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Shotgun Lipidomics on High Resolution Mass Spectrometers

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Despite their compositional complexity, lipidomes comprise a large number of isobaric species that cannot be distinguished by conventional low resolution mass spectrometry and therefore in-depth MS/MS analysis was required for their accurate quantification. Here we argue that the progress in high resolution mass spectrometry is changing the concept of lipidome characterization. Because exact masses of isobaric species belonging to different lipid classes are not necessarily identical, they can now be distinguished and directly quantified in total lipid extracts. By streamlining and simplifying the molecular characterization of lipidomes, high resolution mass spectrometry has developed into a generic tool for cell biology and molecular medicine.

As an independent scientific discipline, lipidomics aims at the quantitative characterization of the full lipid complement produced by cells, organisms, or tissues (reviewed in Wenk 2005; Oresic et al. 2008; Dennis 2009). According to different estimates, eukaryotic lipidomes might comprise from 10,000 to 100,000 species originating from a few hundred individual lipid classes (van Meer 2005; van Meer et al. 2008; Yetukuri et al. 2008). Lipidomics relies heavily on mass spectrometry, which helps to recognize individual molecules of varying molecular lipid structures at the low picomole sensitivity (reviewed in Ivanova et al. 2009; Blanksby and Mitchell 2010; Shevchenko and Simons 2010).

Irrespective of a particular method implementation, mass spectrometry characterizes lipid molecules in two major ways. First, it determines their intact masses. Second, by colliding molecular ions of lipid precursors with neutral gas, tandem mass spectrometry dissociates them into several structure-specific fragments (reviewed in Hsu and Turk 2009) such that individual molecular species can be recognized even in total lipid extracts (reviewed in Han and Gross 2005; Niemela et al. 2009). Top-down lipidomics is a strategy that aims at the

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rapid quantitative characterization of global changes of the lipidome composition and is solely reliant on accurately determined masses of intact lipids (Houjou et al. 2005; Schwudke et al. 2007a). The method operates with the integral abundances of clusters of lipid molecules with exactly the same mass-to-charge ratios (m/z) and does not necessarily identify individual species. In contrast, bottom-up lipidomics strategy aims at the accurate quantification of individual molecular species and relies entirely upon the detection of characteristic fragment ions by tandem mass spectrometry (Ejsing et al. 2006, 2009; Schwudke et al. 2006).

The specificity of both top-down and bottom-up approaches depends on the ability of a mass spectrometer to distinguish the peaks of intact molecular ions or their fragments. It is characterized by the instrument's resolving power (or, synonymously, mass resolution) R, which is usually calculated as a ratio of the ion peak mass to the peak width at the half of its height to the mass of the peak (full width at half maximum [FWHM]) (Gross 2006). To exemplify mass resolution in more "visual" terms, we could assume that two equally abundant peaks having $(m/z)_1 = R$ and $(m/z)_2 = R + 1$ are expected to overlap at 50% of their height (this, however, only holds true for some "ideal" device—in reality, mass resolution depends on the ion mass and also mass range of the instruments is limited). Mass resolution depends on the type of mass analyzer employed in a particular mass spectrometer, as well as actual experiment settings: often, it is intentionally compromised for the sake of achieving better sensitivity or spectra acquisition rate (reviewed in Glish and Burinsky 2008; Griffiths and Wang 2009).

Until very recently it was generally assumed that unit mass resolution, under which a mass spectrometer should be able to distinguish peaks separated by 1 Da (like isotopic peaks of a singly charged ion) is sufficient for the vast majority of lipidomics applications. Indeed, molecules of glycerophospolipids—the most abundant component of both eukaryotic and prokaryotic lipidomes—are often isobaric and the same nominal mass (Yergey et al. 1983;

Brenton and Godfrey 2010) might represent several individual species of different lipid classes. Direct measurement of intact masses did not allow their unequivocal assignment to individual species or even to lipid classes and it was required to fragment the entire cluster of plausible precursors and detect fragment ions specific for each of these isobaric molecules (Ekroos et al. 2002; Ejsing et al. 2006). Therefore, reaching higher than unit mass resolution by compromising the sensitivity might only result in marginal increase of the overall lipid identification capacity. Also, only a decade ago the mass resolution of over 50,000 was only achievable at FT ICR (Fourier transform ion cyclotron resonance) mass spectrometers featured with superconducting magnets that were expensive and complex in operation and maintenance.

Orbitrap mass analyzers, first introduced in hybrid linear ion trap–Orbitrap tandem mass spectrometers (LTQ Orbitrap) (Makarov et al. 2006a,b), have been changing the paradigm of lipidomics analysis. The mass resolution of 100,000 together with sub-ppm mass accuracy (Olsen et al. 2005) has become routinely achievable in both MS and MS/MS modes. Combining Orbitrap and linear ion trap analyzers within a hybrid tandem instrument enabled efficient MS/MS experiments and increased the dynamic range of ion detection to more than 1000-fold (Scigelova and Makarov 2006). However, high mass resolution comes at the price of reduced spectra acquisition rate. It might take up to 2 sec to acquire a spectrum at 100,000 mass resolution, which is impractical for the analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Olsen et al. 2009). However, acquisition time is less important for shotgun lipidomics, in which total extracts are directly infused into a mass spectrometer. Because the composition of infused analyte does not change in the course of analysis, both MS and MS/MS spectra could be acquired at the minute time scale (Schwudke et al. 2006, 2007b).

High mass resolution of modern instruments offers robust practical solutions for both top-down and bottom-up shotgun lipidomics because isobaric precursor ions and

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fragments can now be distinguished and independently quantified (Schwudke et al. 2007a). This enhances both the specificity and throughput of top-down shotgun screens and enables accurate structural characterization of individual molecular species in tandem mass spectrometric experiments.

IDENTIFYING LIPIDS BY ACCURATE MASSES OF INTACT PRECURSORS

Why might species of different lipid classes have isobaric masses? Let us first consider glycerophospholipids (GPL) as a representative example. For clarity, we assume that the chemical structure of GPL molecules is composed of two elements, each of which may independently vary: a head group together with glycerol backbone with two ester or ester and ether moieties attached (here we will term it as a lipid class determinant, or LCD) and an aliphatic complement (AC), comprising methylene (-CH_2-) and methylidene (-CH = CH-) groups (Fig. 1).
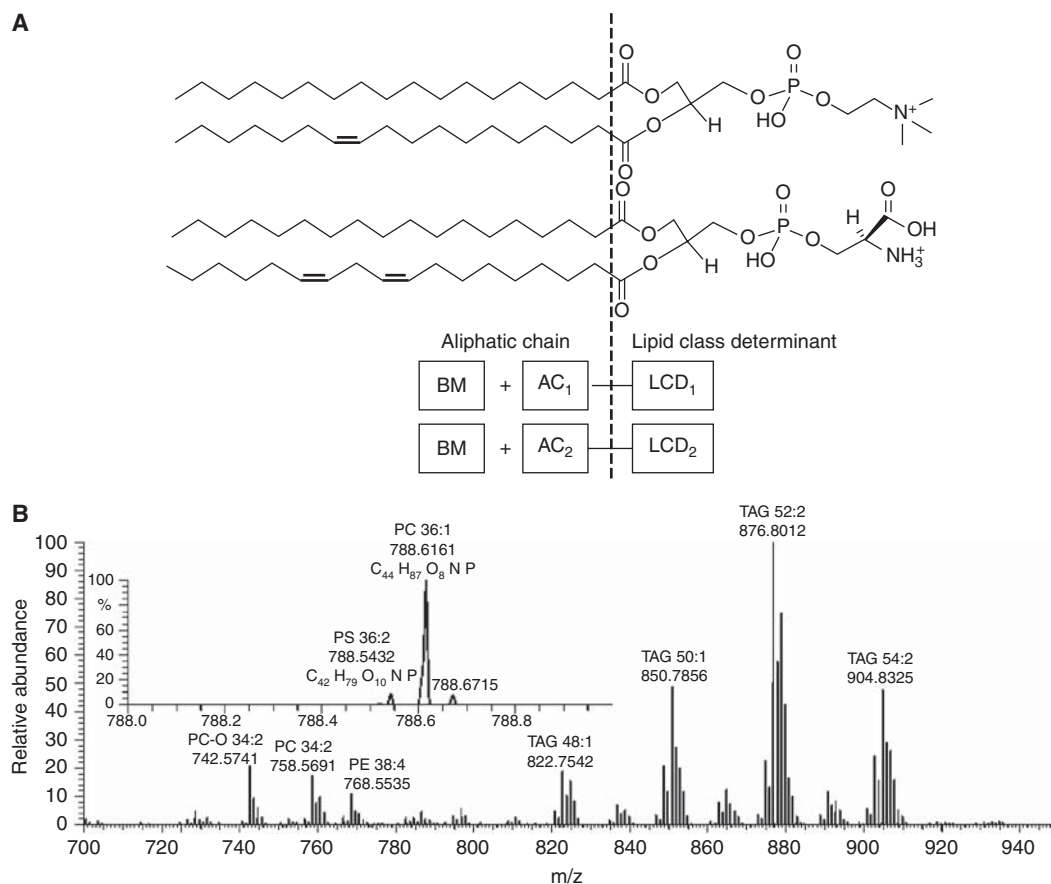
Mass differences between LCDs could be balanced by altering the complementary AC so that masses of certain species from different lipid classes might be isobaric, although their exact masses will still be different. For example, the mass difference between the LCDs of phosphatidylcholine (PC) and phosphatidylserine (PS) species is 1.9429 Da. Apparently, masses of PC and PS species could be isobaric if they have the same number of carbon atoms in their fatty acid moieties and the PS molecule has one double bond more compared to the PC. Although they have the same nominal masses (i.e., they are isobaric), their exact masses will differ by 0.0726 Da and it should be possible to distinguish their peaks in high resolution mass spectra (Fig. 1).

How could we determine whether the exact masses are unique for species of a given lipid class, or if they overlap with masses of species from other classes? With no compositional constraints applied, major lipid classes can be clustered into several groups (Table 1). Exact masses of lipids from these groups do not overlap and their peaks in MS spectra could be attributed unequivocally, no matter what fatty acid or fatty

alcohol moieties they comprise. Each group differs by the unique number of N, O, and P atoms in their molecular cations or anions. Some groups consist of more than one lipid class, implying that species of different lipid classes could have the same exact masses. For example, it might not be possible to judge solely from the accurately measured masses whether a peak belongs to the lipid of PC or phosphatidylethanolamine (PE) class because they are within the same group. At the same time, if a peak in the high resolution spectrum matches the mass of a sphingomyelin (SM) species, its attribution is unequivocal. We underscore that Table 1 presents the composition of heteroatoms of molecular ions, rather than neutral lipid molecules. Several lipid classes (such as, for example, triacylglycerols [TAG]) are barely detectable in the protonated form $[M + H]^+$, but produce abundant adducts with cations of alkaline metals or ammonium $[M + NH_4]^+$ (McAnoy et al. 2005; Schwudke et al. 2006; Hsu and Turk 2010). Note that grouping of lipid classes differs between positive and negative modes, because some lipids are detectable as adducts, whereas others are not. For example, in negative ion mode PCs produce abundant adducts with acetate or formiate anions (Ekroos et al. 2003), whereas PEs are detected as deprotonated anions. Because of the different heteroatom composition of their anionic forms, they fell into different groups and their peaks could be unequivocally distinguished.

Would even higher mass resolution further increase the specificity of top-down lipidomics? A mass difference of 0.009 Da spaces the second isotopic peak of a lipid species and the monoisotopic peak of the species from the same lipid class that lacks one double bond. Computational modeling suggests that although these peaks are not completely resolved even at 100,000 mass resolution, they still could be recognized as individual signals by a conventional spectra processing software. Within the entire space of lipid compositions there are no isobaric pairs with closer exact masses and therefore we speculate that higher mass resolution (e.g., exceeding 500,000) might only result in the marginal improvement of lipid identification.

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A

Aliphatic chain | Lipid class determinant

| BM | + | AC$_1$ | LCD$_1$ |
| BM | + | AC$_2$ | LCD$_2$ |

B

TAG 52:2
876.8012

PC 36:1
788.6161
C$_{44}$ H$_{87}$ O$_8$ N P

PS 36:2
788.5432
C$_{42}$ H$_{79}$ O$_{10}$ N P
788.6715

TAG 50:1
850.7856

TAG 54:2
904.8325

PC-O 34:2
742.5741
PC 34:2
758.5691
PE 38:4
768.5535

TAG 48:1
822.7542

m/z

Figure 1. Molecular ions of isobaric species could be distinguished in high resolution mass spectra. (*A*) Chemical structures of molecular cations of isobaric PC 36:1 and PS 36:2. In general, masses of isobaric species should meet the following requirements:

$$(LCD_1 + AC_1) - (LCD_2 + AC_2 + BM) \rightarrow 0; \quad BM = x_1(14.0153) - d_1(2.0153) \text{ or}$$
$$(LCD_1 + AC_1 - BM) - (LCD_2 + AC_2) \rightarrow 0; \quad BM = x_2(14.0153) + d_2(2.0153)$$

Here LCD stands for the mass of lipid class determinant; AC, mass of the aliphatic complement of the first and second lipid species; BM, balance mass required to form a pair of isobaric lipid species; x_1, x_2, number of methylene groups; and d_1, d_2, the number of double bonds. (*B*) LTQ Orbitrap MS spectrum of a total lipid extract of bovine heart acquired at the resolution of 100,000. Monoisotopic peaks of isobaric PS 36:2 and PC 36:1 are shown in the *inset*.

We underscore that the grouping of lipid classes in Table 1 presumed that no restrictions on the length and number of double bonds in their fatty acid/fatty alcohol moieties were applied. However, some of these moieties might not occur because of the stringent specificity of lipid biosynthesis pathways. Therefore, applying some broad, yet reasonable compositional restrictions (such as limiting the number of double bonds or length of fatty acid/fatty alcohol moieties, restricting fatty acids to those having even number of carbon atoms, etc.) dramatically increases the specificity of mass assignment.

Therefore we conclude that the acquisition of high mass resolution spectra provides sufficiently specific means for taking a rapid

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Table 1. Major lipid classes grouped according to the number of heteroatoms

Lipid class	Positive mode[a]	Negative mode[a]	N	O	P
DAG	–	$[DAG + Ac]^-$	0	7	0
TAG	–	$[TAG + Ac]^-$	0	8	0
PA	–	$[PA - H]^-$	0	8	1
CholFA	$[Chol\text{-}FA + NH_4]^+$	–	1	2	0
Cer	$[Cer + H]^+$	$[Cer - H]^-$	1	3	0
DAG	$[DAG + NH_4]^+$	–	1	5	0
TAG	$[TAG + NH_4]^+$	–	1	6	0
PE-O	$[PE\text{-}O + H]^+$	$[PE\text{-}O - H]^-$	1	7	1
LPE	$[LPE + H]^+$	$[LPE - H]^-$	1	7	1
PC-O	$[PC\text{-}O + H]^+$	–	1	7	1
LPC	$[LPC + H]^+$	–	1	7	1
HexCer	$[HexCer + H]^+$	$[HexCer - H]^-$	1	8	0
PE	$[PE + H]^+$	$[PE - H]^-$	1	8	1
PC	$[PC + H]^+$	–	1	8	1
PA	$[PA + NH_4]^+$	–	1	8	1
PC-O	–	$[PC\text{-}O + Ac]^-$	1	9	1
LPC	–	$[LPC + Ac]^-$	1	9	1
PG	–	$[PG - H]^-$	0	10	1
PS	$[PS + H]^+$	$[PS - H]^-$	1	10	1
PG	$[PG + NH_4]^+$	–	1	10	1
PC	–	$[PC + Ac]^-$	1	10	1
PI	–	$[PI - H]^-$	0	13	1
PI	$[PI + NH_4]^+$	–	1	13	1
SM	$[SM + H]^+$	–	2	6	1
CerPE	$[CerPE + H]^+$	$[CerPE - H]^-$	2	6	1
SM	–	$[SM + Ac]^-$	2	8	1

Abbreviations: DAG, diacylglycerols; PA, phosphatidic acid; Chol-FA, cholesterol esters of fatty acids; FA, fatty acids; Cer, ceramides; HexCer, hexosylceramides; PE-O, PE; PC-O, 1-alkyl-2-acyl PC; LPE, lyso-PE; LPC, lyso-PC; PI, phosphatidylionositols; PG, phosphatidylglycerols; CerPE, ceramide PE; Ac, acetate. Other abbreviations are spelled out in the text.

[a]Molecular ions commonly detectable in the spectra of total lipid extracts using 7.5 mM ammonium acetate in isopropanol/methanol/chloroform 4:2:1 (v/v/v) solvent.

informative "snapshot" of the lipidome composition, even though the exact molecular assignment of certain peaks might remain ambiguous.

HIGH-RESOLUTION MASS SPECTROMETRY FOR TOP-DOWN LIPIDOMICS SCREENS

In top-down shotgun lipidomics the identification and quantification of lipid species relies on accurate masses and abundances of their molecular ions, respectively. Top-down analysis is a high-throughput screening tool that rapidly reveals global changes within the entire lipidome. Although these screens could be accompanied by MS/MS experiments, these would only target a small number of selected precursors. Therefore, for top-down screens high mass resolution is of paramount importance because their specificity and accuracy depend on how many individual species (rather than unresolved clusters of different precursors with close masses) could be distinguished in MS spectra of total lipid extracts.

A typical top-down lipidomics screen encompasses up to 100 individual samples, from each of which five to 10 MS spectra are acquired. LipidXplorer software preprocesses the entire dataset such that individual spectra are organized in a single flat file database that is further interrogated by user-defined queries written in the molecular fragmentation query language (MFQL) (Herzog et al. 2011). In

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each MS spectrum the lipid class-specific MFQL query checks if plausible precursor masses match the elemental composition expected for the corresponding molecular species. If required, optional search criteria, such as the odd or even number of carbon atoms in the fatty acid moieties, expected number of double bonds, etc., are applied. These criteria could also be bundled by logical Boolean operations (AND, OR, NOT) (Schwudke et al. 2007b) helping to resolve ambiguous peaks assignments. In this way, the interpretation does not rely on a preconceived knowledge of the lipid composition of analyzed samples and is not associated with any particular mass spectrometry platform. However, the confidence of lipid identification and the number of unequivocally recognized lipid classes strongly depend on the mass resolution and mass accuracy of the employed instrument.

The top-down lipidomics screen of human blood plasma samples collected from 70 male patients with various manifestations of metabolic syndrome (obesity, insulin resistance, hypertension) serves here as a representative example (Graessler et al. 2009). Internal standards were spiked into plasma samples prior to one-step lipid extraction by methyl-*tert*-butyl ether (MTBE) (Matyash et al. 2008). It was previously shown that MTBE extracts lipids as efficiently as "gold standard" protocol of Bligh and Dyer (1959) or Folch et al. (1957). However, MTBE phase into which lipids are partitioned, is lighter than water and forms an upper layer in the two-phase system. It is convenient that nonextractable residual material (proteins, oligosaccharides, etc.) is collected in the water phase at the bottom of the test tube after gentle centrifuging. Therefore, it is easy to recover a clean lipid extract that is also free from "floating" solid particles disturbing or even completely blocking electrospray from micrometer-size nozzles (Kameoka et al. 2001).

Total lipid extracts were directly infused into a LTQ Orbitrap XL mass spectrometer and survey mass spectra were acquired within less than 3 min time at the target mass resolution of 100,000. High mass resolution, better than 5 ppm mass accuracy and practical

compositional constraints identified lipid species directly solely relying on their accurately determined m/z (Fig. 2).

Within this screen high mass resolution alone distinguished peaks of isobaric species of several classes: ether lipids and ester lipids comprising fatty acids with odd number of carbon atoms; cholesterol esters and DAG; PC-*O* and TAG. Monoisotopic peaks of SM species were also distinguished from first isotope peaks of PCs. Altogether, 28 lipid species were resolved within the isobaric clusters. Unequivocal assignment of elemental compositions to peak masses allowed us to disregard background peaks. Indeed, from approximately 2500 peaks detected in each survey MS spectrum, only approximately 100 (<4%) represented monoisotopic peaks of bona fide lipid species.

To distinguish the species from lipid classes that might have the same exact masses, we imposed several compositional constraints. If the accurate mass (and, hence, unequivocally determined elemental composition) could be assigned to the species with odd and even number of carbon atoms in their fatty acid moieties, the software attributed the peak to the latter species. For example, for the peak with m/z 744.5538 detected in positive ion mode the software suggested the sum composition of $C_{41}H_{79}O_8N_1P_1$ that could be interpreted as PE 36:2 or PC 33:2. The latter would imply that one of the fatty acid moieties must be odd and therefore the peak was recognized as the PE molecule. Of course, such compositional constraints should only be applied in the organism-specific manner: in particular, odd numbered fatty acid moieties are relatively rare in mammalian lipid species, yet are commonly found in major lipids of *Caenorhabditis elegans* (Entchev et al. 2008). Although top-down screens might rely on tentative peak assignments, the molecular identity of peaks showing interesting phenotype-specific abundance changes could be unequivocally established in follow-up MS/MS experiments.

Limited use of "slow" MS/MS, as compared to "fast" MS, is a major time-saving factor. For accurate quantification, MS/MS spectra of minor lipid species are usually acquired for
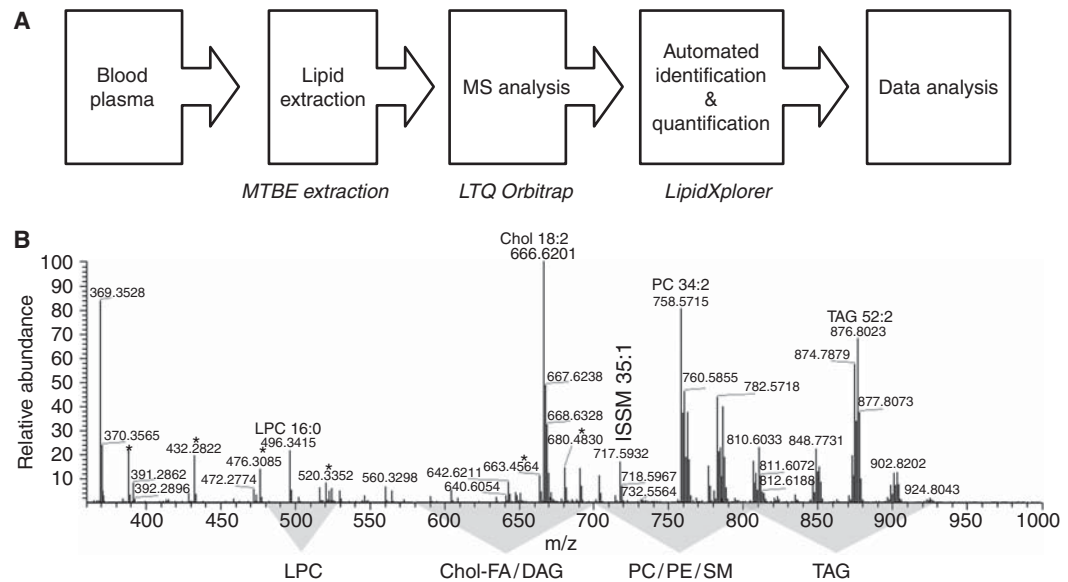
Figure 2. Top-down lipidomics screen of human blood plasma extracts. (*A*) A workflow of a top-down lipidomics screen encompassing blood plasma samples collected from 70 patients. In total, 151 high-resolution spectra were acquired. (*B*) Representative MS spectrum of a total blood plasma extract acquired at the LTQ Orbitrap XL mass spectrometer. Here only the most abundant species are annotated; *m/z* ranges in which peaks of the species of major lipid class were located, are highlighted. Most abundant peaks of chemical noise are designated with asterisks. IS, internal standard spiked into samples prior to lipid extraction.

5–30 sec. Considering that more than 250 precursors are usually detectable in a survey MS spectrum, it might take 30–90 min to compete the analysis, instead of 3 min used in MS-based bottom-up screening. Acquiring MS/MS spectra from all detectable precursors would have reduced the analysis throughput by 10-fold, whereas most of the acquisition time would be spent on fragmenting precursors with unaffected abundances.

Altogether, within the constraints imposed on elemental compositions and detection reproducibility of lipid peaks, we were able to recognize and quantify 95 lipids from 10 major classes: 5 lipids belonging to Chol-FA, 4 to DAG, 5 to LPC, 13 to PC and 13 to PC-*O*, 8 to PE and 5 to PE-*O*, 10 to SM, 31 to TAGs, and free cholesterol. Statistical analysis of the variation of lipid abundances suggested that in male hypertensive patients the content of PE-*O* and PC-*O* species was decreased specifically and independently of other common manifestations of metabolic syndrome, such as

obesity and insulin resistance. The molecular compositions of eight most affected PC-*O* and PE-*O* precursors were established in subsequent MS/MS experiments.

HIGH RESOLUTION MS/MS FOR THE CHARACTERIZATION OF INDIVIDUAL LIPID SPECIES

Lipid fragmentation pathways might look simple, compared to, for example, peptides. Indeed, lipid molecules contain only a few heteroatoms, whose bonds with carbon atoms are cleaved by low-energy collision-induced dissociation (Hsu and Turk 2009). Therefore, it is a common notion that mass resolution might be unimportant for lipid MS/MS because only a few fragments are produced.

In our experience, high resolution MS/MS contributes to the accurate characterization of molecular species in two ways. First, precise masses of fragment ions reveal important details of precursor structures. Second, completely

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resolved peaks of isobaric fragments enable accurate assignment of intensities to the characteristic reporter ions and simplify the quantification of molecular species. Below we substantiate this notion with a few representative case studies.

### Accurate Interpretation of Spectra of Polyunsaturated Species

One problem alleviated by high resolution MS/MS is the quantification of glycerophospholipids comprising moieties of polyunsaturated fatty acids (PUFA). Following collisional activation, acyl anions of PUFA moieties could further lose CO_2 (reviewed in Griffiths 2003). In this way, the acyl anion of docosahexaenoic (22:6) FA produces a fragment with m/z 283.2420, which is isobaric to the acyl anion of abundant stearic (18:0) FA (m/z 283.2632). Their peaks overlap in low resolution MS/MS

spectra and therefore the quantification of individual species relies on a correction factor determined in the series of MS/MS experiments with lipid standards containing 22:6 moieties performed under the fixed collision energy offset (Ejsing et al. 2006). However, in high resolution Fourier transform (FT) MS/MS spectra peaks of $[18:0]^-$ and $[22:6\text{-}CO_2]^-$ anions were completely resolved (Fig. 3) and their individual abundances were accounted for directly.

FT MS/MS also supported the identification of rare species with unusual fatty acid moieties. For example, in shotgun analysis of rat retina extracts we detected a large number of peaks whose exact masses pointed to uncommon PE and PC species comprising very long chain PUFA moieties with more than 26 carbon atoms and more than four double bonds. Abundant peaks of acyl anions along with corresponding products of CO_2 losses were observed in FT MS/MS spectra (Fig. 4).
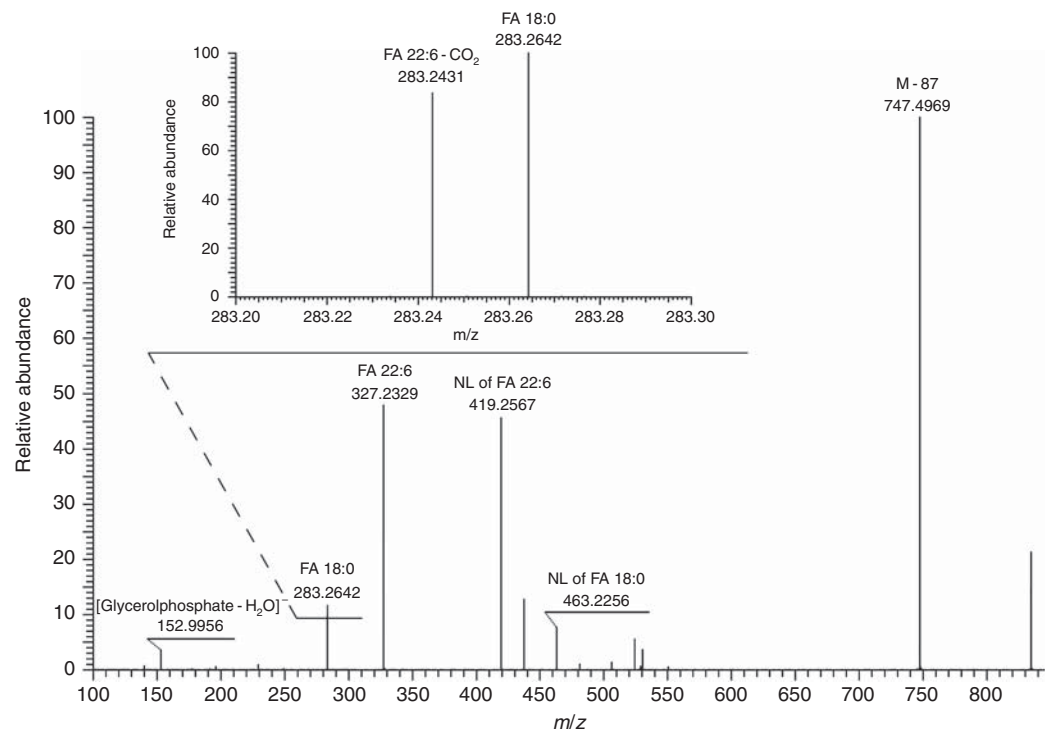
Figure 3. FT MS/MS spectrum of PS 18:0/22:6 acquired in negative mode with the target mass resolution of 30,000 acquired on a LTQ Orbitrap XL instrument. Peaks of the acyl anion of FA 18:0 and the product of CO_2 loss from the acyl anion of FA 22:6 were fully resolved.
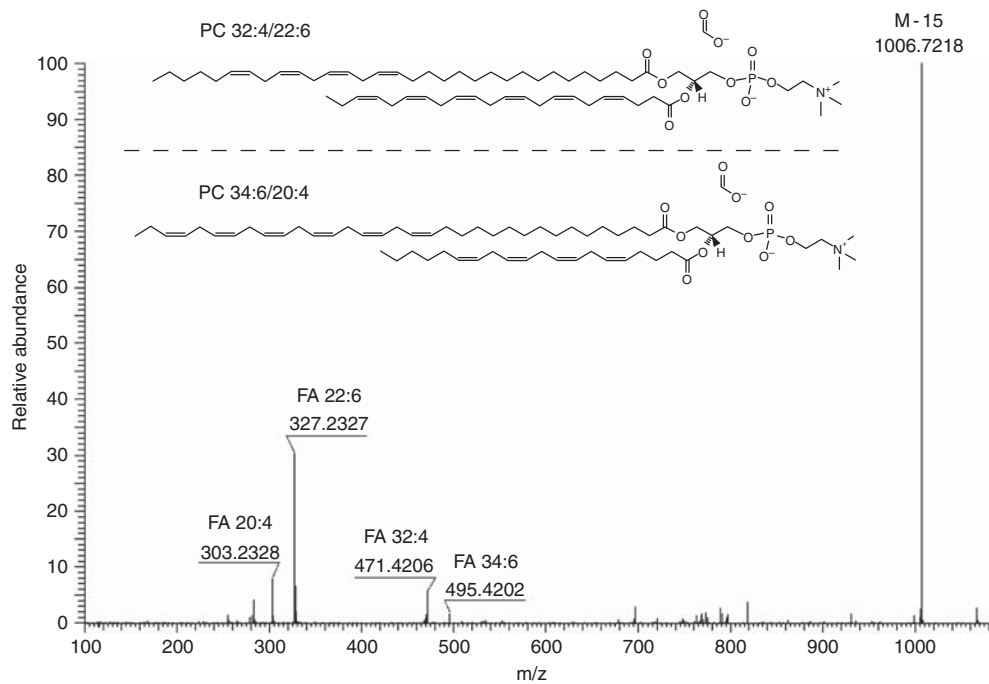
Figure 4. FT MS/MS spectrum of a low abundant precursor ion with m/z 1066.7490 (0.8 ppm) detected by shotgun analysis of a total lipid extract of rat retina in negative ion mode. Spectrum interpretation revealed that this precursor ion represents two very long chain polyunsaturated species of PC 32:4/22:6 and PC 34:6/20:4 ($[M + HCOO]^-$). As judged from the relative intensity of acyl anion fragments, FA 32:4 and 34:6 are at the $sn-1$ position, whereas FA 22:6 and FA 20:4 are at the $sn-2$ position of the glycerol backbone of the corresponding PC species. Masses of fragment ions were determined with the low ppm accuracy: [M-15]- fragment at m/z 1006.7218 (-5.2 ppm), acyl anion of FA 34:6 at m/z 495.4202 (-1.1 ppm), acyl anion of FA 32:4 at m/z 471.4206 (-0.3 ppm), acyl anion of FA 22:6 at m/z 327.2327 (-0.8 ppm), acyl anion of FA 20:4 at m/z 303.2328 (-0.5 ppm), and the product of neutral loss of CO_2 from the acyl anion of FA 22:6 at m/z 283.2431 (-0.1 ppm).

Characteristic fragment masses determined with the low ppm accuracy supported unequivocal identification of these very uncommon species.

High Resolution MS/MS Distinguishes Structurally Related Isobaric Species

In similar way, high resolution MS/MS simplified structural assignments in complex mixtures of isobaric species that are commonly encountered in the analysis of TAG. TAG are major energy storage and free fatty acids resource (reviewed in Gross and Han 2007). It is therefore not surprising that their molecular composition is highly diverse: in higher eukaryotes

(Schwudke et al. 2006; Entchev et al. 2008) and mammals (McAnoy et al. 2005; Bartz et al. 2007) each isobaric cluster of precursors sharing the same number of carbon atoms and double bonds might represent five to 10 individual molecules differing by their fatty acid composition. In mammals TAG often include fatty acids with odd number of carbon atoms and branched fatty acids acquired through the food chain (Quehenberger et al. 2010). TAG might also include species with oxidized fatty acids and also recently identified alkyldiacylglycerols (Bartz et al. 2007).

As an example, let us consider the analysis of molecular composition of precursor ion with m/z 860.7703 (Fig. 5). Note that, within 1.5 Da

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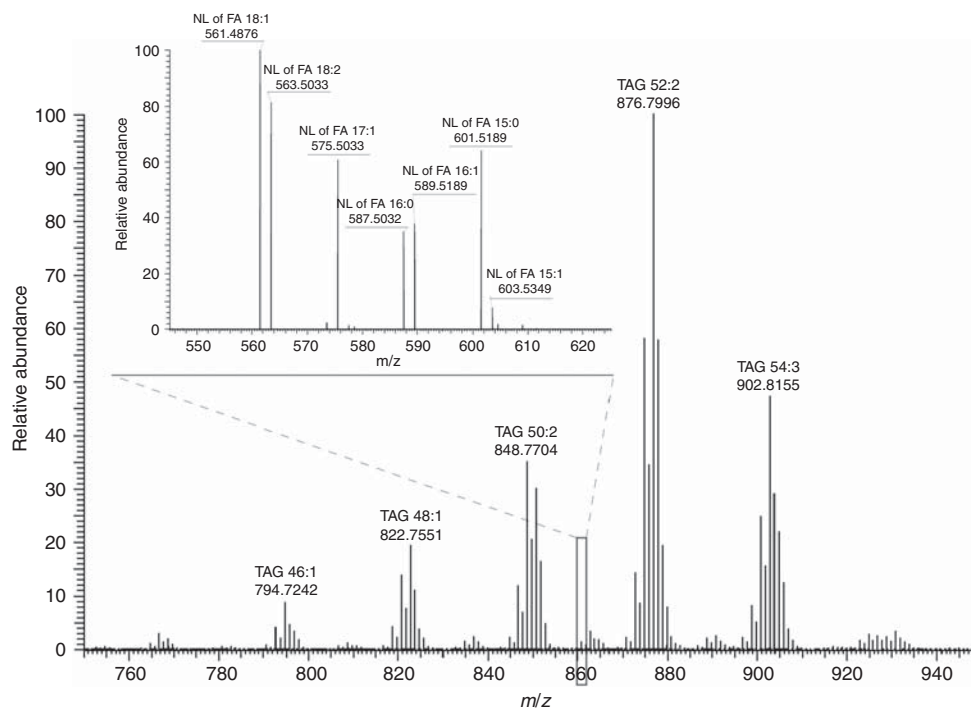
Figure 5. Part of the FT MS spectrum of a total extract of human adipose tissue acquired in positive ion mode at a LTQ Orbitrap XL mass spectrometer. TAG peaks were detected as ammonium adducts $[M + NH_4]^+$ and are annotated by m/z and total number of carbon atoms and double bonds. Following MS/MS fragmentation, TAG precursors underwent neutral losses of fatty acid moieties and ammonium. *Inset* shows a part of FT MS/MS spectrum acquired from precursor(s) having m/z 860.7703. Peaks of neutral loss fragments are designated with their m/z, the acronym NL XX:Y stands for the product of neutral loss of the acid moiety having XX carbon atoms and Y double bonds. Target mass resolution defined at m/z 400 was 100,000 and 30,000 in MS and MS/MS modes, respectively.

precursor isolation window, several species are typically coisolated for MS/MS. Some of them might be obscured by background and remained unrecognized in survey MS spectra, yet their fragments will be detectable in MS/MS spectra because of much reduced chemical noise. In this example, the accurate mass of isolated peak (m/z 860.7703) pointed to TAG 51:3 (mass difference of 0.2 ppm) having an odd numbered fatty acid moiety, whereas a putative isobaric ether lipid TAG−O 52:3 (expected m/z 860.8066; 42.1 ppm) was a less likely candidate. In FT MS/MS spectra fragments were clearly assigned to neutral losses of ammonia and fatty acid moieties of 15:1 (m/z 603.5349; −0.4 ppm); 15:0 (m/z 601.5189; −0.2 ppm); 16:1 (m/z 589.5189; −0.2 ppm); 16:0 (m/z

587.5032; −0.3 ppm); 17:1 (m/z 575.5033; −0.2 ppm); 18:2 (m/z 563.5033; −0.2 ppm); and 18:1 (m/z 561.4876; −0.2 ppm). Considering fragment intensities, TAG 15:0 / 18:1 / 18:2; TAG16:0 / 17:1 / 18:2; and TAG 16:1 / 17:1 / 18:1 are major molecular species among the cofragmented isobaric precursors.

We further inspected the MS/MS spectrum seeking evidences whether putative TAG−O 52:3 was also present among minor cofragmented precursors. However, m/z of expected fragments of FA neutral losses from putative TAG−O 52:3 would have been 60 ppm off of m/z of observed fragments. Despite very low level of chemical noise, fragment peaks corresponding to a putative ether TAG species were undetectable. Both MS and MS/MS spectra

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suggested that the fragmented precursor was indeed TAG 51:3 that contained the odd numbered fatty acids 15:1, 15:0, and 17:1 and there was no evidence of the presence of alternative isobaric ether species. We note that in low resolution MS/MS spectra the presence of odd numbered fatty acid moieties (that, according to a common notion, should not be produced by mammals) could have been a frequent source of confusing structural assignments.

CONCLUDING REMARKS

In this article, we intended to show how the advent of novel high resolution mass spectrometers is changing the basic concept of lipidome characterization. For a long period of time the importance of achieving high mass resolution has been recognized, yet not accorded the attention it really deserves. Part of the problem was that applying high resolution machines to routine lipidomics analysis was technically challenging and required the level of expertise only available in a few dedicated mass spectrometric laboratories. This changed considerably with the introduction of the family of Orbitrap tandem mass spectrometers and routinely achieving the mass resolution of 100,000 both in MS and MS/MS.

The demand for higher mass resolution in lipidomics has one simple rationale: unique sum compositions can be attributed to fully resolved peaks of isobaric precursors or fragments. As shown above, this dramatically simplified the interpretation of MS and MS/MS spectra, which, in turn, streamlined qualitative and quantitative characterization of complex eukaryotic lipidomes. However, even a very high mass resolution does not distinguish molecules with identical masses and does not circumvent other well known analytical limitations, such as dynamic range, signal-to-noise, ion statistics, etc.

Acquiring a quality data set of high resolution mass spectra is only a part of the lipidomics pipeline. Importantly, spectra interpretation algorithms and software should support resolution-dependent interpretations. In the simplest scenario, the same signal could be recognized as an occasional spike and removed from a low resolution mass spectrum, yet it should be treated as a bona fide peak in a high resolution spectrum. Note that there is no clear cut numerical threshold that distinguishes high and low resolution spectra, it is more that the actual mass resolution should be used as an obligatory peak attribute in any spectra interpretation routine. There is an urgent need in developing such algorithms and software. As shown above, a practical solution could be to apply flexible data processing and interpretation rules, rather than building instrument- and project-specific spectra database resources.

We envision that in forthcoming years high resolution mass spectrometry will gradually develop into a "gold standard" lipidomics methodology and efforts should be made to adapt biochemical and analytical routines and software to fulfill its potential.

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