Why Isolate Lipids from Biological Samples? Making Lipidomics by Mass Spectrometry the Standard

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Isolating lipids from biological samples using liquid-liquid extraction is a standard way of preparing these molecules for analysis by mass spectrometry. A number of sample extraction techniques are available today that provide different advantages in terms of reproducibility, speed, compatibility, and coverage of extracted analytes. After obtaining the total lipid extract from a sample, differential mobility separation has proven to be a powerful tool for gas phase fractionation of lipid classes. These components; sample extraction, gas phase separation, and mass spectrometry, form the basis of a novel integrated quantitative lipid analysis solution.

Introduction

The metabolome is an important class of molecules to study as it can lend insight into the health and well-being of an organism and the causative elements associated with disease. It integrates the effects of our genes with the impact of our environment by being the product of both internal factors (our genome and proteome) and external factors (our lifestyle and our environment). In addition, the metabolome is extremely dynamic and always changing. By identifying and quantifying changes in specific metabolites, any changes that are observed can be mapped back to specific pathways for detailed interpretation. Thus, quantitative analysis of the metabolome can provide important information that can help predict the onset of a disease or disorder or characterise its progression.

Lipids are a key part of the metabolome and are involved in the formation of important biological elements such as membranes, lipid droplets, and lipoproteins. The study of lipids as a class of molecules within the metabolome is important as they act as signalling and inflammatory molecules, and are known to be significant players in a number of metabolic disorders, cardiovascular disease, oncology, and other disease areas.

However, lipids are an extremely complex and diverse group of compounds. Lipid molecules are polymers that are formed by combining metabolites from different metabolic pathways and result from the combinatorial products of fatty acids and head groups that can often have the same mass. Thus, using phosphatidylcholines (a phospholipid class) as an example, for 40 different fatty acids and 5 main head groups these 45 components could account for 8000 unique molecular species (Figure 1).

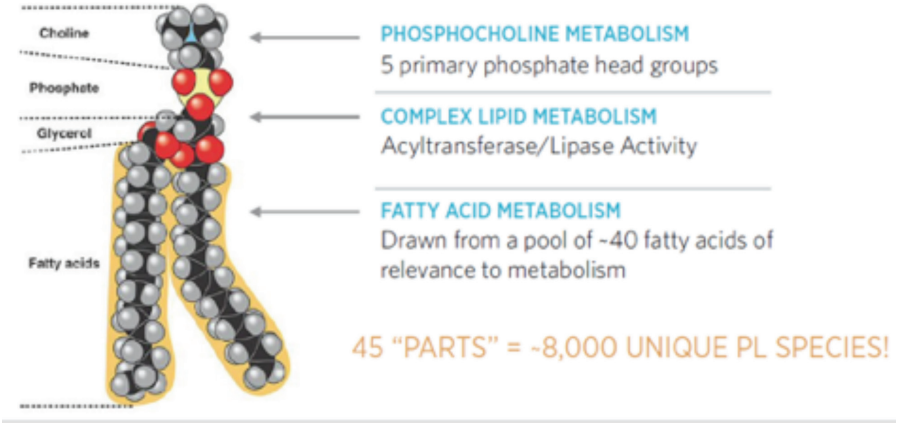

Figure 1. A Phosphatidylcholine Molecule. Lipids are polymeric structures and their individual elements have their own pathways. They breakdown into intermediary metabolites such as fatty acids therefore mapping these molecular species to pathways requires mapping to the right level of metabolism. In this example if you imagine there are 40 different fatty acids and 5 main head groups, these 45 components could account for 8000 unique phospholipid species! This makes pathway mapping difficult.

Lipid Extraction

In order to simplify their analysis and improve the accuracy of identification and quantification, the first step in analysing lipids by mass spectrometry is efficient extraction of the entire class of lipid compounds from the biological sample.

There are several methods which allow the extraction and recovery of total lipid from any kind of organism or matrix. For isolating lipids from a biological sample, there are really only three methods that are readily employed in the field of lipidomics; Folch (Folch, Lees & Stanley, 1957), Bligh & Dyer (Bligh & Dyer, 1959), and the methyl-tert-butyl ether (MTBE) method (Matyash, 2008).

The Folch method was proposed in 1957 by Folch, Lees, and Stanley, and is one of the most widely adopted methods used for lipid extraction today [1]. It describes a 1-step solvent extraction with mixture of water and chloroform:methanol (2:1) followed by washing with potassium chloride

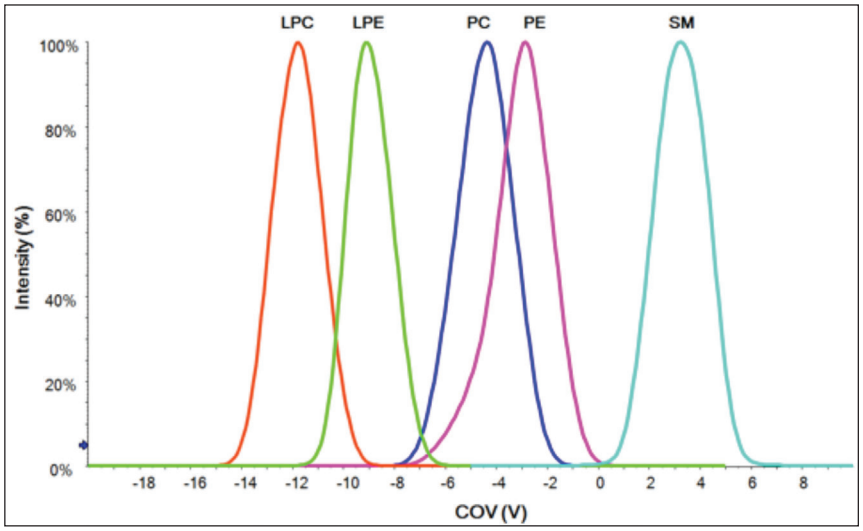

Figure 2. Differential Mobility Spectrometry (DMS). A compensation voltage (COV) trace highlighting a mixture of standards infused into the DMS cell. COV ramped from -25V to 10V separates lipid classes by their head groups in the gas phase. This figure highlights the specificity of the DMS cell to separate lipid classes from complex mixtures. The COV per class is added to the MRM tables in the acquisition method and at any one voltage across the DMS, only that specific lipid class is selected and allowed and passed through to Q1 for subsequent MRM analysis. In this figure the COV tuning mixture is infused at 7µl/min and the software automatically tunes the COV value per class by collecting LPC/LPE/PC/PE MRMs in the negative ion mode and SM MRMs in the positive ion mode.

or hydrochloric acid (KCl or HCl) for the extraction of total lipid from animal tissues. Whilst it is a standard method and allows good extraction of total lipids, it is quite laborious in the filtration steps required, employing large and inconvenient volumes of solvent (10s of ml). It also employs chloroform, which has environmental and toxicity issues.

The Bligh & Dyer method, another very widely adopted extraction procedure, was described in 1959 [2] after it was published that the Folch method was unsuitable for routine investigators to use due to large volumes of solvent handling. The Bligh & Dyer method uses a 3-step solvent extraction: (1) methanol + chloroform (2) chloroform and (3) water are added to the sample all in stages to one test tube. After phase separation, total lipids are found in the bottom chloroform (organic) fraction, and after isolating and drying this fraction, the lipids can be analysed. This is a very simple and straightforward procedure and has been readily accepted in the field for extracting total lipid from serum and plasma samples. The substitution of dichloromethane instead of chloroform has been evaluated by Cequier-Sanchez et al in extracting lipids from different sample matrices [3]. The results indicate that dichloromethane/methanol can replace the commonly employed chloroform/methanol, thus avoiding the health and regulatory problems associated with the use of chloroform.

More recently Matyash and colleagues from the Shevchenko lab (Matyash, 2008) described an accurate lipidome profiling strategy using an MTBE extraction, which allows a faster and cleaner lipid recovery [4]. Because of MTBE's low density, the lipid-containing organic phase forms the upper layer during phase separation. This allows three advantages; (1) it simplifies the fraction collection, (2) minimises dripping losses from the end of the pipette used to collect the fraction and (3) the non-extractable matrix forms a dense pellet at the bottom of the extraction tube and is easily removed by centrifugation. Rigorous testing demonstrated that the MTBE protocol delivers similar recoveries of the molecular species of most major lipid classes compared with the Folch recipe. This extraction technique is also more amenable to automation which is an emerging need as sample cohorts become larger.

Lipid Separation

While extraction creates a clean sample of virtually only lipid compounds, the remaining lipid fraction is highly complex. The extracted lipids exhibit tremendous isobaric overlap with many lipids per class, all within a narrow mass range. Separation prior to analysis is essential. Liquid chromatography (LC) has traditionally been used to separate and simplify compounds prior to mass spectrometry. However, because of the extreme complexity of the lipid fraction one LC method alone doesn't supply the necessary separation power to efficiently resolve all of the lipid compounds for accurate quantification. Traditionally, several methods must be used or a sub-fractionation technique must be added in order to resolve each lipid class and achieve accurate qualitative and quantitative data on the molecular species. This can be done by using solid phase extraction and separating the polar from the neutral lipid components but can be very time and labor intensive.

Differential mobility spectrometry (DMS) has been used as an effective separation tool to couple to mass spectrometry, used most often to reduce interferences, improve signal-to-noise and therefore sensitivity of detection. For lipid analysis it has proven very successful as an orthogonal separation technique by allowing lipids to be fractioned in the gas phase by the dipole moment on their head groups. As the device is located at the front of the mass spectrometer, it helps eliminate isobaric overlap often encountered during any lipid analysis, whether applying accurate mass or nominal mass spectrometry. In DMS, an ionised sample enters a mobility cell that is located at atmospheric pressure in front of the mass spectrometer orifice. An RF voltage is applied across the cell that is cycled between high and low fields. As the ions transit the cell, they are separated based on the difference in their mobility between high and low field, which is impacted by numerous molecular properties. A second voltage (a compensation voltage known as COV) is applied to steer analytes through the cell into the mass spectrometer. Now mass isolation, fragmentation and analysis can be performed on the separated classes yielding simplified, more reliable data for qualitative and quantitative analysis. The DMS is a small planar cell that is added between the source region and the high vacuum region of the instrument.

When DMS is used for lipid analysis, each lipid class will have a different COV that can be used to separate each individual class by head group and allow them to enter into the MS, one class at a time (Figure 2). This is because each lipid class possesses a different head group with differing dipole moments that are affected by the voltages they encounter within the mobility cell. In a paper by Lintonen et al. [5], a linear relationship between the dipole moment of each lipid class and the COV value was found. By applying a specific COV, each lipid class could be directed through the mobility cell into the MS, and all other

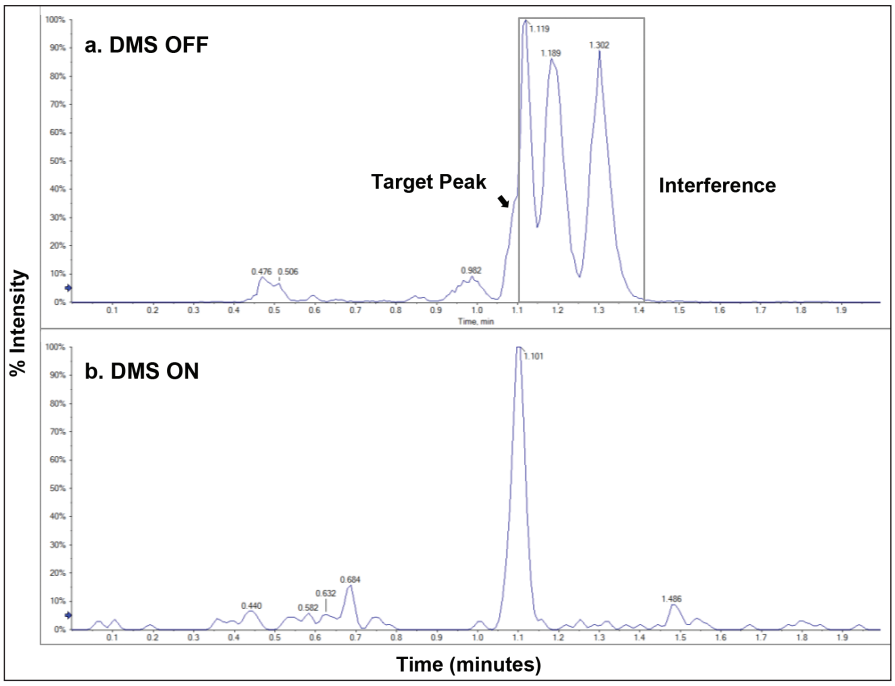

Figure 3: DMS Removes Isobaric Interference. The above figure shows an extracted plasma sample infused into the MS using an MRM transition for PE 40:5 (Q1 = 792.6/Q3 = 283.2). In panel (a) the DMS is fitted but the voltages are turned off and it can be seen that multiple different lipids have the same MRM transition. The box highlights the actual isobaric interference described in this article. The target peak for quantitation is the small shoulder on the left peak. Panel (b) The DMS voltages are on and have been tuned to allow only PE lipid species, at a COV of -3.0, through the DMS and enter the MS for MRM analysis. The isobaric interference has been greatly reduced and allows more accurate quantitation.

classes (and species) will not be transmitted. Alternatively, by ramping the COV, different classes of lipids could, in turn, become stable and transit successfully through the cell, thereby allowing sequential analysis of all lipid classes and respective molecular species. In Figure 2, the separation of the lipids by class is demonstrated by analysing the Lysophosphatidylcholine/ Lysophosphatidylethanolamine/ Phosphatidylcholine/ Phosphatidylethanolamine classes (LPC/ LPE/PC/PE) in the negative ion mode and Sphingomyelin class (SM) in the positive ion mode.

Separating lipid classes by DMS allows us to overcome the confounding factor of isobaric overlap of these molecules. When coupled with targeted multiple reaction monitoring (MRM) on a triple quadrupole or QTRAP® mass spectrometer, this simple separation of lipid classes eliminates the need for any up-front LC, greatly simplifying the workflow. By selecting specific COV values for lipid class transmission that provide the least amount of lipid class overlap, isobaric interference, inherent in lipid analysis, is significantly reduced (Figure 3).

A new LC-MS/MS solution (Lipidyzer™ Platform, Sciex, USA) simplifies and automates the high throughput analysis of lipids by infusion, leveraging the optimised sample preparation procedure and DMS separation power described above. Using flow injection sample introduction, the platform can analyse up to 45 samples per day in a fully automated fashion, including system optimisation and data processing.

The platform employs the Bligh & Dyer extraction protocol (modified to use dichloromethane) because of the acceptance in the field and its simplicity. It had readily been shown to extract the larger proportion (~98%) of lipids and is more efficient in its recoveries. The full extraction can be completed in one test tube without any complicated filtration steps. The optimised protocols are included with the platform.

Finally, novel internal standards strategy has been developed for the Lipidyzer Platform that enables more accurate quantification with reduced bias (Ubhi & Watkins, 2015) [6]. The chemical kits include over 50 labelled internal standards that cover thirteen lipid classes across complex lipid metabolism.

Conclusion

Because of their extreme complexity, lipids have traditionally been challenging to extract, separate, characterise and quantify in a routine and efficient manner. However, through a combination of individual techniques that have been carefully selected and optimised to work synergistically together, a complete solution has been developed. This unique and powerful combination of technologies has enabled a full solution for quantitative lipidome profiling to be provided that bypasses more laborious LC-based analysis methodologies. Combining the Bligh & Dyer extraction protocol for reproducible lipid extraction with the novel internal standard strategy enables profiling of over 1100 lipid species with quantitative measurements. Using DMS for gas phase separation and MRM for targeted analysis enables a flow-injection approach that provides a seamless analysis platform for highly accurate lipid quantitation. Combined with powerful solution-based software, this platform can be easily employed for the high throughput, accurate quantitation of lipid samples on a routine basis.

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