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Recent Advances in Lipid Separations and Structural Elucidation Using Mass Spectrometry Combined with Ion Mobility Spectrometry, Ion-Molecule Reactions and Fragmentation Approaches

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Abstract

Lipids are a vital class of molecules that play important and varied roles in biological processes. Fully understanding lipid roles, however, is extremely difficult since the number and diversity of lipid species is immense, with cells expressing hundreds of enzymes that synthesize tens of thousands of different lipids. While recent advances in chromatography and high resolution mass spectrometry have greatly progressed the understanding of lipid species and functions, effectively separating many lipids still remains problematic. Isomeric lipids have made lipid characterization especially difficult and occur due to subclasses having the same chemical composition, or species having multiple acyl chains connectivities (sn-1, sn-2, or sn-3), double bond positions and orientations (cis or trans), and functional group stereochemistry (R versus S). Fully understanding the roles of lipids in biological processes therefore requires separating and evaluating how isomers change in biological and environmental samples. To address this challenge, ion mobility spectrometry separations, ion-molecule reactions and fragmentation techniques have increasingly been added to lipid analysis workflows to improve identifications. In this manuscript, we review the current state of these approaches and their capabilities for improving the identification of specific lipid species.

Keywords

lipids; isomers; ion mobility spectrometry; mass spectrometry; ozonolysis; photochemical derivatization

Lipids constitute a large class of diverse molecules ranging from fats and oils to glycerophospholipids and glycerolipids. Due to this great diversity, each category of lipids has distinct biological mechanisms and functions, and even lipids within the same class can perform drastically different roles. To define the function of each lipid species, molecular

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and structural characterization is required for evaluating how each changes in disease states and under environmental perturbations. Recent studies have highlighted the importance of lipid presence/absence or abundance changes in the assessment of cancerous tissue margins [1], identification of different bacterial types [2], and evaluation of system stability [3]. However, even with all the lipidomic information acquired over the last 50 years, new species are being discovered [4–6]. Therefore, improved analytical techniques are expected to provide additional insight into novel lipid species, while establishing new lipid mechanisms and functional roles and enabling better disease diagnostics and drug development.

Mass spectrometry-based lipid measurements

Currently, most lipid mixture characterization is performed with mass spectrometry (MS) by either directly infusing the mixture into the mass spectrometer (shotgun lipidomic analyses) or in conjunction with a prior chromatographic separation (Figure 1). Both approaches are often used with tandem MS/MS methods such as collision induced dissociation (CID) and higher-energy collisional dissociation (HCD) since they provide an assessment of the fatty acyl chains and head groups present in each detected lipid. To date, shotgun lipidomic studies makeup the most widely used approaches, providing high throughput assays needed for large-scale patient and environmental perturbation studies [7,8]. However, injecting all lipids simultaneously into the mass spectrometer generally precludes detection of the lowest abundance species, due to issues such as the inherent dynamic range limitations of MS. Therefore chromatographic methods are often used to provide greater lipid coverage.

Lipid chromatography approaches include thin layer chromatography (TLC), GC and LC. TLC is often performed without MS and utilizes iodine vapor and class specific dyes and radioactivity to detect lipid species. In TLC, the solvent systems for most lipid classes have been well established and automated, making it an inexpensive approach for rapid screening [9]. However, TLC lacks sufficient resolution and specificity for many applications; therefore it is often used as an initial method for complex lipid mixture analysis with more complex studies following. GC-MS is used frequently for fatty acid methyl ester (FAME) analyses. However, the derivatization steps (i.e. fatty acids being converted into fatty acid methyl esters) and limitations of amenable ionization approaches (flame ionization or electron impact), preclude full lipid structural information [10–12]. Therefore GC-MS is increasingly being replaced by LC separations coupled to MS with alternative ionization techniques such as electrospray ionization (ESI).

LC-MS studies for lipids can use several types of LC separations such as normal phase, reverse phase or a combination of both. In normal phase LC (NPLC), lipids are separated based on their head groups or lipid classes. Reverse phase LC (RPLC), on the other hand, separates lipids mainly on their fatty acyl composition with lysolipids with one fatty acyl group eluting before triacylglycerides with three. NPLC and RPLC both provide high quality lipid separations and even 2D LC separations can be obtained by collecting the normal phase fractions for reversed phase analyses, or vice versa, to acquire better resolution for isolation of lipid species. More recently, hydrophilic interaction liquid chromatography (HILIC) has even been utilized to separate lipids and it has shown promising results for

lysophospholipid regioisomers [13,14]. All of these LC techniques have been used for qualitative and quantitative lipid analysis, providing important information about complex samples [15–17]. However, while these LC separation have many advantages, they also have challenges such as being time consuming (usually requiring gradients greater than 10 min), expensive (due to column and solvent costs), and unable to separate many of the lipid isomers and species in complex matrices. To address the time consuming aspect of LC, supercritical fluid chromatography (SFC) has recently been explored as a higher throughput method for lipidomic class separations since its separations are inherently faster than LC, and generally involve minimal solvent consumption since they typically use CO₂ as a mobile phase [18–20]. Thus, further exploration of SFC may prove essential in future lipid studies. Lipid isomers still present a great challenge for LC methods as difficulties have been observed in separating isomers having different subclasses (e.g. phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) with the same elemental composition), multiple acyl chain locations possibilities along the lipid backbone (sn-1, sn-2, or sn-3), different double bonds positions and orientations (cis or trans), and unique functional groups orientations along the backbone (R versus S). Thus, ion mobility spectrometry (IMS) separations, ion-molecule reactions and recent fragmentation techniques such as ultraviolet photodissociation (UVPD) have increasingly been added to lipid workflows for better class identification and isomer evaluations. The advantages and challenges of each method are detailed below.

IMS separations for lipid characterization and isomer separation

IMS-MS has recently had an increasing impact on lipid class and isomer identifications, with impressive results seen for multiple different IMS-based techniques including field asymmetric waveform IMS (FAIMS), drift tube IMS (DTIMS) and traveling wave IMS (TWIMS) [21]. FAIMS, also known as differential ion mobility spectrometry (DMS), is an atmospheric pressure separation that distinguishes ions based on their mobility differences at high and low electric fields (Fig. 2a, left panel). FAIMS is one of the most commonly used IMS-based techniques in current lipidomic studies since it is able to baseline separate lipids from different classes and subclasses in <1 s [22]. FAIMS is also able to characterize different lipid classes into specific ‘trend lines’ groupings as illustrated by the FAIMS plot of compensation field versus m/z values (Fig. 2a, middle panel) [23]. In the FAIMS analysis of glycerophospholipids, lysophosphatidylcholine (LPC) separates first followed by phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylserine (PS) (Fig. 2a, right panel) [24]. While FAIMS separations are relatively rapid and easily coupled with MS, to date commercially available devices are unable to distinguish isomers differing in sn-1 and sn-2 fatty acyl positions or cis/trans double bond orientations, so other IMS approaches have been evaluated for these applications.

Two other IMS techniques widely used in lipidomic analyses are drift tube IMS (DTIMS) and traveling wave IMS (TWIMS) since they are easily coupled with LC separations. DTIMS and TWIMS both distinguish ions based on their mobility differences as they move through a buffer gas. Specifically, DTIMS separates ions in a uniform electric field, while TWIMS separations occur when ions are subjected to a dynamic travelling voltage wave (Fig 2b, left panel). The drift time versus m/z plots for both separations illustrate trend lines

for each lipid class similar to the FAIMS results (Fig 2b, middle panel) [25–28]. Moreover, it has been shown that DTIMS with a 4 Torr pressure and 1 m long IMS drift region separated lipid isomers such as sn-1/sn-2 positional isomers, cis/trans double bond orientation isomers and stereochemical isomers (e.g. R versus S) [25]. In the sn-1/sn-2 separations, it was found that if the smaller fatty acyl chain was in the sn-1 position the lipid had a smaller structure. The cis double bonds also were observed to have smaller structures than trans double bonds (Figure 2b, right panel) and when R and S orientations of the 2-hydroxyl group in ceramides was present, the S orientation had a smaller structural size. While these were very promising results for standards, many of the isomers were only partially separated, limiting the utility of this DTIMS platform for their full separation in complex lipid mixtures.

In order to obtain better DTIMS or TWIMS separations, the pressure or length of the drift cell must be increased. Atmospheric pressure DTIMS (AP-DTIMS) recently was shown to achieve better separation for the cis/trans lipid isomers PC(16:1(9Z)/16:1(9Z)) and PC(16:1(9E)/16:1(9E)) than the lower pressure DTIMS techniques (Figure 2c, right panel) [29]. This same cis/trans isomer pair was also recently separated with a homebuilt high resolution FAIMS platform, as well as a TWIMS device created with structures for lossless ion manipulations (SLIM). In the analyses, both platforms showed significantly better separations than the AP-DTIMS platform [30–33]. This enhanced resolution was due to the FAIMS device being constructed to operate at higher voltages than commercial versions [30] and the SLIM TWIMS platform having a compact serpentine ion drift path [31,32] which allowed ultrahigh resolution IMS separation to be achieved due to the long IMS path (30.6 m, in this example) [33]. A final IMS technique to note is the recently developed trapped ion mobility spectrometry (TIMS) method, which also allows high resolution IMS separations and shows promise for future lipidomic separations [34].

Fragmentation approaches and ion-molecule reactions to determine C=C bond locations

Another important challenge for lipidomic studies is the determination and quantitation of carbon-carbon double bond (C=C) locations due to their ubiquity and abundance. While IMS separations have been shown to distinguish fatty acyl position isomers and cis/trans isomer, their ability to identify the location of C=C double bonds is limited unless standards are available to confirm the positions. Further, due to the inability to distinguish C=C bond locations in many MS analyses, their location is rarely reported in lipidomics studies. The main challenge with determining C=C locations is that traditional fragmentation methods such as CID are not effective at breaking the C=C bond due to the high dissociation energy needed for cleavage. A number of fragmentation approaches, including HCD, multistage CID, charge remote fragmentation, and charge transfer dissociation have been explored for this purpose, but have shown limited capabilities [35]. Recently, several novel fragmentation approaches have shown great promise for efficient C=C determination, including electron transfer dissociation (ETD) [36], helium metastable atom activated dissociation (He-MAD) [37,38], electron impact excitation of ions from organics (EIEIO)/electron-induced dissociation (EID) [39,40], radical-directed dissociation (RDD) [41] and ultraviolet photodissociation (UVPD) [42,43]. ETD and He-MAD utilize ions reactions with radical

anions metastable or helium atoms to induce similar radical-based fragmentation pathways. EIEIO/EID uses ion reactions with electrons to generate fragmentation spectrum detailing the lipid class, acyl chain length, regioisomers, and C=C bond locations [39,44,45]. In several reports, EIEIO even distinguished cis/trans isomers in intact complex lipids, but this was only done with pure standards [46]. In the RDD approach, radical product ions are formed upon 266 nm irradiation of a lipid precursor complexed with a molecule containing a photocaged radical initiator via selective cleavage of a carbon–iodine bond. These products are then selected to undergo further low energy CID to yield diagnostic ions for the C=C bond and chain-branching positions as demonstrated for PCs, SMs and PGs [41,47]. Last but not least, UVPD utilizes 193 nm UV to cleave the C-C bonds adjacent to the C=C double bond, pinpointing C=C bond positional isomers [42,43]. These fragmentation techniques have great potential for C=C bond determination in lipids, but are all still in early development. While great progress is expected, three main challenges for each include: (1) the need for specialized instrumentation or setups; (2) some methods only work in positive ion mode, while certain lipids only ionize in negative mode; and (3) data analysis can be exceedingly difficult due to the many resulting peaks and relatively low signal-to-noise levels occurring due to the limited dissociation efficiencies. Thus, advances are still greatly needed for the full potential of each technique to be fully implemented in lipidomic studies.

Specific ion-molecule reactions have also been employed to either directly cleave the C=C bond or convert them to other functional groups that can be easily fragmented during ionization or by low-energy CID to generate specific diagnostic ions. Two main ion-molecule reactions are ozonolysis or the Paterno-Buchi (P-B) reaction (Figure 3). Ozonolysis dates back to 1905 and allows oxidative cleavage of unsaturated compounds by the introduction of ozone into a system [48]. A simplified ozonolysis reaction for a C=C bond is shown in Figure 3a. In this reaction, the ozone molecule attacks the C=C inducing the formation of a primary ozonide, which is very unstable and dissociates into both aldehyde and criegee product ions without the need of any dissociation energy. Since the product ions have a 16 Da difference, they can be used as diagnostic ions for unambiguous lipid double bond identifications. Ozonolysis was first used to determine the double bond positions in FAME in the 1960s [49]. Over the last decade, Blanksby et al. have applied ozonolysis for lipid C=C bond determination within the ESI source of the mass spectrometer (ozone ESI) [50], and inside the mass spectrometer in a trapping region (termed ozone-induced dissociation (OzID)). OzID has been demonstrated on linear ion traps, triple quadrupoles and a TWIMS instrument, illustrating its wide compatibility [51–56]. While most OzID experiments have been implemented with direct infusion workflows, applications with LC have also been illustrated [57]. Limitations have however been observed due to the extended reaction times required in low millitorr pressure trapping areas, which can take as long as 1 s for reaction due to the maximum concentration of ozone achieved without affecting instrument performance (mass selection or mass analysis). Therefore, higher pressure trapping areas are being analyzed to provide higher ozonolysis efficiencies in shorter time frames (millisecond timescale) for easier coupling with LC.

The P-B reaction is another method of directly cleaving the C=C bond through ion-molecule reactions and was first introduced to identify lipid C=C bonds in 2014 by Y. Xia et al. [58]. As shown in Figure 3b, the P-B reaction involves the activation of the carbonyl group within

the aldehyde or ketone often using acetone. UV excitation is then required to give rise to radicals, which subsequently react with the C=C. Two isomeric oxetane ring compounds can be formed depending on the relative positions of the carbonyl and the C=C bond. Therefore, applying CID following the reaction leads to the cleavage of the C-C bond at the initial C=C bond position and the C-O bond of the initial carbonyl group. These cleavages form a new lipid and an aldehyde/ketone diagnostic pair with a mass difference of 26 Da, which can be used to identify the C=C bond locations. P-B reactions coupled with CID have been successfully utilized for the identification and quantitation of unsaturated lipids in lipidomics studies and have even illustrated the importance of C=C positions in patients with breast cancer [59–63].

Future directions for IMS-MS based lipidomic measurements

While the IMS separations, ion-molecule reactions and fragmentation techniques reviewed allow the acquisition of novel lipidomic information, we believe this is just the beginning of the developments needed to perform more effective lipid measurements. At present, no single method is able to fully characterize all lipid species and the numerous isomers cannot be baseline separated in most cases by chromatography or IMS due to their structural similarity. Therefore in the future, we expect combined approaches (e.g. LC-IMS-MS/MS) will be increasingly recognized as essential for the improved and broadly effective lipid characterization. For example, by performing, LC, IMS, OzID, CID, and MS simultaneously on the same sample, each lipid would have five different characteristics for its species classification. These multidimensional characterizations however, are not without challenges as advanced data processing and bioinformatics tools will be needed for spectral deconvolution. Furthermore, techniques such as LC require time to perform, so developing faster gradients, while avoiding ionization suppression will be needed to increase throughput in screening studies. Thus, while a fully optimized analytical technique to characterize every lipid species still has a long way to go, new developments are greatly advancing capabilities and we expect major impacts in understanding lipid roles in biological and environmental systems over the next decade.

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Highlights

- Isomeric lipids have made full lipid characterization extremely difficult.
- Ion mobility spectrometry separations, ion-molecule reactions and fragmentation techniques are readily being added to lipid analyses.
- The current state of these approaches for improving the identification of lipid species is detailed in this review.

Figure 1.

Different techniques utilized in lipid characterization and how each is broken down for class identification and isomer evaluations. Lipid characterization is normally performed with MS analyses, chromatography techniques, fragmentation approaches, ion mobility spectrometry (IMS) methods and ion-molecule reactions. The further identification of the lipid classes and specific structural information then requires specific techniques depending on the information desired. Mass spectrometry (MS); fragmentation approaches such as collision induced dissociation (CID), electron impact excitation of ions from organics (EIEIO) and ultraviolet photodissociation (UVPD); IMS methods including field asymmetric waveform IMS (FAIMS), drift tube IMS (DTIMS), traveling wave IMS (TWIMS), atmospheric pressure DTIMS (AP-IMS), and structures for lossless ion manipulations based TWIMS (SLIM TWIMS); and ion-molecule reactions include ozonolysis and the Paternò–Büchi (P-B) reaction are all illustrated in this manuscript.

Figure 2.

Different IMS techniques for lipid separations and structure elucidation including a) FAIMS/DMS, b) DTIMS and TWIMS, and c) higher resolution IMS approaches. Three features of the FAIMS/DMS analyses are shown by the left panel - schematic of FAIMS separation; middle panel - FAIMS compensation voltage versus m/z trend lines of lipid classes (Adapted from *Journal of The American Society for Mass Spectrometry*, Separation and Classification of Lipids Using Differential Ion Mobility Spectrometry. 22, 2011, 1146–1155, Shvartsburg A.A. et al., With permission of Springer); and right panel - separation of lipid classes in FAIMS shotgun lipidomics (Adapted with permission from Lintonen TPI, et al. Differential Mobility Spectrometry-Driven Shotgun Lipidomics. *Analytical Chemistry* 2014, 86:9662–9669, Copyright (2014) American Chemical Society). Three features of the DTIMS and TWIMS separations are shown by the left panel - schematic of DTIMS and TWIMS separation; middle panel - drift time versus m/z trend lines of lipid classes separation in DTIMS or TWIMS (Adapted from Kyle J.E. et al.: Uncovering biologically significant lipid isomers with liquid chromatography, ion mobility spectrometry and mass spectrometry. *Analyst* 2016, 141:1649–1659, with permission from The Royal Society of Chemistry); and right panel - cis/trans double bond orientation isomer separation by DTIMS (Adapted from Kyle J.E. et al.: Uncovering biologically significant lipid isomers with liquid chromatography, ion mobility spectrometry and mass spectrometry. *Analyst* 2016, 141:1649–1659, with permission from The Royal Society of Chemistry). Finally, the cis/trans isomer separations of PC(16:1(9Z)/16:1(9Z)) and PC(16:1(9E)/16:1(9E)) are illustrated with c) AP-DTIMS (Adapted from Groessl M, Graf S, Knochenmuss R: High resolution ion mobility-mass spectrometry for separation and identification of isomeric lipids. *Analyst*

2015, 140:6904–6911. With permission from The Royal Society of Chemistry), d) high resolution FAIMS (Adapted from *Journal of The American Society for Mass Spectrometry*, Broad Separation of Isomeric Lipids by High-Resolution Differential Ion Mobility Spectrometry with Tandem Mass Spectrometry. 28, 2017, 1552–1561, Bowman A.P. et al., With permission of Springer) and e) SLIM TWIMS (Adapted from, *International Journal of Molecular Sciences*, Wojcik R, et al. Lipid and Glycolipid Isomer Analyses Using Ultra-High Resolution Ion Mobility Spectrometry Separations. 2017, 18:183, open access). The schematics for the FAIMS, DTIMS and TWIMS separations were adapted from *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1811, Kliman M, et al. Lipid analysis and lipidomics by structurally selective ion mobility-mass spectrometry, 935–945, Copy right (2011), with permission from Elsevier.

Figure 3.

Ion-molecule reactions allowing the determination of C=C bond locations in lipids are a) ozonolysis and the Paternò-Büchi (P-B) reaction. Ozonolysis occurs in the gas phase upon introduction of ozone, thereby generating aldehyde and criegee diagnostic ions with a mass difference of 16 Da. The P-B reaction is a photochemical derivatization which takes place in the solution phase to generate an aldehyde/ketone and new lipid as diagnostic ions with a mass difference of 26 Da.