# Standardizing and increasing the utility of lipidomics: a look to the next decade

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**Abstract**

*Introduction:* We present our views on the current application of mass spectrometry (MS) based lipidomics and how lipidomics can develop in the next decade to be most practical use to society. That is not to say that lipidomics has not already been of value. In-fact, in its earlier guise as metabolite profiling most of the pathways of steroid biosynthesis were uncovered and via focused lipidomics many inborn errors of metabolism are routinely clinically identified. However, can lipidomics be extended to improve biochemical understanding of, and to diagnose, the most prevalent diseases of the 21$^{st}$ century?

*Areas covered:* We will highlight the concept of "level of identification" and the equally crucial topic of "quantification". Only by using a standardised language for these terms can lipidomics be translated to fields beyond academia. We will remind the lipid scientist of the value of chemical derivatisation, a concept exploited since the dawn of lipid biochemistry.

*Expert opinion:* Only by agreement of the concepts of identification and quantification and their incorporation in lipidomics reporting can lipidomics maximise its value.

**Keywords**

Clinical chemistry, identification, in-born errors of metabolism, imaging, lipids, mass spectrometry, medicine, quantification

**Article Highlights**

- Lipidomics requires clear reporting, using commonly accepted concepts especially those for identification and quantification
- Lipidomics requires the use of common standard compounds and standard reference material
- Lipidomics will enhance our fundamental understanding of basics bioscience
- Lipidomics has the potential to enhance our understanding of diseases prevalent to 21$^{st}$ century
- Lipidomics can become a key technology in clinical chemistry

**1. Introduction**

At the beginning of the third decade of the twenty first century lipidomics is at a crossroads. Will it continue straight on, largely as the preserve of scientists in academia, or will it branch towards clinical science or perhaps towards the agricultural sector? If lipidomics is to have a meaningful impact in society beyond academia there are a number of aspects that the community will need to agree on and adopt [1-3]. In this perspective we give our views on the key areas that these might be. The authors will focus this article on biomedical lipidomics (Table 1) but lipidomics is likely to have equally important impacts in the agriculture and food, cosmetics and perfumery industries. The article will be biased towards mass spectrometry (MS)-based lipidomics, which is the research interest of the authors.

The term lipidomics was first popularised around the beginning of this century [4], following on from the other molecular "omics" i.e. metabonomics/metabolomics [5] and proteomics [6]. Lipidomics is essentially lipid-focused metabolite profiling, an area of research which has been explored since the early days of gas-chromatography (GC) [7,8], GC-MS [9-11] and fast atom bombardment (FAB)-MS [12]. Lipidomics is generally regarded as the quantitative determination of the entire lipid content of

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a cell, tissue, biofluid or an organism. Determining the lipidome of a cell type is a daunting prospect. When one considers all the potential lipids that may be present in a more complex sample, determining its lipidome is going to be orders of magnitude more challenging. Taking plasma as an example, there are likely to be many thousands of discreate lipid species present, ranging in concentrations from pM (e.g. eicosanoids) to mmol/L (free cholesterol) [13-15].

### 1.1. Identification

It is important to consider what is meant by identifying and subsequently quantifying a lipid. While the identity of a lipid can be determined by a combination of accurate mass measurement by MS and through NMR, very often providing the relevant stereochemistry, many lipidomic studies exploit MS with tandem MS (MS/MS) where the degree of structural identification is incomplete. MS when combined with chromatography can give accurate mass information and suggest chemical formulae for discreate lipids and if MS/MS or multistage fragmentation (MS$^n$) is included information regarding the building blocks of a lipid may be forthcoming [16,17]. However, in the absence of more specialised adaptions e.g. keV collision-induced dissociation (CID) [18,19], ozoneolysis [20] or derivatisation [21-23], determination of the position of double bonds can be difficult if not impossible, enantiomers cannot be differentiated in the absence of chiral chromatography and even diastereoisomers are difficult to differentiate in the absence of authentic standards [24]. Hence, it is important to define at what degree an identification is made in any lipidomic study [2,3,25]. While databases such as those provided by Lipid Maps are of huge value [26,27], their widespread use also provides a danger in that a measured *m/z* may be reported with a Lipid Maps ID in the absence of additional evidence, implying to the non-expert that the molecule defined by the Lipid Maps ID has been definitively identified when often this is not the case. There are many examples of this in the literature and it is a trap easy to fall into. To minimise miss communication and over reporting, a shorthand notation for lipid structures derived by MS has been proposed and has just been updated [2,3], adoption of this nomenclature by the community will minimise the number of misleading identifications made. It is important to be aware that even when all the chromatographic and MS data are considered it is still unlikely that an absolutely definitive identification will be made. To take an extreme example, while accurate mass measurement, GC retention time and electron - ionisation (EI) fragmentation pattern will identify cholesterol, we need to use a chiral column to confirm we have the natural enantiomer not its synthetic equivalent. Of course, if the sample comes from a biological source it is legitimate to use our "biological intelligence" and identify the lipid as cholesterol. We do, however, need to be careful in less obvious cases in the use of "biological intelligence" and state clearly any assumptions that are made. By LC-MS/MS it would be very easy to confuse the isomers lathosterol and cholesterol and make the assumption that an appropriate peak, e.g. in a plasma sample, consisted exclusively of cholesterol. In all probability, this identification based on "biological intelligence" would be correct, but not if the sample came from a child suffering from lathosterolosis, where the plasma concentration of lathosterol is elevated [28].

### 1.2. Quantification

Quantification can be another subject that leads to misunderstandings. In clinical chemistry MS is used to quantify lipids e.g. cholesterol, 25-hydroxyvitamin $D_3$, in units of moles/unit volume, usually using isotope-labelled standards [29,30]. Isotope-dilution techniques should provide the best accuracy for a quantitative MS method, however, purchasing and using an internal standard for each analyte in a global lipidomics experiment is not practical. However, absolute quantification with one standard per analyte can be performed in targeted lipidomic studies, where one specific class of lipids is analysed [13,31-35]. In global lipidomic studies it is more common to use one or two internal standard per lipid class. This is now made easier by the commercial availability of accurately prepared mixtures of

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isotope labelled lipids suitable for quantification of different lipid classes [36,37]. Rather than presenting quantitative values in moles or gram per unit volume, or per unit wet weight or per unit mass of protein, some authors give % quantities relative to that measured in a control sample (100 %). This is not recommended, but it is appreciated that this may be the most practical way to present results following retrospective analysis of experimental data. Alternatively, this may be appropriate where the goal is simply to determine only relative quantification following perturbation of a system.

If one accepts that analytical studies are aimed at obtaining the true value of a particular invariable, then the truth will best be reached if multiple different analytical methods are employed to determine the same invariable. That is not to say that the mean or median value of these analyses is closest to the truth or that outlier values are necessarily incorrect. However, if multiple different analytical methods converge to a common value, then the observer is given some confidence that this value is "likely" to be correct. As such there is considerable value in standard reference materials that are prepared in bulk and aliquoted into small volumes that can be analysed by many independent laboratories [30,38]. Such a standard reference material is NIST SRM 1950 which is a plasma sample representative of the US population [30]. A lipidomic study has been performed in which many different laboratories analysed the NIST SRM 1950 plasma sample using different MS methods [14] and further studies are planned targeting on specific lipid groups. Similar studies have already been performed with locally prepared plasma samples distributed to collaborating groups for sterol and oxysterol analysis [39,40]. While these "ring" trials will not necessarily lead to "true" quantitative values, they will lead to "consensus" values which are very important when comparing data sets from different laboratories. With respect to ring-trials, one way to minimise the spread of values from different participating groups is for all participants to use the same set of standard compounds.

## 2. Instrumentation

At the beginning of the 2000's when lipidomics was in its infancy, the dominant instruments were tandem quadrupoles [41], quadrupole and linear ion-traps (QIT or LIT) [17,42], the hybrid quadrupole-time-of-flight (Q-TOF) [43] and the hybrid quadrupole – ion-trap (Q-Trap) [13]. Electrospray ionisation (ESI) was, and still is, the most common ionisation mode, although some studies were conducted with matrix-assisted laser desorption/ionisation (MALDI) mostly on TOF instruments [44]. Ultra-high resolution Fourier transform ion cyclotron resonance (FTICR) instruments have also been used in lipidomic studies [45], but in more recent times hybrid Orbitrap type instruments have become popular [46-48]. Table 2 provides typical figures of merit for different MS instruments utilised in lipidomics. While MALDI has its place in lipidomics, especially in MS imaging (MSI) [49,50], ESI is the most widely used ionisation method. When combined with liquid chromatography (LC) i.e. high performance-LC (HPLC) or ultra-high performance (UHPLC), ESI is usually used at conventional flow rates (100s µL/min), although there are some studies using low-flow-rates [51-54]. ESI is often used in a low-flow-rate format for direct infusion (DI)- (also called shotgun) lipidomics studies which are performed in the absence of chromatography [46]. Ion-mobility spectrometry (IMS) has more recently being been incorporated into lipidomic experiments, this can add an extra-dimension of separation and measured collisional cross-section values can provide additional evidence for identifications [55-58].

## 3. Lipidomics Methods

To-date, lipidomics has largely been an endeavour driven by research laboratories in Universities and industry [13,32,41,43,46,59]. There are essentially two lipidomic strategies, those based on category/class specific separations and LC-MS or GC-MS and those based on shotgun- or DI-MS in the absence of chromatography [13,32,41,46,60,61]. Both have their strengths and weaknesses (Table 3).

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It is beyond the scope of this article to discuss these two strategies in detail. However, it is of value considering the task at hand in measuring entire lipidomes.

If plasma lipidomics is taken as an example, concentrations of different lipids range from the oxylipins at pM levels to cholesterol and its esters at μmole/L – mmole/L levels and within the plasma lipidome there are thousands of individual lipid species [13,14,37,46]. Lipids at the high end of this concentration scale i.e. cholesterol esters (CE), glycerophospholipids (GP) and glycerolipids (GL) can be readily analysed by both strategies [41,46,62,63]. For identification, both shotgun- and LC-MS methods depend on measurement of precursor ion *m/z* and MS/MS fragmentation spectra which in combination allow definition of structure, at least at the sum composition level. There are well-established fragmentation rules for the different classes of lipids which aid in their structural identification. Below we highlight a few fragmentation mechanisms [64] and illustrate how this knowledge can be used to achieve identification at different levels [2,3].

**4. MS/MS Fragmentation and Structure Determination of Complex Lipids**

Complex lipids include the neutral lipid class of sterol esters (SE) and the lipid category glycerolipids (GL), and the phospholipid categories of glycerophospholipids (GP) and sphingolipids (SP) (Table 1) [1]. Cholesterol esters (CE) and triacylglycerols (TAG) are particularly abundant in plasma (1 - 2 mmole/L) where they are carried in lipoprotein particles. CE and TAG are also abundant in sub-cellular organelles called lipid droplets. Diacylglycerols (DAG) are important in intracellular signal transduction while the monoacylglycerol 2-arachidonoylglycerol has cannabinoid-like pharmacology. Phospholipids play a central role in cell and subcellular membranes, allowing cells and subcellular organelles to separate from one another.

Whether LC-MS/MS or DI-MS/MS is used, the determination of structures of complex lipids is dependent on their MS/MS spectra. Structure determination is aided by high mass accuracy, but this is not a pre-requisite, however an understanding of MS/MS fragmentation is a requirement, either at the level of the analyst or the developer of lipid-centric MS/MS databases. Bearing in mind the importance of fragmentation mechanisms we discuss some of those which we consider most useful in the sections below.

*4.1. Fragmentation Mechanism and Analysis of Complex Lipids*

Table 4 summarises key fragment-ions useful for the determination of structure at the species level (sum composition) and above in shotgun and targeted lipidomic studies of complex lipids  [2,3].

*4.1.1 Cholesterol Esters*

Cholesterol esters are abundant in plasma, and despite being neutral molecules can be ionised in positive-ion ESI as the ammonium adduct $[M+NH_4]^+$ [64]. The $NH_4^+$ cations may be present in the ESI solution, added to the LC mobile phase, or added post LC column to the infusion solvent.  When fragmented by MS/MS the dominant fragment-ion is formally the dehydrated protonated molecule of cholesterol $[Chol+H-H_2O]^+$ at *m/z* 369.35 (Figure 1A, see MS/MS spectrum provided by Lipid Maps at [65]). A very useful resource providing MS/MS spectra of numerous lipid standards can be found at the Mass Bank of North America  (e.g. [66]). The ion at *m/z* 369.35 is an ideal target for precursor-ion scanning on a tandem quadrupole instrument or for MS/MS$^{ALL}$ scans, or variants thereof, on hybrid Q-TOF or hybrid Orbitrap instruments to reveal $[M+NH_4]^+$ ions of cholesterol esters [62,63,67,68]. It should be noted here, that "biological intelligence" tells us that the fragment-ion at *m/z* 369.35 is almost certainly derived from the cholesterol ester, but if the plasma was from a patient suffering from lathosterolosis [28], the ion could be derived from a lathosterol or perhaps zymostenol ester.

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Thus, according to Liebisch et al's nomenclature the structure in Figure 1A should be annotated at the molecular species level as SE 27:1/18:1, where SE refers to sterol ester, as most likely determined by MS/MS, or as CE 18:1 based on "biological intelligence" assuming the sterol to be cholesterol, and at the full structural level to be CE 18:1(9Z) where the two components are cholesterol and oleic acid [2,3,25]. If the Lipid Maps ID LMST01020003 is to be used, then any assumptions regarding "biological intelligence" should be indicated. Double bond location or geometry within the fatty acyl group is not determined by routine MS/MS methods, however, the Paternò-Büchi PB reaction has been successfully exploited towards this aim [69]. The PB reaction is a photochemical [2+2] cycloaddition between a carbonyl group and an alkene. The reaction can be performed prior to or during ESI and the PB reaction products fragmented by CID to reveal double bond location [21,22,69].

### 4.1.2 Triacylglycerols (TAG)

Like other neutral lipids TAG can be ionised as $[M+NH_4]^+$ ions. MS/MS of the $[M+NH_4]^+$ ion leads to the loss of fatty acids ($RCO_2H$) and $NH_3$ leaving a series of $[M+H-RCO_2H]^+$ ions [64]. These ions will reveal the fatty acyl groups attached to the glycerol backbone, although not their positions or location of double bonds. This is illustrated in Figure 1B - D where the $[M+NH_4]^+$ ion at 874.79 will fragment to give $[M+H-R_1CO_2H]^+$, $[M+H-R_2CO_2H]^+$ and $[M+H-R_3CO_2H]^+$ at $m/z$ 603.53, 573.49 and 577.52. This allows molecular species level identification as TG 16:1_18:0_18:2. Loss of $R_2CO_2H$ at the second carbon of glycerol is often, but not always, less facile than at other positions, allowing a tentative identification of this acyl group. Diacylglycerols (DG) fragment through similar mechanisms to TAG. If the alkali metal adducts $[M+Na]^+$ or $[M+Li]^+$ are fragmented the dominant fragment-ions correspond to the loss of the fatty acids with the metal attached to the residual ion. However, fragmentation of $[M+Na]^+$ ions is less facile than of $[M+NH_4]^+$ ions.

Analysis of TAGs from either animal or plant origin is complicated by multiple isobaric species. Rather than having just three product ions as in the example above, it is not uncommon to observe nine or ten $[M+H-RCO_2H]^+$ ions [17]. This complicates unambiguous identifications. One solution is reversed phase LC. An alternative is exploiting multiple MRM or multiple neutral loss scans.

### 4.1.3. Glycerophospholipids (GP)

While glycerphosphocholines (PC) are most commonly analysed in the positive-ion mode, other phospholipids are often analysed as $[M-H]^-$ ions, although they can also be analysed as $[M+H]^+$ or $[M+Cat]^+$ ions, where Cat is a metal cation.

Glycerophospholipid $[M-H]^-$, or in the case of PC, $[M-15]^-$ ions, fragment in MS/MS to give carboxylate ions derived from the fatty acyl groups attached to the glycerol backbone (see below).

### 4.1.3.1. Glycerophosphocholine (PC)

In the positive-ion mode, PC readily give $[M+H]^+$ ions in ESI which fragment upon MS/MS to give the characteristic and abundant fragment-ion at $m/z$ 184.07 (Figure 2A and for reference spectra [70] and [71]). Note, sphingomyelin also gives this fragment-ion. While a combination of accurate mass and the fragment-ion at $m/z$ 184.07 can lead to identification at the species level (sum composition) e.g. PC 34:1 in Figure 2A, more information is required for fatty acyl group identification. This can be provided by minor fragments in the MS/MS spectrum. These are observed following the loss of the $sn$-1 or $sn$-2 fatty acyl group as the neutral ketenes $R_1CH=C=O$ and $R_2CH=C=O$ (Figure 2B - C). This will then allow identification at the molecular species level e.g. PC 16:0_18:1 in Figure 2. It may be possible to achieve identification at the $sn$-position level by ion mobility, while the double bond position could be determined by exploiting the Paternò-Büchi reaction and MS/MS [21,22].

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Fragmentation of [M+Li]$^+$ and [M+Na]$^+$ ions is very different from [M+H]$^+$. Both of the alkali metal adducts fragment with the loss of 59.07 Da (N(CH$_3$)$_3$) and 183.07 Da (HO$_3$POC$_2$H$_4$N(CH$_3$)$_3$) while the [M+Li]$^+$ also gives a neutral loss of 189.6 Da (LiO$_3$POC$_2$H$_4$N(CH$_3$)$_3$).

### 4.1.3.2. Glycerophosphoethanolomines (PE)

PE when ionised in the positive-ion mode gives [M+H]$^+$ ions which upon MS/MS give the characteristic fragment-ion at [M+H-141.02]$^+$ (see Figure 3A, and for reference spectra [72] and [73]). This, when combined with data from MS/MS of the [M-H]$^-$ ion, can define the PE class and the fatty acyl composition. The MS/MS spectra of [M-H]$^-$ ions of PE, like other glycerophospholipids, gives fragment-ions corresponding to [R$_1$CO$_2$]$^-$ and [R$_2$CO$_2$]$^-$ allowing structure determination at the molecular species level (Figure 4A - B, for reference spectra see [74]).

The alkali metal adducts of PE show major fragment ions corresponding to [M+Li-147.03]$^+$ and [M+Na-163.00]$^+$ which are chemically identical to the [M+H-141.02]$^+$ ion.

### 4.1.3.3. Glycerophosphoserines (PS)

The [M-H]$^-$ ion of PS fragments in MS/MS to give a characteristic neutral-loss of 87.03 i.e.[M-H-87.03]$^-$, besides the [R$_1$CO$_2$]$^-$ and [R$_2$CO$_2$]$^-$ ions defining the acyl groups attached to the glycerol back bone (see Figure 3B and 4A - B, [75]). When ionised in the positive-ion mode the [M+H]$^+$ ion fragments to give a major neutral-loss of 185.01 Da, i.e. [M+H-185.01]$^+$ (see Figure 3C, and for reference spectra [76] and [77]). The [M+Li]$^+$ and [M+Na]$^+$ give the equivalent [M+Li-191.02]$^+$ and [M+Na-206.99]$^+$ fragment-ions and also the lithiated or sodiated head group [H$_2$O$_3$POCH$_2$CH(NH$_3$)CO$_2$Li]$^+$ (*m/z* 192.02*)* or [H$_2$O$_3$POCH$_2$CH(NH$_3$)CO$_2$Na]$^+$ (*m/z* 208.00).

### 4.1.3.4. Glycerophosphatidic acids (PA)

MS/MS spectra of [M-H]$^-$ ions of PA give abundant carboxylate anions characteristic of the fatty acyls groups (Figure 4A - B, see [78]). A useful resource provided by Lipid Maps is a library of theoretical MS/MS spectra (e.g. [79]) giving predicted fragment ions and their structures. In the positive-ion mode [M+H]$^+$ ions fragment to give a major ion corresponding to the loss of HPO$_4$, i.e. [M+H-95.96]$^+$.

### 4.1.3.5. Glycerophosphatidylinositols (PI)

PI in the negative-ion mode give [M-H]$^-$ ions that fragment in MS/MS to give carboxylate anions characteristic of the fatty acyls and also a PI characteristic ion at *m/z* 241.01 (Figure 3D, for reference spectra see [80] and [81]). In the positive-ion mode MS/MS of [M+H]$^+$ ions give a major fragment at *m/z* [M+H-260.03]$^+$ corresponding to the loss of the neutral head group.

### 4.1.3.6. Phosphatidylglycerol (PG)

MS/MS of [M-H]$^-$ ions of PG give the characteristic [R$_1$CO$_2$]$^-$ and [RCO$_2$]$^-$ fragment-ions.

### 4.1.4. Shingomyelin (SM)

The MS/MS spectra of SM [M+H]$^+$ ions give a prominent ion at *m/z* 184.07, just like PC (Figure 4C, for reference spectra see [82]). The MS/MS of the [M-15]$^-$ ion yields a major product ion at *m/z* 168.04.

As discussed for sterol esters, to obtain information on the location of double bonds the Paternò-Büchi reaction can be exploited [21,22] or alternatively gas-phase ozonolysis [20].

## 5. Fatty Acids, Oxylipins, Sterols, Oxysterols, Steroids and Bile Acids

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While the complex lipids discussed above are evident in global shotgun type experiments, for the less abundant lipids more targeted approaches are most appropriate. These may be on a shotgun-MS, LC-MS or GC-MS format [33,35,83-86].

*5.1. Free Fatty Acids (FA)*

Simple fatty acids have been analysed by GC-MS for decades [87] and were one of the first lipid classes to be analysed by FAB-MS in the 1970's [19,88]. For analysis by GC-MS the carboxylic acid group is usually converted to an ester. Many different types of fatty acyl esters have been generated for GC-MS analysis each with their own merits [23,85,89,90]. Besides the popular derivatisation to methyl esters (fatty acyl methyl esters, FAME), two other derivatisations with particular merit are to picolinyl esters [23] and to pentafluorbenzyl esters [85]. Picolinyl esters fragment in the EI source through a charge-mediated mechanism to give a series of fragment-ions resulting from cleavage of successive carbon-carbon bonds, this allows the determination of the position of double bonds, cyclic groups and with trimethylsilylether derivatisation of alcohol groups, the site of hydroxylations [23]. The advantage of derivatisation to pentafluorobenzyl esters is that with negative chemical ionisation dissociative electron capture occurs to give $[RCO_2]^-$ ions allowing detection of chromatographically separated fatty acid derivatives at high sensitivity [85].

*5.2. Eicosanoids and Oxylipins*

In plasma, the other end of the concentration range to cholesterol esters and triacylglycerides are the eicosanoids and oxylipins. These are present in plasma as free acids at the pM level [13]. While strictly speaking, according to chain-length eicosanoids are based on a $C_{20}$ chain, historically the term eicosanoids has also embraced related polyunsaturated fatty acids of longer and shorter chain length. More recently the term oxylipins referring to oxygenated fatty acyls has come into use to cover eicosanoids and their shortened and elongated relatives.

Oxylipins, including eicosanoids, can exist as free acids or as components of complex lipids. In an impressive study based on UHPLC-MS/MS Wang et al were able to monitor the levels of about 140 free oxylipins from only 20 µL of human plasma [33]. To do this a targeted approach was employed both to sample preparation, exploiting solid phase extraction (SPE), and MS/MS utilising time-defined multiple reaction monitoring (MRM). To achieve quantitative data the "gold standard" isotope labelling approach was followed where isotope-labelled internal standards were added to correct for analyte loss and provide MS/MS response factors. As only 26 deuterated standards were available, strictly speaking this provided absolute quantification for only the corresponding non-deuterated endogenous analytes, but it was possible to use these internal standards to quantify other analytes by generation of standard curves using commercially available non-labelled pure standards.

To analyse total oxylipins i.e. the sum of non-esterified and esterified molecules, it is necessary to perform a hydrolysis step. This is problematic as some oxylipins, particularly the prostaglandins are sensitive to basic conditions. Never-the-less, Quehenberger et al developed a method incorporating mild-base hydrolysis suitable for oxylipin analysis in plasma [34]. By the use of isotope-labelled and commercial standards it was possible to quantify 38 oxylipins in plasma over the concentration range of 5 – 40,000 pmol/mL and interestingly on average about 70% of all oxylipins were esterified.

*5.3. Sterols including Oxysterols, Steroids and Bile Acids*

Like fatty acids sterols have been analysed by MS for decades [9-11,86-88,91].

*5.3.1. Cholesterol, its Precursors and Oxysterols*

In recent years there has been a reawakening of interest in cholesterol precursors and metabolites on account of their myriad of biological activities [92-98]. Non-esterified cholesterol is the most abundant single molecular species in plasma, being present at 200 – 500 μg/mL (0.5 – 1.3 mmol/L) and most of its precursors and oxysterols are present at levels of less than 0.1% of that of cholesterol [13]. Oxysterols, cholesterol and its precursors are also present in biological samples esterified to fatty acids and to a lesser extent sulfuric acid and oxysterols can also be found bound to glucuronic acid through an acetal condensation [99-103]. These sterols are usually analysed following saponification to give values for the combination of esters and free analytes [35,40] but have also been analysed in their non-esterified free forms [98,103-105]. The sterol esters are often about 10-fold more abundant than the free analytes [13,99].

One oxysterol receiving increased attention is 25-hydroxycholesterol (25-HC). This can be formed from cholesterol enzymatically by the enzyme cholesterol 25-hydroxylase (CH25H) [106], but also by free radical oxidation *ex vivo*, it is normally found at low levels (ng/mL) in body fluids and tissues (< ng/mg) [107]. However, around 2009 it was discovered that CH25H is upregulated in macrophages in response to lipopolysaccharide (LPS, endotoxin), an integral component of the cell wall of Gram-negative bacteria, with the consequent increase in 25-HC biosynthesis [32,97,108]. These studies were performed by analysis of cells and plasma following class-specific extraction and purification, saponification to hydrolyse oxysterol-fatty acyl esters, then derivatisation to trimethylsilyl ethers and GC-MS utilising isotope-labelled standards for quantification [108], or by LC-MS/MS in the absence of derivatisation exploiting MRM with isotope labelled standards for quantification [97,109]. Cyster and colleagues have suggested that 25-HC supresses interleukin-1 driven inflammation by repressing the processing of SREBP-2 (sterol regulatory element-binding protein 2) transcription factor [95]. Interestingly, a patient with deletion of the *CH25H* gene shows susceptibility to BCG abuses supporting the hypothesis of Cyster and colleagues that 25-HC is anti-inflammatory [110]. CH25H is also up regulated as a consequence of viral infection and its anti-viral activity has also been explained by repression of SREBP-2 processing [111]. Blanc et al used a derivatisation technology to enhance the LC-MS/MS detection of 25-HC, which also aided in the identification of this oxysterol by MS³ [111]. Using Girard P derivatisation each oxysterol isomer gives its own unique MS³ fragmentation pattern and or retention time (Figure 5) [98,103,105]. Importantly, *CH25H* is an interferon (IFN) stimulated gene and it has been suggested that SARS-CoV-2 (COVID-19) may be treated by exogenous IFNs [112]. The beneficial effects of IFNs may be through upregulation of CH25H, and 25-HC has been shown to suppress SARS-CoV-2 replication by blocking membrane fusion [113,114]. 25-HC is metabolised by cytochrome P450 (CYP) 7B1, and the product 7α,25-dihydroxycholesterol (7α,25-diHC), which does not supress SARS-COV-2 replication [114], is normally present at low levels in plasma (ng/mL) [35,59,115]. Its level has not yet been measured in plasma from COVID-19 patients. Interestingly, 7α,25-diHC is a ligand to the G protein-coupled receptor, GR183, also known as Epstein-Barr virus induced gene 2 (EBI2), and acts as a chemoattractant to immune cells expressing this receptor [96]. So while 25-HC can be anti-inflammatory, its metabolite 7α,25-diHC can be pro-inflammatory [116].

Another important oxysterol is 24S-hydroxycholesterol (24S-HC). In human, 24S-HC is the dominant diastereoisomer, although 24R-HC is also present in plasma at much low levels (Figure 5B). 24S-HC and 24R-HC are often unresolved in GC-MS and LC-MS experiments and "biological intelligence" is invoked to characterise the oxysterol identified as the 24S-epimer. The geometrical similarity of 24S-HC, 24R-HC, 25-HC, (25R)26-hydroxycholesterol ((25R)26-HC, also known as 27-hydroxycholesterol) and of 22R-hydroxycholesterol, all of which are present in plasma at different levels makes their chromatographic separation challenging (see Figure 5 and also [109]). This challenge is accentuated in LC-MS/MS studies by the similarity of their MS/MS spectra which, in the absence of derivatisation, are dominated by the loss of one or two molecules of water (see [117] and [118] for reference spectra).

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An advantage of the Girard P derivatisation method used by Blanc et al [111], as developed by Griffiths and Wang [53,98,103,105,119,120], is that the derivatising group which "charge-tags" the analyte directs fragmentation resulting in $MS^3$ spectra that are different for different oxysterol isomers. This in combination with enhanced solubility in reversed-phase solvents allows the determination of essentially all monohydroxycholesterol isomers (Figure 5).

24S-HC is generated from cholesterol by CYP46A1 expressed in neurons [121]. It acts as a transport molecule, removing excess cholesterol from brain [122]. Despite the comparatively mild phenotype of the *Cyp46a1* knock-out mouse [123,124], CYP46A1 appears to be an essential enzyme in human as no inborn error of metabolism resulting from its deficiency has been reported and neither has an individual been found lacking in 24S-HC. Total (unesterified plus esterified) 24S-HC is a marker of neurodegeneration, patients with advanced multiple sclerosis have decreased plasma levels of 24S-HC, but patients in an early stage have increased levels [125]. This can be explained by acute episodes of demyelination causing transient increase in 24S-HC [126]. Similarly, patients with advanced Alzheimer's disease (AD) have significantly reduced levels of plasma 24S-HC [127]. In both AD and multiple sclerosis, the reduced 24S-HC with advanced disease stage can be explained by the loss of CYP46A1 expressing neurons. This concept can also explain reduced plasma levels during the progression of Huntington's disease [128]. With respect to Parkinson's disease the prevailing data suggests that total 24S-HC increases in cerebrospinal fluid of patients with early Parkinson's disease explained by release of cholesterol during cell death providing enhanced quantities of substrate for CYP46A1 [129]. Interestingly CYP46A1 gene therapy has been suggested as a treatment for Huntington's disease [130], Alzheimer's disease [131] and spinocerebellar ataxia [132].

### 5.3.2. Bile Acids Profiling

Excellent methods for the GC-MS profiling of bile acids were developed in the 1970's and 1980's and since these halcyon days the field has possibly regressed at the Alter of simplicity [133-135]. As GC-MS is not generally suitable for analysis of conjugated bile acids sample preparation is paramount involving, separation of different conjugation types followed by hydrolysis, derivatisation and ultimately GC-MS analysis [133,134]. The separation methods make extensive use of ion-exchange chromatography for group fractionation, this still has value for modern day bile acid profiling using LC-MS/MS [102]. A lipidomic study of bile acids is challenging based on the huge range of polarity and solubilities extending from almost water insoluble lithocholic acid (stone bile acid) to highly water soluble taurocholic acid sulfate, and the huge number of different forms of conjugation i.e. at $C_{24}$ a free acid, glycine or taurine amidate or a glucuronic acid ester, and at ring hydroxy groups esters with sulfuric acid or acetals with glucose, glucuronic acid or *N*-acetyl glucosamine [86]. Note, it is unlikely that the exact hexose sugar, will be identified by LC-MS methods. Additionally, hydroxylation can be at almost any carbon when the activities of the gut microflora and the enterohepatic system are considered. The picture is further complicated when $C_{27}$ and *nor* bile acids are considered . Never-the-less there are currently excellent methods for bile acid analysis if it is accepted that they are not fully comprehensive [136-140].

### 5.3.3. Steroid Profiling

Steroid profiling can be thought of the analysis of $C_{21}$ – $C_{18}$ steroids, usually by MS analysis of body fluids [9-11]. Importantly, urinary steroids are not the secreted hormones or their precursors, but end products of hepatic or renal metabolism. As illustration, 3-oxo-4-ene structures of typical steroid hormones or their precursors are reduced to form 3α-hydroxysteroids with either a 5α- or 5β-hydrogen at the A/B ring junction. Commonly a carbonyl at C-20 is converted to a hydroxy group, while hydroxy groups at 17β and 11β are converted to carbonyls. Metabolites with a 3α-hydroxy group are

usually excreted as glucuronides while those with a 3β-hydroxy-5-ene are excreted as sulfates [11]. Following de-conjugation essentially all neutral sterols can be profiled by GC-MS following methyloxime protection of carbonyl groups and trimethylsilylation of hydroxyls. When steroid conjugates are of interest then LC-MS/MS is the method of choice [141].

## 6. Pitfalls and Traps During Analysis

In the above sections we have discussed the different MS-based methods employed to dissect the lipidome. Even when employed with care there are pitfalls for both the novice and experienced scientists to fall into. These include isotopic overlap both in shotgun and LC-MS methods, analyte loss, oxidation and unconsidered enzymatic activity, during sample storage and preparation, and impurities in internal standards. Many of these dangers are discussed by the Lipidomics Standards Initiative [25]. Two of the major shortcomings evident when comparing quantitative data from different laboratories are the efficiencies of different extraction methods and the use of different internal standards. While the second issue can be solved by the use of a common set of standards, both isotope-labelled and non-labelled, the first issue is more problematic. For instance, addition of acid or base may enhance the extraction of certain lipids but it may result in the decomposition of others. It is also essentially impossible, without radioactive monitoring, to determine true extraction efficiency from a biological sample.

Even if we fall into a trap, it is of value to make our data accessible to all. Lipidomics data can be made available through the Metabolights database [142], Metabolomics Workbench [143] and the Center for Open Science [144] to name just a few.

## 7. Top Tips or Personal Bias

Simple microchemical reactions have been used to aid structure determinations since the emergence of lipids as a class of biomolecule. There is still a value for simple chemistry in lipidomics today, whether it is GC-MS [23,90], for charge reversal as in Han and colleagues "acidomics" for analysis of fatty acid by LC-MS [83] or Girard derivatisation to enhance ionisation and fragmentation of lipids with a carbonyl group [52,53,105,119], and also for their MSI [50,145], or for double bond location using ozonolysis [20] or the Paternò-Büchi reaction [21,22,69]. In fact there are a myriad of simple chemical reactions that the lipidomics analyst can explore in an effort to solve different problems [146]. We recommend this as a route to consider when routine lipidomics methods fail.

## 8. Towards Clinical Chemistry

Lipid analysis is well established in clinical chemistry laboratories for targeted analysis to diagnose in-born errors of metabolism [147]. For many years, acylcarnitines have been analysed by ESI-MS/MS from dried blood spots to diagnose organic acid disorders and 17-hydroxyprogesterone is routinely analysed in newborn screening laboratories to diagnose 21-hydroxylase deficiency resulting in congenital adrenal hyperplasia. Disorders of bile acid metabolism are diagnosed in screening laboratories by ESI-MS [140,148,149], as are in-born errors of cholesterol biosynthesis [150]. 25-Hydroxyvitamin $D_3$ is now commonly measured by LC-MS/MS to asses vitamins D deficiency [29]. These are mostly single analyte assays, where a high or low level of metabolite can be used to diagnose a disorder.

A metabolite profile or lipidome will provide even more information to the clinician, but the immediate need of the clinician is simply to make the diagnosis in the quickest and simplest possible way. For clinical science to fully embrace lipidomics the field is going to have to demonstrate that it can diagnose one of the multifactorial diseases of the twenty first century, such as non-alcoholic fatty

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liver disease progressing to non-alcoholic steatohepatitis, atherosclerosis, one of the neurodegenerartive disease or even COVOID-19.

Standard reference materials have been used for some time by external quality assessment schemes for clinical chemistry laboratories [29]. The NIST SRM 1950 plasma has clinical chemistry data associated with it provided by NIST, including values for total cholesterol, estradiol, progesterone, cortisol, dehydroepiandrosterone and testosterone determined by immunoassay, values for 25-hydroxyvitamin $D_3$, cortisol, testosterone and progesterone determined by LC-MS/MS and values for free fatty acids and total cholesterol determined by GC-MS [30,38]. This material has been extensively analysed in lipidomic studies. In their 2010 study Lipid Maps provided quantitative data for over 500 distinct molecular species [13] and more recently a community effort measured the levels of 1,527 lipids identified at the sum composition level (species level) in this sample [14]. The results of the second study could be summarised by stating that there was consistently inconsistent results between different sites and platforms [37]. There is now another community effort lead by Singapore Lipidomics Incubator to reanalyse the SRM 1950 in a targeted lipidomic manner to provide absolute quantitative data for defined analytes. While there are dangers in determining consensus values as an "all laboratory trimmed mean" (ALTM), perhaps this strategy is the best one to move lipidomics into clinical laboratories. Even if ALTM values are not truly accurate, by providing a list of reference values in a standard reference material that all laboratories can access will at least allow clinical laboratories to "calibrate" their value to a reference value and allow interlaboratory comparison of data which will be necessary for lipidomics to use its full data sets for disease diagnosis and prognosis.

## 9. Concepts in Lipidomics, Metabolomics and Proteomics

The study of proteomics has driven impressive developments in MS technology. Motivated by the need for enhanced sensitivity Wilm and Mann developed nano-ESI [151], Emmett and Caprioli developed LC compatible micro-ES [152] and Caprioli and colleagues developed MSI [153], while instrument manufacturers have made huge developments through the introduction of Q-TOF [154], hybrid ion-trap FTICR [155] and hybrid Orbitrap instruments [156]. However, some concepts in proteomics are very different to those understood by lipid chemists e.g. identification. In top-down proteomics hundreds to thousands of proteins are identified in a single MS/MS experiment through searching a protein/gene database with peptide data derived by MS/MS following proteolytic digestion. Each identified protein will have a probability of being correct. The identification does not require that the MS data is sufficient to match the entire amino acid sequence of the protein, just to provide a probability that this identification is the most likely one from the entries in the data base and above a defined false discovery rate. It is very possible that the proteins confidently identified do not exactly match the translate gene sequences within in the data base. They may be isoforms or variants of the deposited sequence, or the protein actually analysed may be post translationally modified. Hence the mindset of the scientist with a proteomics background is very different from one of a lipid chemist who regards identification as the definition of an exact chemical structure.

The metabolomics expert comes at identification from yet another angle. In many metabolomics experiments the goal is to identify what has changed in a system as a result of perturbation of that system. Here multivariant statistics are used to discover which MS features have changed as a result of the perturbation. The goal is then to identify these features, this is usually done by searching a metabolite database which is often compiled from many other databases. Unless the database to be searched is carefully curated this process is fraught with dangers. It is not uncommon that metabolites in a database are only there as a consequence of their chemical synthesis and patenting or are completely theoretical. In addition, identifications in metabolomics are made to different levels which may not be understood by all. Identification can be simply made by the matching of a measured *m/z*

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with a certain tolerance to a metabolite entry in a database, or the identification may include the matching of a second parameter, such as specific fragments in an MS/MS spectrum to those of standard compounds.

As long as the nomenclature used for identifications made by scientists with a proteomics, metabolomics or lipid biochemistry background are understood, each concept of identification is perfectly reasonable. The problem arises where the disciplines cross and the nomenclature of identification is not clearly defined in published works [25,36,157].

Quantification is another term that can mean different things to different scientists. As with the concept of identification the best way to avoid confusion is by clarity of reporting. In proteomics and metabolomics the goal is very often to determine relative quantitative changes. This can be aided by the use of derivatisation and differential isotope-coded tags [158,159] and in proteomics through stable-isotope labelling of whole proteins [160]. To the lipid chemist the goal is absolute quantification of a defined species. In targeted lipidomic studies this is perfectly possible and can be achieved by the incorporation during sample preparation of isotope-labelled standards, ideally exactly matching the compound to be quantified [33,35,99]. For many classes of lipid it is impractical to have isotope-labelled standards for each exact lipid, in which case it is reasonable to use one or two isotope-labelled standards per lipid class. It is preferable to quote lipid concentrations in moles per unit volume, moles per unit wet or dry weight or moles per unit of protein.

## 10. Future

What of the future of lipidomics? To our minds lipidomics will be of most value when accurate quantification is linked to reliable identifications and to biological location. This goes beyond biofluids into tissues. Exciting data has been obtained by MALDI-MSI [49,50,161], desorption-ESI (DESI)-MSI [162,163] and also through liquid extraction for surface analysis (LESA)-MSI [163,164]. We are also excited by the combination of LESA – chromatography – $MS^n$ as recently developed by Yutuc et al to quantitate cholesterol and its metabolites in brain tissue (Figure 5F & G) [145]. Yutuc et al linked LESA with LC-$MS^n$. It will be exciting in the future to see if ion-mobility can match the separation power to LC .

## 11. Conclusion

In this centenary, lipidomics as a discipline has taken huge strides forward. Much of this is based on basic gas-phase ion chemistry, our improved understanding of it to explain fragmentation and its application to help determine structure. From the conceptually simple but beautiful shotgun lipidomics of Han and Gross to the much more complex class-specific lipidomics of Lipid Maps the methodologies are available to solve many of the next-generation problems in biology.

## 12. Expert Opinion

- *A real world impact in disease diagnosis and monitoring response to therapy*

In the short-term class-specific lipidomics is most likely to have an impact in medicine. It is not difficult to envisage large panels of target lipids analysed in a single run being used to "metabolically diagnose" numerous in-born errors of metabolism simultaneously. In academic laboratories this is already routine, the challenge will be translating it to a clinical environment. Similarly, with a knowledge of pre-existing metabolic markers response to therapy can be monitored.

It is unlikely that there will be similarly definitive markers for multifactorial diseases, in which case multivariant statistics is likely to be key. The challenge will be for laboratories throughout the world

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to be able to reproduce the lipidomic measurements for hundreds of lipids necessary for cross-laboratory diagnosis.

- *Overcoming the problems of miss-communication*

As discussed above, a clarity in reporting of identification and quantification is required. This is absolutely necessary when data from published papers is trawled by computer algorithm which will be oblivious to the nuances of tone of language. It is better to have fifty correct identifications than two hundred which may or not be correct.

- *Reference values as a guide but not a definition of fact*

Perhaps the only way for lipidomics to become accepted fully into the clinical chemistry laboratory is in combination with standard reference materials, where ALTM values will be provided for many hundreds of individual species. Each laboratory will then be able to compare its data to that provided along with the reference material. It should be noted that the ALTM value is not necessarily the true value. For example, there is no guarantee that the most popular method of sample extraction is the best one and it is almost impossible to assess the true extraction efficiency of an endogenous molecule.

- *Evolution or revolution?*

Where will lipidomics be in five years. Certainly, the newest MS instruments will be faster, more sensitive and have higher resolution. But will these provide "game breakers" in the discipline? Perhaps the greatest development in our understanding of lipid biology will come in the marriage of lipidomics with MSI allowing the observation of lipidomes at almost the cellular level. There is also the exciting prospect of determining tumour margins by lipidomics in theatre.

**Acknowledgement**

**Figure and Table Captions**

Figure 1. MS/MS fragmentation of the $[M+NH_4]^+$ ions of (A) the cholesterol ester CE 18:1(9Z) and of (B - D) the triacylglycerol TG 16:1(9Z)/18:0/18:2(9Z,12Z). Shorthand notation for identification at the molecular species level and full structural level is shown.

Figure 2. MS/MS fragmentation of the $[M+H]^+$ ion of the glycerophosphocholine PC 16:0/18:1(9Z). Formation of the fragment ion (A) at *m/z* 184.07 and of (B) $[M+H-R_2CH=C=O]^+$ and (C) $[M+H-R_1CH=C=O]^+$. Shorthand notation for identification at the species level, molecular species level and full structural level is shown.

Figure 3. MS/MS fragmentation of the $[M+H]^+$ ion of (A) glycerophosphoethanolamine PE 16:0/18:1(9Z), of the (B) $[M-H]^-$ ion and (C) of the $[M+H]^+$ ion glycerophosphoserine PS 16:0/18:1(9Z), and (D) of the $[M-H]^-$ ion of glycerophosphoinositol PI 16:0/18:1(9Z). Shorthand notation for identification at the species level, molecular species level and full structural level is shown.

Figure 4. MS/MS fragmentation of (A – B) $[M-H]^-$ ions of glycerophospholipids, (C) $[M+H]^+$ and (D) $[M-H]^-$ of sphingomyelin SM 18;O2/16:0.

Figure 5. Analysis of oxysterols following charge-tagging with Girard P (GP) reagent. (A) Separation of oxysterols in plasma. As a consequence of derivatisation each oxysterol gives *syn* and *anti* conformers which may or may not be resolved. (B) MRM ([M]$^+$→[M-Py]$^+$]→353.3) targeting 24-HC, Py is pyridine. The top chromatogram shows the 24S-HC and 24R-HC epimers in plasma, the bottom chromatogram shows [$^2$H$_7$]-labelled epimers eluting slightly earlier. MS$^3$ ([M]$^+$→[M-Py]$^+$]→) spectra of (C) 24S-HC, (D) 25-HC and (E) (25R)26-HC. LESA-LC-MS analysis of a sagittal section of mouse brain, quantification of (F) cholesterol and (G) 24S-HC. Panels (F) and (G) are reproduced from reference [145] distributed under Creative Commons Attribution License 4.0 (CC BY).

Table 1. Six of the eight lipid categories as defined by Lipid Maps and some common classes within these categories[1] [1-3].

Table 2. Typical figures of merit for different MS/MS instruments used in lipidomics.

Table 3. Simplified comparison of shotgun and category/class specific strategies in lipidomics.

Table 4. Key fragment-ions useful for the determination of structure at the species level and above in shotgun and targeted lipidomic studies of some complex lipids.

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Table 1. Six of the eight lipid categories as defined by Lipid Maps and some common classes within these categories[1] [1-3].

| Fatty Acyl (FA)[2] | Glycerolipids (GL) | Glycerophospholipids (GP) | Sphingolipids (SP) | Sterol Lipids (ST) | Prenol Lipids (PL) |
|---|---|---|---|---|---|
| Fatty acids (FA01)[2][FA][3] | Monoradylglycerols (GL01)[MG] | Glycerophosphocholines (GP01)[PC] | Spingoid bases (SP01)[SPB] | Sterols (ST01)[ST] | Isoprenoids (PR01) |
| Eicosanoids (FA03) | Diradylglycerols (GL02)[DG] | Glycerophosphoethanolamines (GP02)[PE] | Ceramides (SP02)[Cer] | Steroids (ST02) | Polyprenols (PR03) |
| Fatty alcohols (FA05)[FOH] | Triradylglycerols (GL03)[TG] | Glycerophosphoserines (GP03)[PS] | Phosphosphingolipids (SP03)[SM] | Seco-sterols (ST03) | |
| Fatty aldehydes (FA06)[FAL] | | Glycerophosphoglycerols (GP04)[PG] | | Bile acids (ST04)[BA] | |
| | | Glycerophosphoglycerophosphates (GP05)[PGP] | | Sterol esters (ST0102)[SE] | |
| | | Glycerophosphoinositols (GP06)[PI] | | | |
| | | Glycerophosphates (GP10)[PA] | | | |

[1]The other two categories of lipids are saccarolipids (SL) and polyketides (PK).

[2]Lipid Maps category, class abbreviations and codes are shown in parentheses.

[3]Common abbreviations are in brackets.

Table 2. Typical figures of merit for different MS/MS instruments used in lipidomics.

| Instrument | Mass Resolution (FWHM) | Scan Speed | Mass Accuracy (ppm) | Sensitivity | Quantification | MS/MS Fragmentation | Identification | Cost | Size |
|---|---|---|---|---|---|---|---|---|---|
| **Hybrid FTICR** | >1,000,0000 | # | <2 | # | ### | Multiple modes | #### | #### | Floor standing |
| **Hybrid Orbitrap** | 100,000 - 1,000,000 | ## | <5 | ## | ### | Multiple modes | #### | ### | Floor standing / Bench top |
| **Q-TOF** | 10,000 – 80,000 | #### | <5 | ## | ### | Low energy CID | ### | ### | Floor standing/ Bench top |
| **Tandem Quadrupole** | 1,000 | ### | 100 | #### | #### | Low energy CID | # | # | Bench top |
| **LIT** | 2,000 | ### | 100 | ### | ## | Resonance excitation | ### | # | Bench top |

Key – High ####, Low #

Table 3. Simplified comparison of shotgun and category/class specific strategies in lipidomics.

| | Shotgun | Category/Class Specific |
|---|---|---|
| **Sample Preparation** | Often Bligh and Dyer, Folch or MTBE[1] | Extraction tailored to lipid category/class of interest |
| **On-line chromatography** | No | Reversed-, normal- or HILIC-phase, HPLC or UHPLC with ESI-MS, GC-MS |
| **MS and MS/MS** | Usually tandem quadrupole or hybrid-Orbitrap | Any tandem instrument |
| **Analysis speed** | Minutes | Many minutes |
| **Identification** | Species (sum composition) or molecular species level | Molecular species level and above |
| **Major Advantages** | Fast, multiple categories simultaneously analysed | High dynamic range, retention time provides additional evidence for identification |
| **Major Disadvantage** | Isomers not separated at MS level, discriminates against minor metabolites | Time consuming, data generated more complex |

[1]The Lipidomics Standards Initiative provides a useful website with links to sample preparation methods [25]. Lipid Maps provides a similerly useful resource [26,27].

Table 4. Key fragment-ions useful for the determination of structure at the species level and above in shotgun and targeted lipidomic studies of some complex lipids.

| Lipid Class | Precursor-ion | Head Group Containing Fragment | Acyl Chain | Comment |
|---|---|---|---|---|
| **Cholesterol ester** | $[M+NH_4]^+$ | $[Chol+H-H_2O]^+$ $m/z$ 369.35 | | Molecular species level e.g. [65,66] |
| **Triacylglycerol** | $[M+NH_4]^+$ | $[M+H-RCO_2H]^+$ | | Molecular species level |
| **Diacylglycerol** | $[M+NH_4]^+$ | $[M+H-RCO_2H]^+$ | | Molecular species level |
| **Glycerophosphocholine** | $[M+H]^+$ | $[H_2O_3POC_2H_4N(CH_3)_3]^+$ $m/z$ 184.07 $[M+H-RCH=C=O]^+$ | | Species level - Molecular species level e.g. [70,71] |
| **Glycerophosphocholine** | $[M-15]^-$ | | $[R_1CO_2]^-$ $[R_2CO_2]^-$ | Molecular species level[1] |
| **Glycerophospho-ethanolamine** | $[M+H]^+$ | $[M+H-141.02]^+$ | | Species level e.g. [72,73] |
| **Glycerophospho-ethanolamine** | $[M-H]^-$ | | $[R_1CO_2]^-$ $[R_2CO_2]^-$ | Molecular species level[1] e.g. [74] |
| **Glycerophosphoserine** | $[M+H]^+$ | $[M+H-185.01]^+$ | | Species level e.g. [76,77] |
| **Glycerophosphoserine** | $[M-H]^-$ | $[M-H-87.03]^-$ | $[R_1CO_2]^-$ $[R_2CO_2]^-$ | Molecular species level e.g. [75] |
| **Glycerophosphatidic acid** | $[M+H]^+$ | $[M+H-95.96]^+$ | | |
| **Glycerophosphatidic acid** | $[M-H]^-$ | | $[R_1CO_2]^-$ $[R_2CO_2]^-$ | Molecular species level e.g. [78,79] |
| **Glycerophosphotidylinositol** | $[M+H]^+$ | $[M+H-260.03]^+$ | | Species level |
| **Glycerophosphotidylinositol** | $[M-H]^-$ | $m/z$ 241.01 | $[R_1CO_2]^-$ $[R_2CO_2]^-$ | Molecular species level e.g. [80,81] |
| **Sphingomyelin** | $[M+H]^+$ | $[H_2O_3POC_2H_4N(CH_3)_3]^+$ $m/z$ 184.07 | | e.g. [82] |
| **Sphingomyelin** | $[M-H]^-$ | $[HO_3POC_2H_4N(CH_3)_2]^-$ $m/z$ 168.04 | | |

[1]When combined with positive-ion data gives identification at the molecular species level.

$[Chol+H-H_2O]^+$ corresponds to the dehydrated protonated molecule of cholesterol.

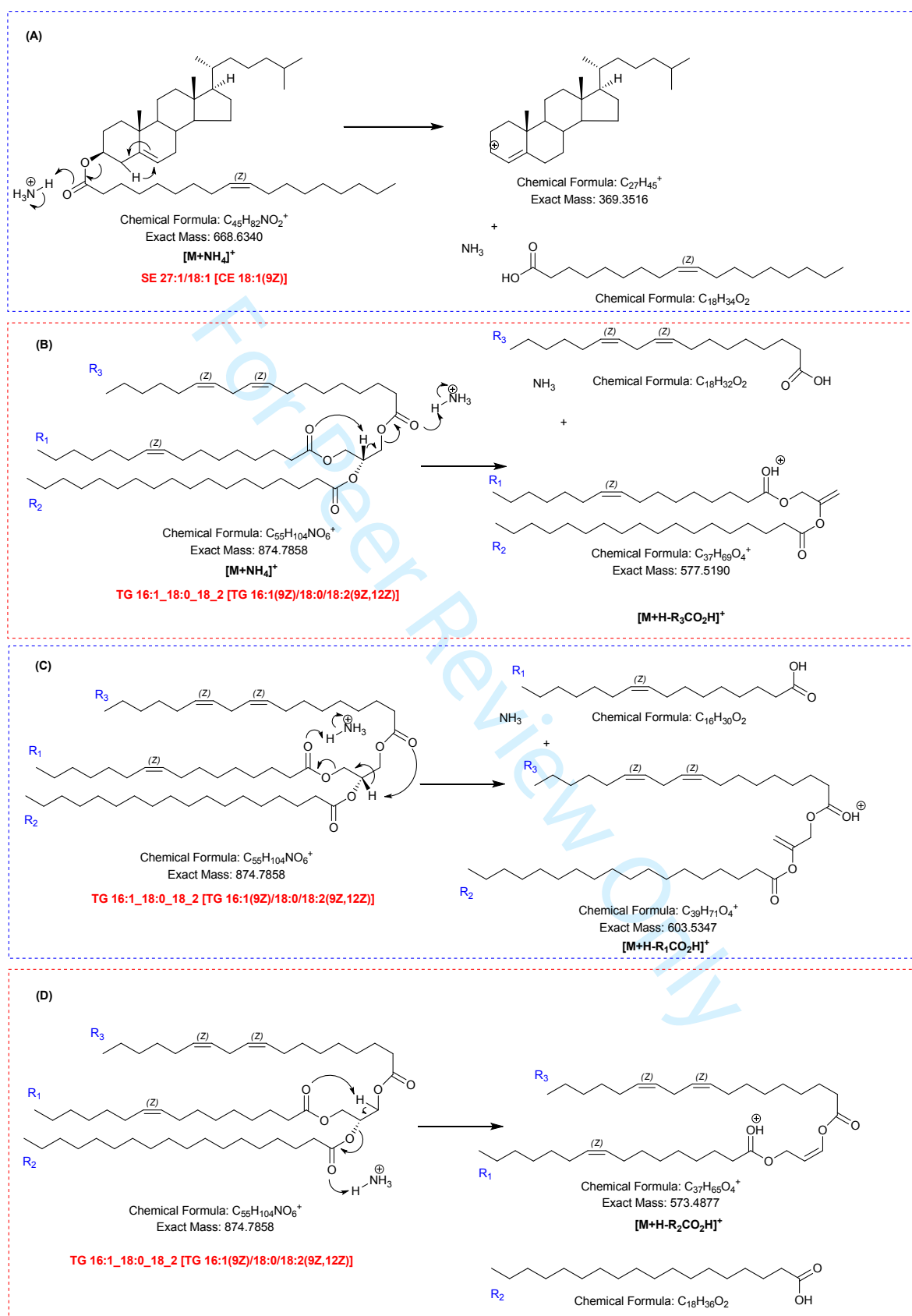See the resources section on the Lipidomics Standards Initiative website for additional useful information [25].
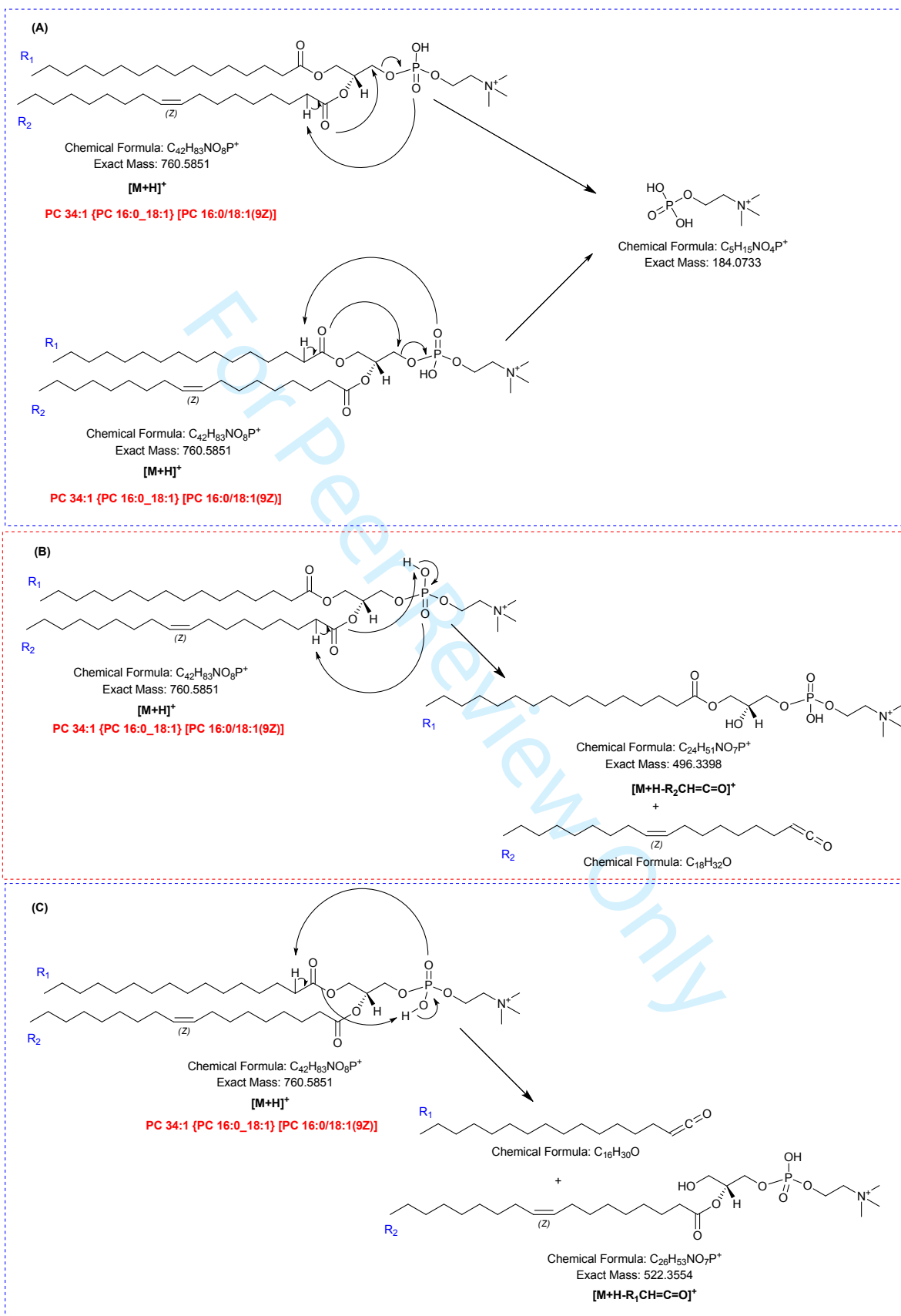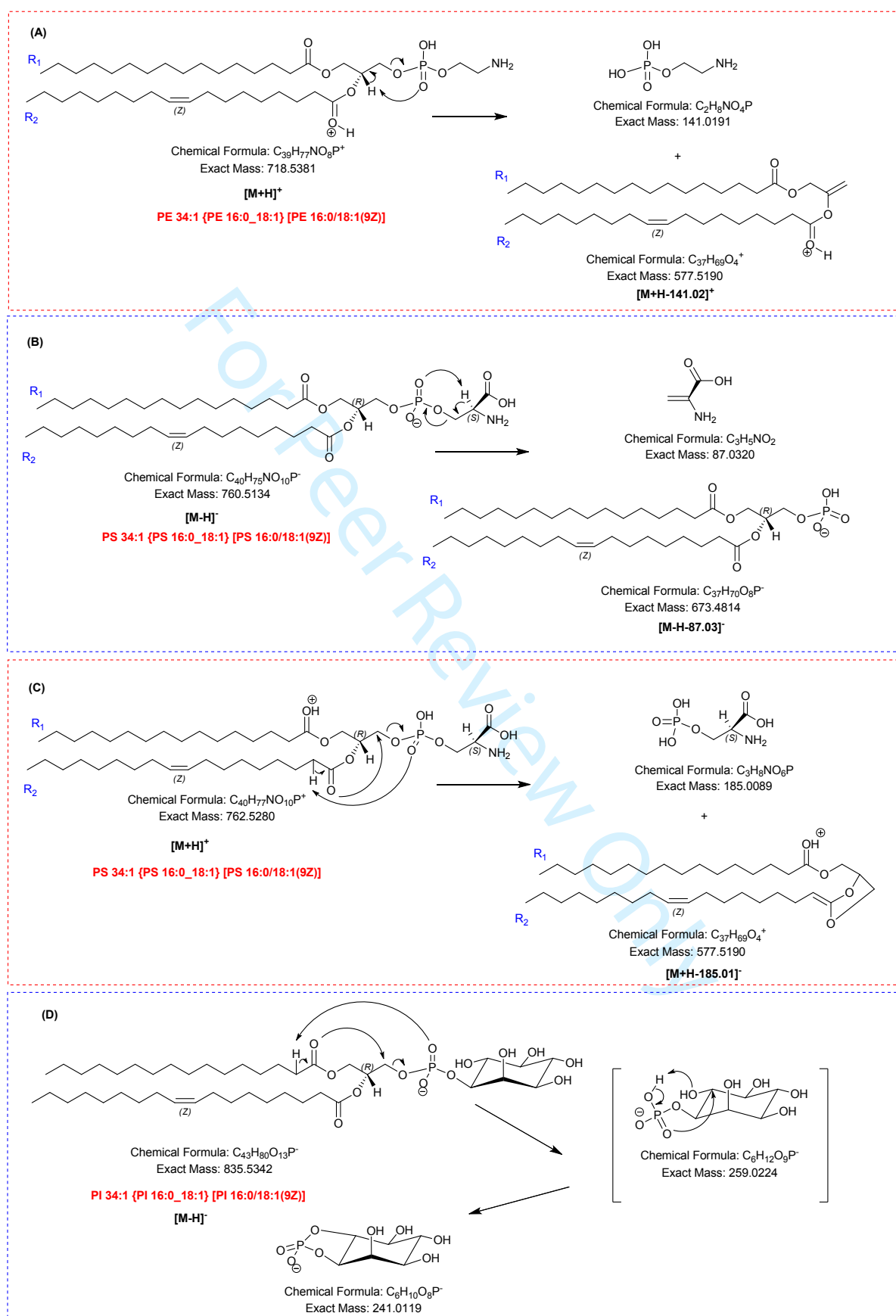
Figure 1

Figure 2

Figure 3

Figure 4