57], [60], [61], [64], [72], [75], [79]
Agilent	**Masshunter series**	**y/y/n/n**	Multiple tools	[7], [25], [34], [40], [54], [68]
GraphPad	**GraphPad Prism**	**n/n/y/y**	Statistics, Data visualization	[56], [57]
KBSI	**iLipid**	**n/y/n/n**	In-house	[14]
Molecular Discovery	**Lipostar**	**n/y/y/y**	Identification only	[65]
N/A	**LiPilot**	**n/y/n/n**	In-house	[24], [42]
Open-source	**CEU Mass Mediator**	**n/y/n/n**	Adduct/s and RT, website	[32], [63]
Open-source	**Cytoscape**	**n/n/n/y**	Visualization	[79]
Open-source	**MetaboAnalyst**	**n/y/y/y**	Multipurpose, website	[10], [11], [17], [27], [32], [34], [37], [40], [46], [48], [59], [60], [61], [63], [65], [77], [79]
Open-source	**Greazy**	**n/y/y/y**	Chemometrics included	[96]
Open-source	**KniMet**	**n/n/y/n**	Visualization	[68]
Open-source	**Lipid Data Analyzer**	**n/y/y/y**	Standardization, statistics	[9]
Open-source	**LipidFrag**	**n/y/n/n**	In silico	[21]
Open-source	**LipidMatch**	**n/y/n/n**	In silico, fragmentation DBs	[3], [17], [63]

A/IQ/S/V: Acquisition/Identification & Quantitation/Statistical analysis/data Visualization

*According to Waters' website, MassLynx is discontinued and replaced with Progenesis QI

DB: database, **KBSI**: Korea Basic Science Institute

Availability	Program	A/IQ/S/V	More	Used in Citation
Open-source	**MassTRIX**	**n/y/n/y**	KEGG/API pathway analysis	[21]
Open-source	**MultiExperiment Viewer**	**n/n/n/y**	Chemometric visualization	[56]
Open-source	**MZmine**	**n/y/n/n**	MS **DB** search	[63], [123]
Open-source	**Skyline**	**n/y/n/n**	MS/MS **DB** search	[7], [34], [45], [69]
Premier Biosoft	**SimLipid**	**n/y/n/n**	In silico structure MS/MS	[10], [32], [61]
Riken Prime	**MS-DIAL**	**n/y/n/n**	SWATH	[35], [58], [73], [75-77]
Sartorius	**SIMCA**	**n/n/y/y**	Statistics and visualization	[5], [26], [33], [35], [36], [68], [79], [124]
Sartorius	**SIMCA-P**	**n/n/y/y**	Same as SIMCA	[7], [19], [30], [37], [52], [54], [56], [62]
Sartorius	**EZinfo**	**n/n/n/y**	Chemometrics	[4], [13], [34], [60], [69]
SAS	**JMP**	**n/n/y/y**		[56]
SCIEX	**Analyst TF**	**y/n/n/n**		[44], [33], [36]
SCIEX	**LipidView**	**n/y/n/n**	Fragment **DB** search	[16], [38], [43]
SCIEX	**MultiQuant**	**n/y/n/n**	Quantitation tool	[75], [76]
SCIEX	**PeakView**	**n/y/n/n**	Peak comparison & analysis	[76]
SCIEX	**MarkerView**	**n/n/y/y**		[38], [73], [76]
Thermo	**Xcalibur**	**y/n/n/n**		[2], [3], [5], [18], [26], [45], [46], [50], [58], [60], [62], [65], [124]
Thermo	**Compound Discoverer**	**n/y/y/y**	Pathway analysis	[46]
Thermo	**LipidSearch**	**n/y/n/n**	Relative quantification	[22], [26], [30], [35], [46], [50], [57], [62], [65], [79], [124]
Thermo	**SIEVE**	**n/n/y/y**	Biomarkers, comparative analysis	[19]
Thermo	**SIEVE**	**n/n/y/n**	Biomarker discovery	[19]
Thermo	**Chromeleon**	**y/n/n/n**		[65]
Waters	**MassLynx MS**	**y/y/n/n**		[7], [13-15], [28], [34], [60], [61], [69]
Waters	**MarkerLynx***	**n/n/n/y**	Chemometrics	[4], [6], [16], [34], [43], [67]
Waters, Nonlinear Dynamics	**Progenesis QI**	**n/y/n/n**	Small molecule protein/lipid analysis	[69]

A/IQ/S/V: Acquisition/Identification & Quantitation/Statistical analysis/data Visualization

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Figures:

Figure 1. Simple triglyceride (TG) structure and shorthand notation. The stereospecific numbering (sn) of fatty acids on a glycerol-molecule are named sn1 (upper carbon of the glycerol body, if k>n), sn2 (middle carbon of the glycerol body) and sn3 (lower carbon of the glycerol body, if k>n) respectively, when the analyte can be stereoisomerically determined (i.e. on the stereo-molecular species level). If individual chain lengths (and their positions) cannot be determined, the number of the carbon atoms and double bonds are expressed as separate sums.

Figure 2. (A) Glycero- and glycerophospholipid structure variations according to sub-class and (B) regular and plasmalogen structures (O- for plasmanyl and P- for plasmenyl, older notation *e* and *p*). The characteristic molecules of lipid classes are typically esterified to the sn3 position. Structures derived from ref. **[93]**

Figure 3. Typical sphingolipid structures. R_1 is an amine that usually has a fatty acid linked to it with an amide bond. R_2 on the other hand is either a free hydroxyl group as in ceramides but occupied with a characteristic phosporylated molecule in SMs. Some structures also Structures derived from ref. **[93]**

Figure 4. '(a)' Nonpolar and '(b)' polar lipid subclass separation techniques. Reprinted from ref **[85]**, DOI: 0958-1669/© 2016 S. Tumanov and JJ Kamphorst. Published by Elsevier Ltd, an open access article under the CC BY license.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: