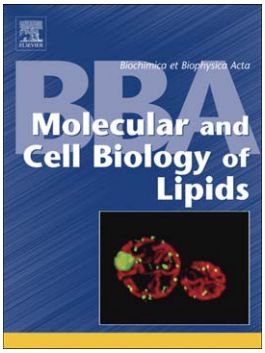

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PII: S1388-1981(17)30042-2
DOI: doi:10.1016/j.bbalip.2017.03.002
Reference: BBAMCB 58125

To appear in: *BBA - Molecular and Cell Biology of Lipids*

Received date: 16 December 2016
Revised date: 7 March 2017
Accepted date: 9 March 2017

Please cite this article as: Joost Brandsma, Andrew P. Bailey, Grielof Koster, Alex P. Gould, Anthony D. Postle, Stable isotope analysis of dynamic lipidomics, *BBA - Molecular and Cell Biology of Lipids* (2017), doi:10.1016/j.bbalip.2017.03.002

Stable isotope analysis of dynamic lipidomics

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Abstract
Metabolic pathway flux is a fundamental element of biological activity, which can be quantified using a variety of mass spectrometric techniques to monitor incorporation of stable isotope-labelled substrates into metabolic products. This article contrasts developments in electrospray ionisation mass spectrometry (ESI-MS) for the measurement of lipid metabolism with more established gas chromatography mass spectrometry and isotope ratio mass spectrometry methodologies. ESI-MS combined with diagnostic tandem MS/MS scans permits the sensitive and specific analysis of stable isotope-labelled substrates into intact lipid molecular species without the requirement for lipid hydrolysis and derivatisation. Such dynamic lipidomic methodologies using non-toxic stable isotopes can be readily applied to quantify lipid metabolic fluxes in clinical and metabolic studies *in vivo*. However, a significant current limitation is the absence of appropriate software to generate kinetic models of substrate incorporation into multiple products in the time domain. Finally, we discuss the future potential of stable isotope-mass spectrometry imaging to quantify the location as well as the extent of lipid synthesis.

Keywords
Dynamic lipidomics; stable isotopes; mass spectrometry; lipid metabolism

Introduction

Omics technologies have the capacity to characterise biomolecular compositions in great detail, yet they are almost universally applied in a 'snapshot' manner. Measuring molecular abundances at one or more defined time points ignores the fact that these are actually the net product of constantly adjusting rates of synthesis, remodelling and catabolism. Metabolic fluxes are a fundamental expression of biological activity and their quantification should be a priority for studies into the regulation of biological processes to complement and extend analysis of steady equilibrium compositions.

Stable isotopes have been used to study lipid synthesis and turnover ever since the pioneering work of Shoenheimer and colleagues in the 1930s, which essentially set the course for isotopic labelling approaches over the next seven decades. Whilst there have been significant improvements in instrumentation and sensitivity almost all studies have relied on precursors of acetyl CoA, including deuterated water ($^{2}H_2O$), ^{13}C-glucose or ^{13}C-acetate, to follow synthesis of cholesterol or glyceride fatty acids. Such studies have fuelled our basic understanding of *de novo* lipogenesis in response to dietary changes and disease processes [11], but the acetyl CoA labelling approach also has a number of inherent limitations. First, in mammalian cells only saturated and monounsaturated fatty acids can be synthesised *de novo* from acetyl CoA, meaning that the synthesis *de novo* of unsaturated fatty acids with two or more double bonds cannot be monitored in this way. Chain elongation of dietary 18 carbon unsaturated fatty acids involves incorporation of a single or at most two acetyl CoA moieties, resulting in a low level of label incorporation into long chain 20 or 22 carbon fatty acids compared with the eight acetyl CoA units that comprise synthesis of palmitate. Second, the gas chromatography-mass spectrometry (GC-MS) or gas chromatography-isotope ratio mass spectrometry (GC-IRMS) techniques used require sample derivatisation, typically through glyceride saponification and the subsequent generation of methyl esters that are sufficiently volatile to be resolved in the gas phase. Consequently, it is difficult to measure synthesis rates for the majority of intact lipid molecules using GC-based techniques.

The introduction of electrospray ionisation mass spectrometry (ESI-MS) approximately two decades ago has facilitated novel analytical approaches for measuring stable isotope-labelled precursor molecules into complex lipid species. This technology offers advantages in terms of specificity, sensitivity, speed of analysis and simpler sample preparation, although it does have of its own limitations. In this article, we will first discuss some of the salient differences between traditional *de novo* lipogenesis and ESI (tandem) mass spectrometry methodologies, illustrated with examples from our own experience. We will then discuss the current methodological limitations and bottlenecks, and finally outline potential future developments.

***De novo* lipogenesis**

Incorporation of ^{13}C-labelled or $^{2}H_2O$-derived acetyl CoA units into glyceride fatty acids increases the abundance of their M_{+1}, M_{+2}, …M_{+n} mass isotopomers above the natural background. In turn, the distribution of such isotopomers depends on the rate of flux through the fatty acid synthesis pathway (i.e., the amount of label that has been incorporated). Using this enrichment distribution information, rates of lipogenic flux can thus be quantified with methodologies such as the elegant mass isotopomer distribution

analysis (MIDA) [7]. For any given rate of synthesis, the final distribution of product isotopomer enrichment will depend on label enrichment in the substrate acetyl CoA pool. Consequently, this substrate enrichment can be back-calculated directly from the product isotopomer distribution which, when combined with product enrichment, can be used to determine rates of net pathway flux. It is important to note that MIDA is a general principle for incorporation of multiple substrate units into any product, and is not specific to just *de novo* lipogenesis.

A variety of studies have used ESI-MS to extend this experimental approach involving incorporations of $^{2}H_2O$, ^{13}C-glucose or ^{13}C-palmitate; examples range from measuring enriched hydrolysed fatty acids by single ion monitoring [5], through to incubating cultured cells with ^{13}C-palmitate in either an untargeted approach [9] or specifically into sphingolipid molecular species [6]. For the untargeted study, tandem MS/MS and Orbitrap-based high mass resolution analysis were used to search for ion pairs that differed by the accurate mass of ^{13}C-palmitate and its potential chain elongation and desaturation products. This approach is undoubtedly an impressive tour-de–force, but it also requires complex data analysis and a relatively high substrate enrichment that would be difficult to achieve in *in vivo* labelling studies. Furthermore, since only a single time point was included the kinetic rates of synthesis, turnover and catabolism could not be determined.

The power of tandem MS/MS to provide detailed insights into lipogenic pathways is demonstrated by experimental data from our own group using larvae of the genetic model organism *Drosophila melanogaster*. These rapidly growing animals were fed for four days on diets containing either 5% unlabelled glucose or 5% 1-^{13}C-glucose, and the intact lipid compositions of whole larval extracts were analysed. Figure panel A shows clear isotopomer redistribution in the phosphatidylethanolamine (PE) molecular species of labelled versus unlabelled larvae. However, overlapping *m/z* values due to differences in double bond numbers make it difficult to interpret these spectra in a comprehensive manner. To enable this, the collision energy was increased to induce fragmentation of all PE precursor ions, yielding the fatty acyl product ions shown in panels B and C. The palmitoyl fragment at *m/z* 255 (panel B) demonstrates a clear redistribution of ^{13}C-labelled isotopomers from the M_0 to higher mass ions. In contrast, there is no such redistribution for the linoleoyl ion at *m/z* 279 (panel C), as this 18-carbon di-unsaturated fatty acid cannot be synthesised *de novo* and has to be provided by the diet. The oleoyl isotopomers show a decreased abundance of the M_0 ion at *m/z* 281 compared to the linoleate M_0 ion (Panel C), reflecting the increased abundance of higher mass isotopomers and confirming the different metabolic origins of oleate and linoleate.

ESI-MS/MS data such as those shown in the Fig permit MIDA calculations of the ^{13}C-acetyl CoA substrate enrichment in intact phospholipids, although in this particular case fast growing *Drosophila* larvae do not represent an equilibrium state and therefore calculation of absolute synthetic rates is not possible. This methodology is easily extended to the analysis of fatty acyl labelling enrichments for other individual lipid species by selecting the appropriate precursor ion. For instance, precursor scans of *m/z* 279 (panel D) will distinguish all negatively charged phospholipid species containing unlabelled linoleate. Since the ion at *m/z* 738 (PE18:2/18:2) contains two linoleoyl groups, neither of which incorporated label (panel C), it was used to normalise the two spectra. The increased abundance of the ion at

m/z 739 in the labelled spectrum is due to ^{13}C-labelling of the glycerophosphate backbone, which complicates interpretation of the spectrum to some extent. However, as the added label was 1-^{13}C-glucose, there can by definition only be one ^{13}C in this glycerophosphate moiety, which in this case has about 25% enrichment. The ion at *m/z* 766 (PE18:2/20:2) contains both linoleate and its unlabelled chain elongation product eicosadienoic acid (20:2). The abundance of this ion relative to PE18:2/18:2 did not change in the ^{13}C-labelled larval extracts suggesting that 20:2 was also provided largely from the diet. In direct contrast, the redistribution of labelled stearoyl groups in PE18:0/18:2 (*m/z* 742) as the M_{+1}, M_{+2}, M_{+3} and M_{+4} isotopomers (panel D) clearly demonstrates the high rate of *de novo* synthesis of this fatty acid. By performing a comprehensive panel of precursor ion scans combined with MIDA assessment of label enrichment in the substrate acetyl CoA pool, the relative contributions of diet and *de novo* synthesis for all PE species can thus be calculated.

Synthesis of individual phospholipid molecular species

A limitation of the approaches using stable isotope label incorporation into fatty acids is that they can only provide information about the origins of those phospholipid species that actually contain the labelled fatty acid (or its metabolites). Moreover, incorporation of *de novo* lipogenesis substrates tends to generate multiple products for each substrate molecule. This greatly complicates analysis of the synthesis of complex lipid species, especially when more than one esterified acyl chain can contain multiple and varied numbers of incorporated stable isotope atoms. The challenge for such studies is therefore to resolve labelled from unlabelled lipid species, while at the same time maintaining sufficient sensitivity to monitor often low isotopic enrichments. One possible solution is to determine elemental compositions by high mass resolution MS. This is the approach adopted by the ^{13}C-palmitate incorporation study described above [9], which in effect performs a highly accurate neutral loss scan of the labelled fatty acid. This methodology does rely on costly Orbitrap or FT-ICR-type instruments with a resolution greater than 200,000. By contrast, we and others have found that tandem MS/MS scans on commonly available triple quadrupole mass spectrometers operating at unit mass resolution provide a robust alternative. For instance, isotopically labelled phospholipid head groups rather than fatty acids (or their precursors) can be used to determine enrichments, and in some circumstances flux rates, for all the species in a lipid class by combining diagnostic precursor and/or neutral loss scans. Methodologies have been established for the incorporations of *methyl*-9^{2}H-choline chloride, 4^{2}H-ethanolamine, 6^{2}H-*myo*-inositol and 3^{2}H-serine into their respective phospholipid classes (for reviews see [4,12]). The incorporation of *methyl*-9^{2}H-choline into phosphatidylcholine (PC) species is discussed below to illustrate some of the underlying principles, together with an assessment of its strengths and limitations.

PC species that are synthesised *de novo* via the Kennedy pathway directly incorporate a choline moiety (in the form of phosphocholine), which is normally derived from the diet. Providing *methyl*-9^{2}H-choline will yield PC (and sphingomyelin) species that are nine mass units heavier than their unlabelled counterparts. To monitor incorporation of *methyl*-9^{2}H-choline, all that is needed are parallel ESI+ precursor ion scans of *m/*z 184 and *m/*z 193, which correspond to the diagnostic phosphocholine fragment in respectively its unlabelled and labelled form. It is important to recognise that the intrinsic high sensitivity of this analytical approach is dependent on the mass difference between unlabelled and labelled ions of the same composition. As the M_{+9} isotopomer resulting from natural ^{13}C in a typical

phospholipid molecule is very small (about 0.0025%), the theoretical minimum detection limit for label enrichment is 0.0087%. In practice, this value is so low that considerations of the dynamic range of the detector impose an instrument limit on achieving this level of enrichment. Consequently, practical achievable minimal label enrichments are in the order of 0.05 to 0.1%.

The importance of being aware of potential isotopomer overlap between natural and experimentally labelled ions is clearly demonstrated by our work on metabolic flux through the phosphatidylethanolamine *N*-methyltransferase pathway (PEMT) [13]. PEMT expression is largely confined to the liver where it catalyses three methylation steps to convert PE into PC, and it is the only route for choline synthesis in mammals. The PEMT pathway can be monitored by administration of *methyl*-9^{2}H-choline *in vivo* in experimental animals and in human studies. A portion of the administered choline is oxidised to betaine, which then donates one methyl group to homocysteine to form methionine through the action of betaine-homocysteine *S*-methyltransferase (BHMT). Methionine is then converted to *S*-adenosylmethionine (SAM), which is the universal methyl donor, including provision of the methyl groups for PEMT action. Starting with *methyl*-9^{2}H-choline, one methyl moiety will be transferred to SAM, in turn generating 3-^{2}H-PC from PE. In addition to this singly labelled methyl group, a proportion of PC synthesised by PEMT will contain two labelled methyl groups. This then permits a MIDA calculation of pathway flux, as the ratio of doubly to singly labelled PC ions will depend at any given pathway flux rate on the label enrichment in the substrate SAM. However, a major consideration for these calculations is the contribution of natural M_{+3} isotopomer to the measured abundances of putative 3-^{2}H-PC molecules, and a specific step must be included in any PEMT pathway analysis to account for this. Although this effect is negligible at high PEMT flux rates and can be ignored for direct incorporation of *methyl*-9^{2}H-choline into PC via the Kennedy pathway, it may significantly affect results in scenarios with low PEMT expression, such as for example in liver cancers [15],

Shotgun versus LC-MS analysis

While the tandem MS/MS methodology described above is robust, it has all the limitations of ion suppression inherent in shotgun lipidomics. Furthermore, the lack of specific information about the fatty acyl compositions of molecular species can result in uncertainty of molecular assignments for isomeric lipid species of identical *m/z*. This identification issue can largely be resolved by using reversed phase liquid chromatography (LC) prior to mass spectrometry, but that in turn raises the quantification issues inherent to LC-MS. While enrichments can be readily calculated from the intensities of co-eluting labelled and unlabelled lipid species, this approach is poorly quantitative in the absence of authentic internal standards for all species. This is due to a combination of ionisation differences during a gradient LC run, and the problem of variable charge distribution when different numbers of species elute at the same time. This problem disappears at limiting sample dilution, but such conditions also intrinsically limit the detection of trace amounts of incorporated label into lipid species. By contrast, shotgun lipidomics has the advantage that scans for unlabelled and labelled lipid species are performed under identical ionisation conditions, which permit accurate quantification and enrichment calculations. Such experiments are typically performed with alternating scans for labelled and unlabelled species, with additional sensitivity made possible by scheduling longer scan times for the labelled species. As with all shotgun approaches the quality of quantification depends on

the appropriate choice and combination of internal recovery standards, which should be representative of the selected mass range. In practice, a good compromise would be to perform LC-MS/MS analyses of stable isotope incorporations into identified lipid species on a limited number of samples representative of the various experimental conditions. For clinical *in vivo* studies, these analyses would be performed for time points of maximal enrichment. The shotgun approach can then be used for high throughput analysis of the remaining samples by either loop injection or automated nanoflow infusion. The latter provides maximal sensitivity and at low flow rates (<100 nl/min) data can be acquired for up to 4 hours from a final sample volume of only 25 µl.

Mass spectrometry software

Another major bottleneck in dynamic lipidomics is the lack of robust and readily available data analysis software for stable isotope incorporation studies. Most software platforms supplied by instrument manufacturers are designed for the detailed analysis and characterisation of unknown lipid compositions. Such untargeted software approaches have been extended to studies tracking stable isotope incorporations into metabolites and lipids in cultured cells. For instance, X^{13}CMS was used to provide a differential analysis of U-^{13}glucose labelled versus unlabelled cell cultures in the presence or absence of lipopolysaccharide treatment [8]. However, this study only looked at low mass metabolites without the complexity of lipid compositions and reported only a single incorporation time point. These methodologies have great potential for *in vitro* studies of cells cultured in defined conditions in response to an insult or pharmacological agent, but have not yet been adapted to the very different data sets generated by lipid kinetic studies *in vivo*. For the majority of such dynamic lipidomic studies, the identities of the lipid species that are being analysed are already well known, and the data sets typically consist of time courses of labelled substrate incorporations into multiple product lipid species. Consequently, relevant software must be able to consistently identify and quantify the same lipid species in a large number of samples, and allow for the extracted data to be entered into kinetic metabolic models. For example, a two compartment kinetic model was proposed for the turnover of lung surfactant labelled with ^{13}C-dipalmitoyl PC in preterm infants who develop neonatal chronic lung disease [3], but this model for a single product has not yet been extended to encompass the multiple product data sets generated by dynamic lipidomics. In addition, the introduction of time as an extra dimension to an already complex analytical processes will provide its own bioinformatics challenges.

Future perspectives

There are three aspects of dynamic lipidomics currently under development. The first is the introduction of novel applications of existing and new stable isotope-labelled probes. One example of this is a recent study using incorporation of U-^{13}C-glucose to determine the turnover of glycosphingolipid species in Hep-2 cells [14]. Such an approach has the potential to monitor both glycosphingolipid and fatty acid metabolism at the same time, although in this particular instance the products of *de novo* lipogenesis were not reported. The second aspect is the development of better models of lipid kinetics for integration with parallel transcriptomic and genomic analyses. This is an important challenge as it promises to bring together studies of the potential for pathway activity (transcriptomics) with direct measurements of pathway flux (dynamic lipidomics). Systems biology requires mathematical models to be validated using real-world data of pathway activities and fluxes,

and in turn such information will help refine the models themselves [16]. Ultimately this will yield a deeper understanding of metabolic regulation (in health) and dysregulation (in disease) and pave the way for predictive models that can be used in applied systems-based medicine. The third, and potentially very exciting, development is the combination of dynamic lipidomics with MS imaging to determine the locations where lipids are synthesised in addition to their concentration distribution. Only a handful of studies to date have reported MS imaging of lipid dynamics. One approach has been to employ secondary ion mass spectrometry (SIMS), in which an ionic beam 'sputters' the sample surface to generate secondary ions. These are then detected simultaneously at atomic level to provide a map of labelled and unlabelled atoms over the sample surface [17]. Such multi-isotope imaging mass spectrometry (MIMS) has the advantage of sub-micron spatial resolution, down to as little as 50 nm in the case of NanoSIMS [18, 19], but comes at the expense of a lack of molecular compositional data as it is restricted to analysis of elemental isotope compositions. This was shown clearly by a time course study of ^{13}C-palmitate incorporation into the gut and fat bodies of *Drosophila* larvae [17], and by a report on *de novo* synthesis of lipids from 1-^{13}C-glucose and 1-^{13}C-acetate, together with incorporation of U-^{13}C-linoleate into *Drosophila* lipids [1]. In the latter study MS imaging was used to show a significant re-distribution of newly synthesised lipids, especially linoleoyl-containing triacylglycerols, into glial lipid droplets in the central nervous system of *Drosophila* larvae exposed to hypoxic conditions (2.5% O_2 for 22 days). In contrast to SIMS, laser-based ionisation techniques can provide comprehensive molecular ion distributions, but currently at only 10-50 micron spatial resolution [2, 20], which is inadequate to resolve individual cells or sub-cellular locations. For instance, NIMS-ToF/ToF (nanostructure-initiated mass spectrometry using samples mounted on a clathrate nanostructured surface) was used to image the distribution of lipids synthesised *de novo* from $^{2}H_2O$ in mice bearing a solid mammary tumour [10]. While this provided an elegant demonstration of the differential distribution of newly synthesised phospholipids between tumorous and healthy tissue, an incubation time of 5 days with a high enrichment of $^{2}H_2O$ (8% in the drinking water) was required to obtain enough product enrichment for satisfactory imaging.

In addition to these ongoing developments, the main challenge for the future of dynamic lipidomics will be to develop novel instruments that: a) combine increased spatial resolution and improved discrimination between labelled and unlabelled lipids, and b) have sufficient sensitivity to quantify isotopic enrichments that are compatible with clinical applications. New laser configurations will be required to image single cells or sub-cellular distributions, perhaps for instance by combining laser dissection microscopy with ESI-MS [2], while FT-ICR or Orbitrap-type instruments will be required to resolve labelled lipid species on high mass resolution alone. Alternatively, the potential for increased specificity of detection using tandem MS/MS has not yet been explored for MS imaging of dynamic lipidomics. Finally, one major future application for this technology could be to image lipid metabolism *in vivo* for diagnostic purposes in clinical biopsies or surgical resection tissue, for instance in healthy compared to tumorous tissue regions in patients with cancer.

Acknowledgments
JB, GK and ADP were supported grants from the Wellcome Trust (057405), the EU Innovative Medicines Initiative (UBIOPRED project) and the National Institutes of Health Biomedical Research Unit in Respiratory Medicine at University Hospitals Southampton. APB and APG

are supported by the Francis Crick Institute, which receives its core funding from Cancer Research UK, the UK Medical Research Council, and the Wellcome Trust, and previously by the UK Medical Research Council, National Institute for Medical Research (U117584237).

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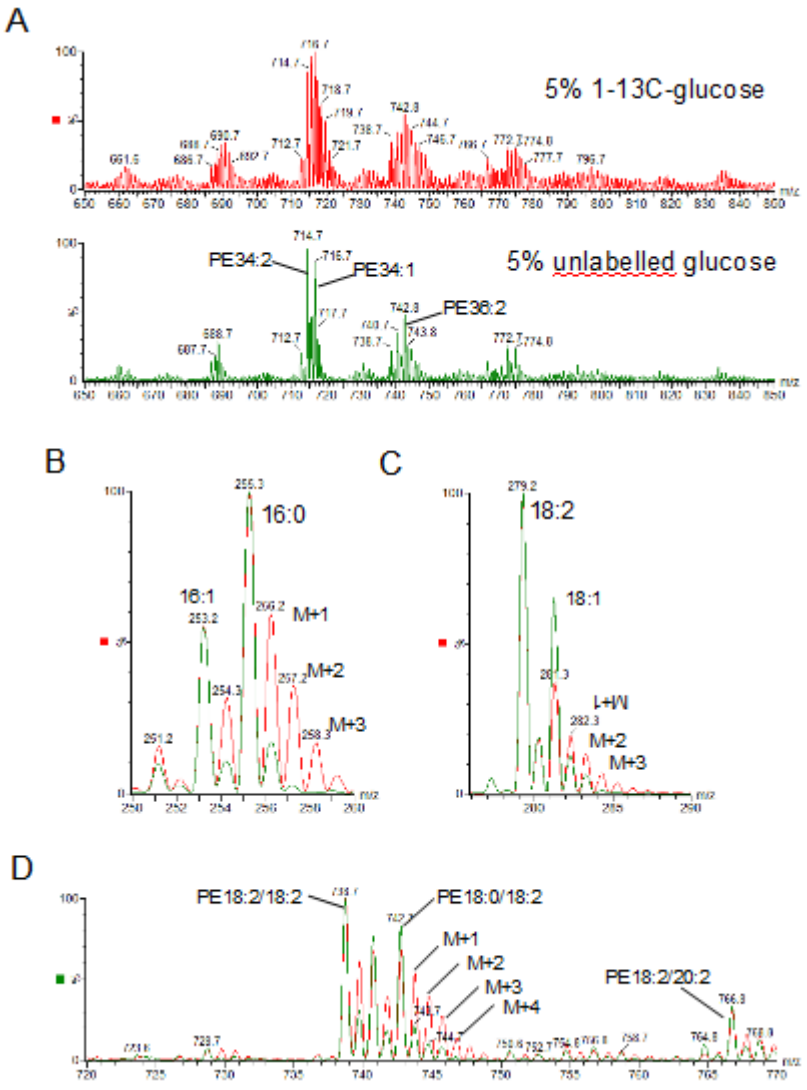
Figure legend

Drosophila larvae were fed diets containing 5% glucose for 4 days, either unlabelled or labelled with 1-^{13}C-glucose. Total larval lipids were extracted with organic solvents and analysed by direct infusion electrospray tandem mass spectrometry (ESI-MS/MS) using a combination of untargeted and targeted scans. The green spectra in all panels are representative of lipid extracts from unlabelled larvae, whereas the red spectra are derived from 1-^{13}C-glucose fed larvae. Panel A displays a portion of the full scan ESI- *m/z* range (no fragmentation), highlighting the increased and complex isotopomer distribution in the labelled larvae; Panels B and C illustrate selected fatty acyl product ions after ESI- high energy CID fragmentation, normalised to the most abundant ion. The unchanged M_{+1} ion at *m/z* 280 confirms that linoleate (18:2) is derived from the diet and not synthesised *de novo,* while the altered isotopomer distributions of all other fatty acyl ions demonstrate substantial label incorporation; Finally, Panel D shows ESI- precursor ion spectra of *m/z* 279. As all the ions in these spectra contain unlabelled linoleate, the isotopomer redistributions reflect the enrichments of ^{13}C in the complementary esterified fatty acyl moiety.



Highlights

- Stable isotopes and mass spectrometry can monitor lipid synthesis and turnover
- Metabolism of intact lipid molecular species
- Software developments required for kinetic modelling of dynamic lipidomics
- Potential of mass spectrometry imaging to visualise location of lipid synthesis
- Future integration into systems biology and systems medicine