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Lipidomics biomarker studies: Errors, limitations, and the future

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ABSTRACT

Lipidomics is an ever-expanding subfield of metabolomics that surveys 3000 to 5000 individual lipids across more than 56 lipid subclasses, including lipid peroxidation products. Unfortunately, there exists a large number of publications with poor quality data obtained with unit mass resolution leading to many lipid misidentifications. This is further complicated by poor scientific oversight with regard to recognition of isobar issues, sample collection, and sample storage issues that inexplicably requires more detailed attention. Inadvertent or intentional obfuscation of *relative* quantification data represented as *absolute* quantification is a subtle but profound difference that may readers outside of the field may not realize, therefore, instigating disservice and unnecessary distrust in the scientific community. These issues need to be addressed aggressively as high quality data are essential for the translation of biomarker research to clinical practice.

1. Sample collection, extraction, and storage

The investigation of lipids via mass spectrometry offers in-depth insights into the molecular underpinnings of cellular function. Lipids have a wide functional diversity from structural components within cell walls and organelles to that of signaling transduction molecules. Therefore, it is no surprise that lipid identification can provide critical information in the understanding of typical or pathological states. The identification of one or more lipids that exemplify a pathological state is generally referred to as "biomarker screening." Screening for biomarkers in the serum and more reliably, the plasma, is advantageous in that the procedure is typically routine. It is important to note that serum and plasma hold critically different properties that will be explored herein. However, collectively we and the literature argue that plasma is the most reliable and accurate means for lipid identification via mass spectrometry.

By definition serum is whole blood following the clotting process. Whereas plasma is whole blood with an anticoagulant such as EDTA (ethylenediaminetetraacetic). EDTA is a chelator that sequesters metal cations such as that of Ca^{2++}. The sequestration of calcium preempts the start of the anticoagulation process and hence no coagulation results from blood phlebotomized into EDTA lined tubes. Both plasma and serum are generally clarified by centrifugation to precipitate red blood cells, white blood cells, and platelets.

It is important to note that while plasma and serum are similar, they are not the same. Therefore, different metabolomic profiles can be elucidated from each respectively. For instance, it is reported that 104 metabolites existed at significantly higher concentrations in serum and concluded that there is higher reproducibility in plasma samples [1]. The authors concluded that the major differences were based on products of the coagulation cascade present in serum samples. These conclusions are supported by others' observations that the intrinsic clotting cascade resulted in higher abundance of lysophosphatidylinositol as measured by liquid chromatography-mass spectrometry (LC-MS) [2]. Similar metabolomic differences were also measured by gas chromatography/time-of-flight- mass spectrometry (GC-TOF MS) [3]. The authors were able to additionally conclude that various incubation times of plasma and serum led to pronounced effects on analyte peak areas, especially for plasma [3]. Other specific metabolomic markers of the clotting cascade such as thromboxane B2 (TXB_2), 12-hydroxy-eicosatetraenoic acid (12-HETE), and 12-hydroxy-eicosapentaenoic acid (12-HEPE) were significantly elevated in serum more than plasma due to their release by activated platelets during coagulation [4].

Most convincingly, one report went on to describe a number of conclusions regarding the lipidomics of healthy Caucasians. Namely lysophosphatidylcholines (lysoPCs), diacylglycerols (DAG), free

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polyunsaturated fatty acids (PUFA), and several oxidized fatty acids (oxFAs) were different in plasma and serum samples regardless of subject's age and gender [5]. They also implicated that the elevation of DAG and inositol 1,4,5-phosphate (IP3) was primarily due to the activation of phospholipase C (PLC). The consequent activation of PLC leads to the enzymatic degradation of phosphatidylinositol 4,5, bisphosphate (PIP2). This is an especially important observation due to the often-ubiquitous role of the DAG & IP3 pathway in various cell signaling pathways. Such masking of "artifactually" elevated signaling molecules can obfuscate appropriate interpretations of biological states. Expectedly, these authors also advocate the use of plasma over serum [6].

Additionally, small peptide fragments were noted to exist in serum samples as measured by UPLC-ESI-QTOF/MS [7]. Hypoxanthine and xanthine were among the molecules elevated and concluded to have been released during clot formation. Interestingly, heparin was most recommended for plasma samples as the use of LC-ESI/MS demonstrated little to no plasma interference or matrix effect.

Conceptually, these conclusions are intuitive. The resultant coagulation cascade that takes place within serum can, and as evidence supports, will confound the interpretation of results. Especially when considering lipid biomarkers, one needs to anticipate the potential interference of their target lipid and the biological cascades that may or may not be underway once the sample is collected. Additionally, it should also be noted that many of these reactions and other enzyme-mediated reactions can take place during the serum extraction protocol wherein serum is incubated at room temperature for 20–30 min. The choice of serum vs. plasma sample use is the decision of the primary investigator, however, advocacy and support for the utilization of plasma samples over serum is prevailing. This is in large part due to the inherent properties surrounding plasma and serum collection, coagulation cascades, and enzyme activation.

1.1. Extraction

Analysis of lipids requires more than the obvious consideration of plasma and serum. Few extraction protocols have remained the "gold standard" for lipid extraction but one extraction procedure, in particular, is noted to provide a robust and clean methodology especially when considering mass spectrometry. Evidence for the use of methyl-*tert*-butyl ether (MTBE) in mass spectrometry, especially for high-throughput lipidomics and direct infusion MS is apparent and reviewed herein.

While the MTBE extraction procedure is our primary recommendation, it is important to understand why this methodology is superior (in most cases) to the "gold standards" such as the Folch, Lees and Sloane Stanley [7], or Bligh Dye [8,9] extractions. Principally, these extraction protocols necessitate the extraction of lipids in a chloroform phase [10]. This extraction process forms an aqueous phase and an organic phase. Because chloroform layer has an inherently higher density than the aqueous phase of methanol and water, the organic phase will exist on the bottom layer. Though this may not seem to implicate any harm the process of separating the (bottom) organic layer containing the lipid of interest does introduce several problems.

Once the phases have separated with centrifugation the top layer is a relatively large volume that one must insert a pipette tip through, penetrate the interface of the aqueous and organic phase, aspirate the organic phase, and then remove the tip back through the aqueous phase. At the interface exist many insoluble compounds. Even seemingly anecdotal contaminates can precipitate enough to clog the electrospray ion source, not to mention introduce a confound of molecular species that can further provide additional ion suppression. It is important to note that the viscous nature of chloroform impedes the ability of contaminants to be effectively removed by centrifugation [10]. The use of chloroform methods does provide a robust means of lipid extraction but at the expense of time and potential contamination. Therefore, MTBE-based extraction is found to be better suited for MS.

MTBE extraction provides a faster means of extraction, especially when considering high-throughput lipidomic analysis. Although MTBE is an overall "faster" extraction procedure it was originally sought for its safety. The analysis of anaerobic bacteria was modified intentionally to use a safer solvent MTBE in the application of gas-liquid chromatography [11]. This concept was further adopted when MTBE was sought for large scale industrial biosurfactant extractions because MTBE demonstrated low toxicity, biodegradability, low flammability, and was explosion safe. Chloroform, used in "gold standard" methods, on the other hand has severe limitations: it is a known carcinogen [12] and is known to decompose into phosgene and hydrochloric acid [13]. Both phosgene and HCl have the potential to modify lipids.

Further advocacy for MTBE came from a comparative study wherein 400 lipid species across 12 major lipid classes demonstrated convincing evidence that MTBE protocol delivered the same or better recoveries verses the Folch or Bligh and Dryer extractions with no limitations [10]. Though the methodology of MTBE extraction was faster and sometimes better than the "gold standards" the highest advocacy for MTBE was due to its inherent chemical properties. MTBE is less dense than a methanol/water mixture and consequently forms the top phase. Unlike chloroform which forms the bottom phase. The less dense organic layer provided by MTBE means that the risk of contamination due to human or robotic pipetting error is substantially lower as there is no need to plunge through an aqueous layer or an organic aqueous interface. Subsequently, MTBE utility is found to fostering of high-throughput lipid profiling [14–16].

Though appealing, MTBE does have some inherent limitations. For instance, ganglioside extractions are nearly impossible with MTBE. Though gangliosides are lipids, they are soluble in water due to their polar nature. One means to circumvent this is using a Folch extraction followed by dialysis of the upper aqueous phase (which removes ions and other low molecular weight molecules) followed by lyophilization [17].

1.2. Storage

Careless storage of lipids can lead to detrimental effects on the data obtained and subsequently interpretations made [18–21]. These changes are due to a multitude of reasons. For instance, some investigator may elect to collect a sample in an organic solvent with the expectation that this will inactivate enzymes. While this is certainly a means to deactivate enzymes, hydrolytic enzymes that degrade lipids in organic solvents have been reported to be active in organic solvents [22,23]. Other aspects of storage are related to the exposure of atmospheric oxygen during the storage process. Polyenoic lipids will undergo oxidation more readily when exposed to oxygen or peroxides which can be found in old solvents i.e. old chloroform. Whereas, esters ethers (e.g. plasmalogens) and amide bonds are sensitive to exposure of various acids and bases [24].

While contamination and exposure to oxygen are obvious causes of storage error, freeze-thaw-cycles are seemingly the most robust. Core temperatures of samples stored in liquid nitrogen can change up to approximately 40 °C when samples are exposed to ambient temperature for only 90 s. This is a sufficient enough temperature change to allow for the formation of liquid phase on a microscale located nearest the tube wall which elicits enzymatic activity [25]. Additionally, freezing and multiple freezes have the

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