

## **The potential of Ion Mobility Mass Spectrometry for high-throughput and high-resolution lipidomics**

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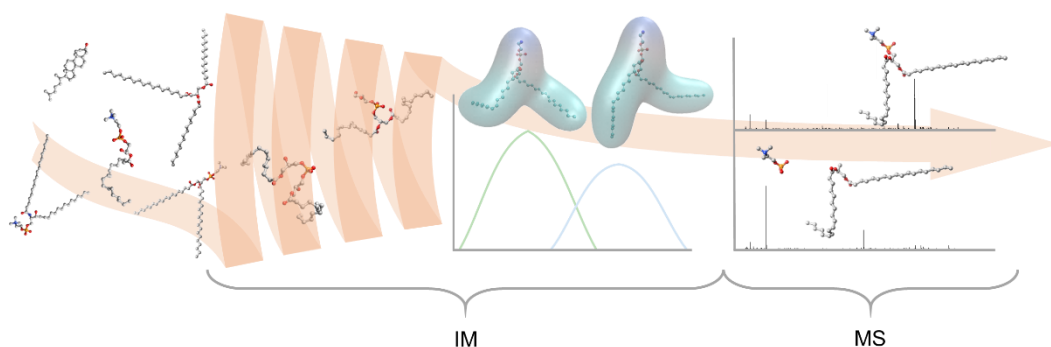
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### **Abstract**

Lipids are a large and highly diverse family of biomolecules, which play essential structural, storage and signalling roles in cells and tissues. Although traditional mass spectrometry (MS) approaches used in lipidomics are highly sensitive and selective, lipid analysis remains challenging due to the chemical diversity of lipid structures, multiple isobaric species and incomplete separation using many forms of chromatography. Ion mobility (IM) separates ions in the gas phase based on their physicochemical properties. Addition of IM to the traditional lipidomic workflow both enhances separation of complex lipid mixtures, beneficial for lipid identification, and improves isomer resolution. Herein, we discuss the recent developments in IM-MS for lipidomics.

### **Graphical Abstract**



## Highlights

- We review the current advantages of IM-MS spectrometry in lipidomics.
- Collision Cross-Section (CCS) values enhance confidence in lipid identification.
- **IM separation coupled with fragmentation of precursor ions results in cleaner product ion spectra, allowing improved data interpretation.**
- IM-MS improves separation of isomeric lipids.

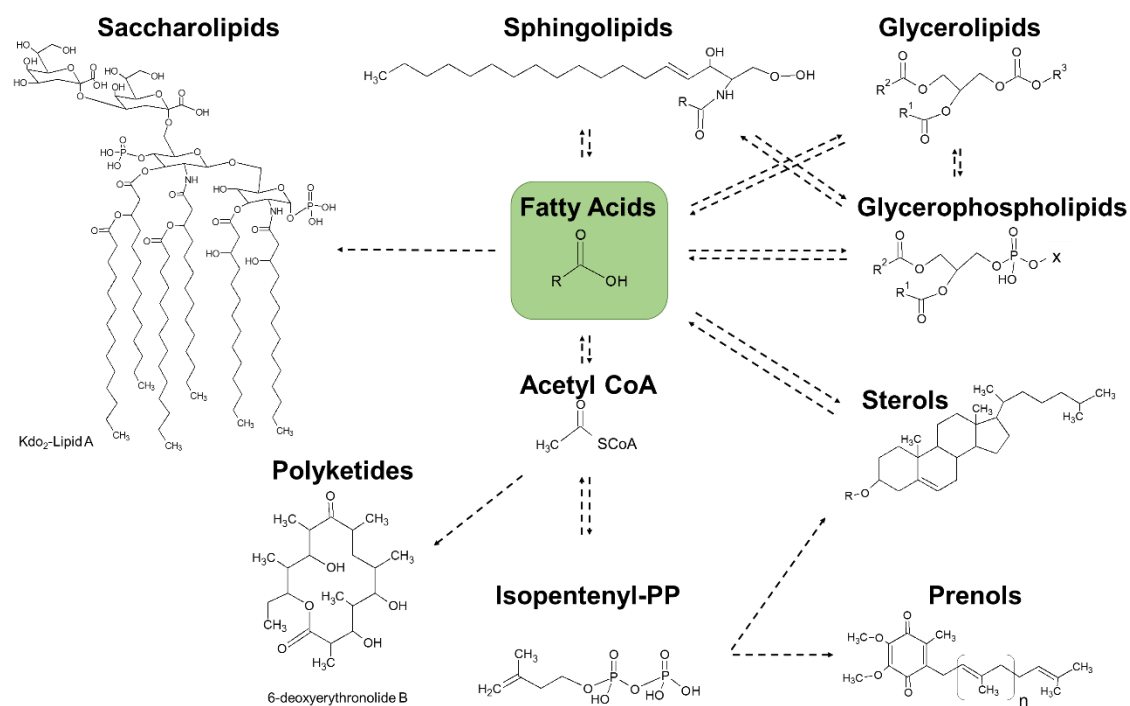
## Abbreviations/Glossary

BMP	bis(monoacylglycerol) phosphate
CCS	Collision Cross-Section
CE	cholesteryl ester
DAG	diacylglycerides
FA	fatty acid
GL	glycerolipid
GP	glycerophospholipid
IM	ion mobility
LC	liquid chromatography
Lyso-PL	lyso-phospholipid
MS	mass spectrometry
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine

SM sphingomyelin  
TAG triacylglycerides

## Introduction

Lipids are a highly diverse group of biomolecules comprising several complex lipid categories (detailed in **Figure 1**) which form an enormous network of structural, storage and signalling molecules within living organisms. Online databases such as LipidBlast [1], and LIPID MAPS [2] attempt to cover this large amount of information. However, the actual number of structurally distinct lipids is unknown. Today, the largest database is the LIPID MAPS Structure Database, which contains nearly 41,000 unique biologically relevant lipid structures, out of which approximately half is manually curated and half computationally generated (<http://www.lipidmaps.org/data/structure/index.html>, last accessed 25/07/2017).



**Figure 1: Fatty acid derived lipid categories.** Fatty acids (FAs), which contain 2-40 carbon atoms and different degrees of unsaturation, are the smallest building blocks for much more complex lipid categories. These categories include sphingolipids, glycerolipids (GLs), glycerophospholipids (GPs), sterols, prenols, saccharolipids, and polyketides. Each category can be further divided into classes such as GPs into phosphatidylglycerols (PGs), phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), phosphatidylserines (PSs), and phosphatidylinositols (PIs) depending on the head group (indicated with X).

The vast chemical space covered by the various lipid categories, along with the structural similarities within the same class, represents a significant analytical challenge in lipidomic studies, aiming to profile the entire lipid content (lipidome) of the studied samples. Currently,

high-resolution mass spectrometry (MS) approaches either alone (shotgun MS) or coupled with liquid chromatography (LC/MS) are commonly used. However, insufficient separation of isomeric and isobaric species, very common in complex biological samples, is a major challenge. Therefore, further analytical separation methods are required to resolve isobaric lipid species, uncover the isomeric distribution of lipids, and to improve the confidence in lipid identification in biological samples.

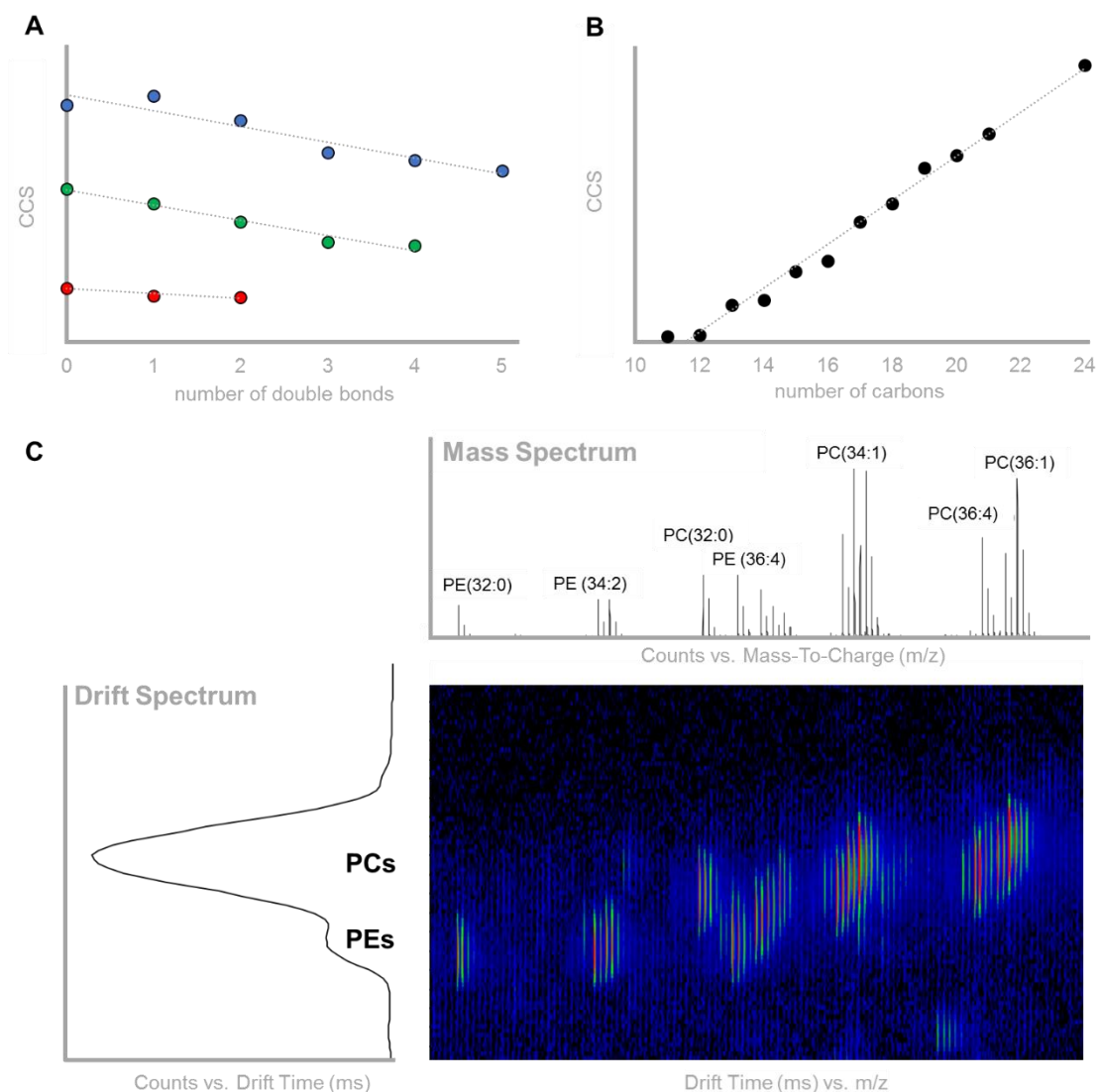
Ion mobility (IM) is a gas-phase technique allowing the separation of ions based on their mobility through an inert gas (typically helium or nitrogen) under the influence of an electric field [3–6]. A number of different technologies in which IM is coupled with MS (IM-MS) have been developed, including the commercially available (1) Drift Time Ion Mobility Spectrometry (DTIMS), developed by Agilent Technologies [7], (2) Field Asymmetric Ion Mobility Spectrometry/Differential Mobility Spectrometry (FAIMS/DMS) approach provided by AB Sciex [8,9], (3) Travelling Wave Ion Mobility Spectrometry (TWIMS) developed by Waters [8,10], and (4) Trapped Ion Mobility Spectrometry (TIMS) developed by Bruker [11–13]. A detailed description of the theoretical concepts behind ion separation in these different technologies is not within the scope and space of this brief review, and hence we refer the reader to recent reviews in the field [4–6]. IM-MS has been shown to provide significant analytical improvements for a variety of chemical classes. Compared to MS only, using IM-MS as additional separation reduces interfering signals in the gas phase, thus increasing spectral clarity, enhancing peak capacity, and improving both selectivity and sensitivity [14–19]. In addition, the Collision Cross-Section (CCS), a physicochemical descriptor correlated to shape, size and charge of ions, can be calculated with most commercially available instruments by considering the experimental conditions and the observed mobility of ions. Both CCS values and accurate mass are highly reproducible (< 2%, < 5 ppm, respectively) [17,19,20] and can be used to discriminate closely related species by both size and mass. In addition, coupling LC to IM-MS [6] allows compound characterisation in multiple dimensions, making LC/IM-MS a promising approach for the comprehensive analysis of complex lipid mixtures [17,19].

Due to the emerging developments in both hardware and software, IM-MS has recently been implemented in a variety of lipidomic workflows, which are reviewed in the following sections.

### **Improved lipid identification by IM-MS**

Currently, compounds discovered in a lipidomic workflow are putatively identified based on accurate mass match with online databases such as LIPID MAPS or LipidBlast. However, as accurate mass only provides the molecular formula, it could correspond to a number of species belonging to different lipid classes. Therefore, further information about the compound's

physicochemical characteristics are required to allow a more accurate identification. It has been shown that the CCS values of FAs and PCs are highly correlated with both the lipid chain length and the degree of unsaturation [21–23] (**Figure 2 A and B**). In particular, larger CCS values are found for saturated species, whose acyl chains extend under the electric field, while unsaturated bonds confer a bent structure in the acyl chain resulting in smaller CCS values [21,22]. The CCS is influenced not only by the degree of saturation, but more generally by the structural characteristics of compounds. Thus, different lipid categories, such as GPs and sphingolipids, have distinct mobility behaviours by which they can be separated. This allows a more selective analysis of lipids belonging to different categories but sharing an identical accurate mass [24]. In addition, the distinct mobility trends observed not only between lipid categories but also among different biomolecular families (such as lipids, peptides and oligonucleotides) improve data interpretation in imaging MS [19,25–32]. In this technique, lipids are analysed directly from tissue slices without additional extraction and/or purification steps, allowing simultaneous CCS evaluation and lipid localisation in tissue [33]. Similarly, different lipid classes belonging to the same category can be separated by mobility based on their head groups (shown for PEs and PC in **Figure 2 C**); however, separation is less efficient than between lipid categories, with some overlapping CCS values between certain species [19,26]. Overall, these lipid category/class specific mobility behaviours decrease the false discovery rate providing a higher degree of confidence in lipid identification [17,22,24,26,34].

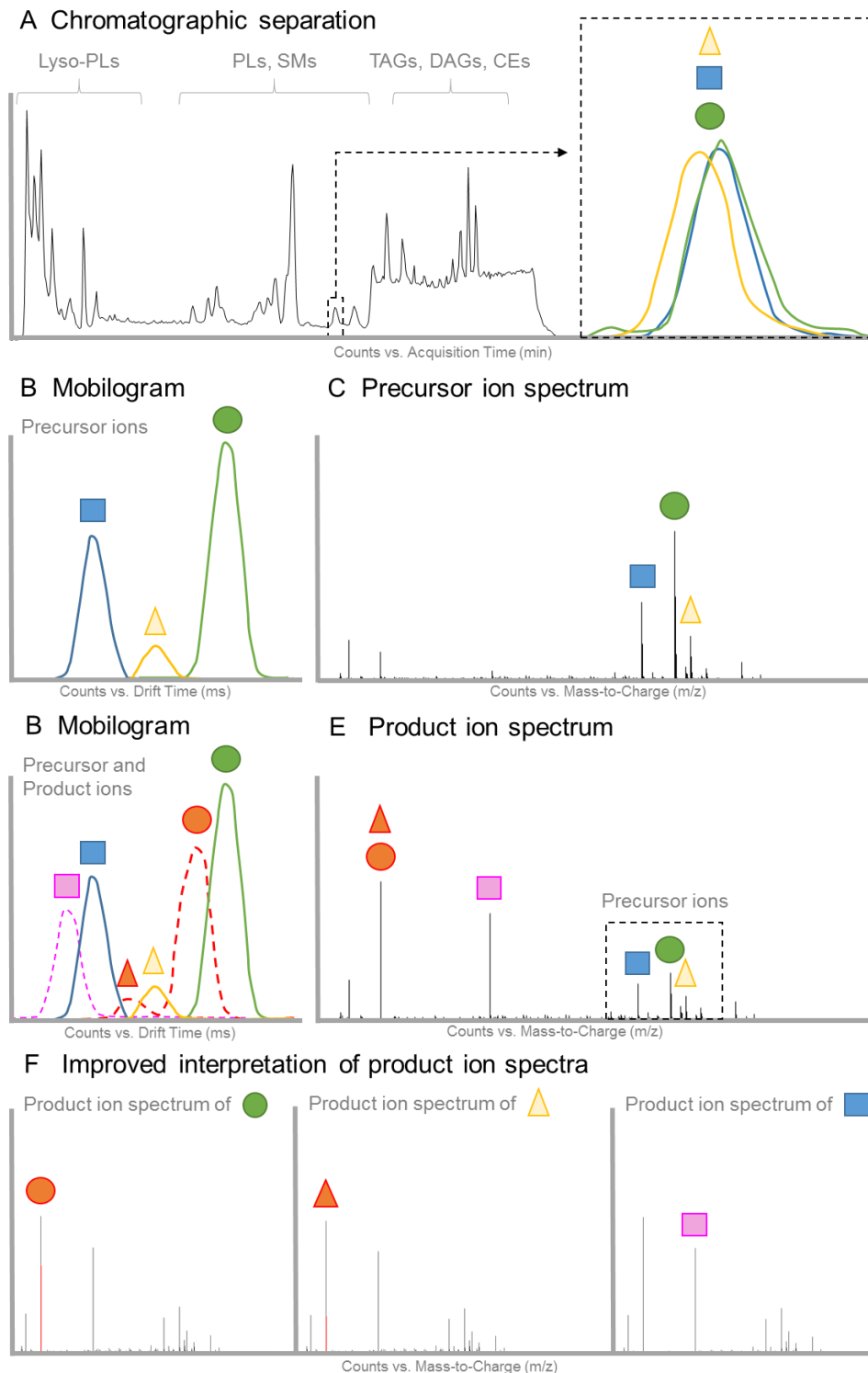


**Figure 2. Improved identification of lipids by IM-MS.** The CCS values of fatty acids increase with both (A) number of double bonds (C16-C20 indicates the number of carbon atoms and x the number of double bonds) and (B) lipid chain length. (C) PEs and PCs are mobility separated based on their head group using a DTIMS-MS instrument.

In order to validate experimentally obtained CCS values, several research groups have developed their own CCS databases by analysing synthetic lipid standards [17,19,35]. However, due to the limited number of commercially available standards, *in silico* CCS determination may represent a promising approach to cover the lipidome of complex biological matrices, as demonstrated for lipids and other biomolecules [36–40].

Traditionally, lipids are identified using either targeted or untargeted fragmentation of precursor ions to obtain diagnostic product ions in high-resolution spectra [41–46]. The addition of IM to targeted workflows, in which precursor ion masses are selected for fragmentation, has been shown to reduce background noise, simplifying interpretation of the spectra and improving the overall detection limits in lipid mixtures [14,16,30]. This is very important when analysing low abundant but biologically relevant species. On the other hand,

targeted methods may struggle with poor duty cycles and decreased sensitivity when too many ion masses are fragmented. Untargeted fragmentation can overcome these sensitivity issues as no precursor ion masses are selected. However, data interpretation is challenging due to presence of multiple product ion masses deriving from co-eluting species in the same spectrum. This is especially true for lipids, as their fragments share a high degree of similarity [47]. It has been demonstrated that addition of IM to untargeted lipidomics allows mobility-alignment of fragment ions to their precursors when fragmentation takes place following mobility separation [48–51] (**Figure 3**). In contrast, fragmentation prior to IM separation allows CCS evaluation of diagnostic product ions as an additional identifying factor [52–54]. Therefore, both approaches improve data interpretation and enhance lipid identification in untargeted workflows.

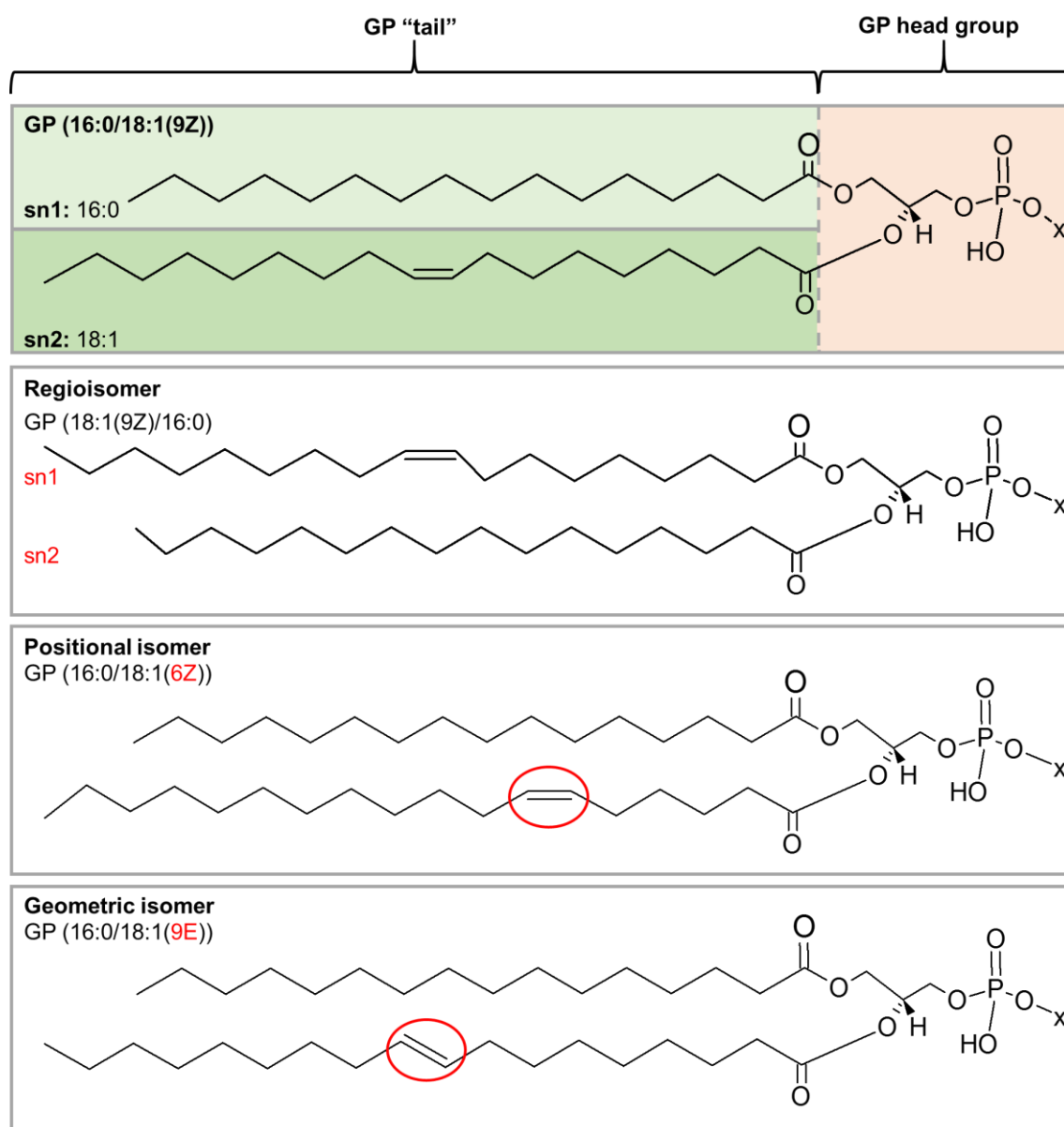


**Figure 3: Ion mobility separation of co-eluting lipids and their product ions.** (A) Chromatographic separation of a complex mixture of lipids such as tissue extracts containing lyso-phospholipids (lyso-PLs), glycerophospholipids (GPs), sphingomyelins (SMs), triacylglycerides (TAGs), diacylglycerides (DAGs) and cholesteryl-esters (CEs) with the extracted ion chromatogram of insufficiently separated lipids species magnified in yellow, blue, and green. These species can be separated by (B) ion mobility and (C) accurate mass. For lipid identification collision-induced fragmentation is applied. However, untargeted fragmentation of all precursor ions present in a sample results in a mix of both precursor (solid) and product (dashed) ion (D) mobilities in the mobiligram and (E) ion masses in the product ion spectrum. As IM separation occurs before fragmentation, product ions can be mobility-aligned to their precursor ions consequently improving interpretation of product ion spectra (F). In addition, identical product ions deriving from different precursor ions (such as red deriving from green and yellow) can be assigned to their precursors in correct proportions (marked in red).



## Isomer separation

One of the major challenges in lipid analysis is isomer separation, with regio- (such as sn1 or sn2 for GPs), positional (position of double bond), or geometric (cis/trans conformation of the double bond, also known as Z/E) isomers commonly found in complex lipid mixtures (



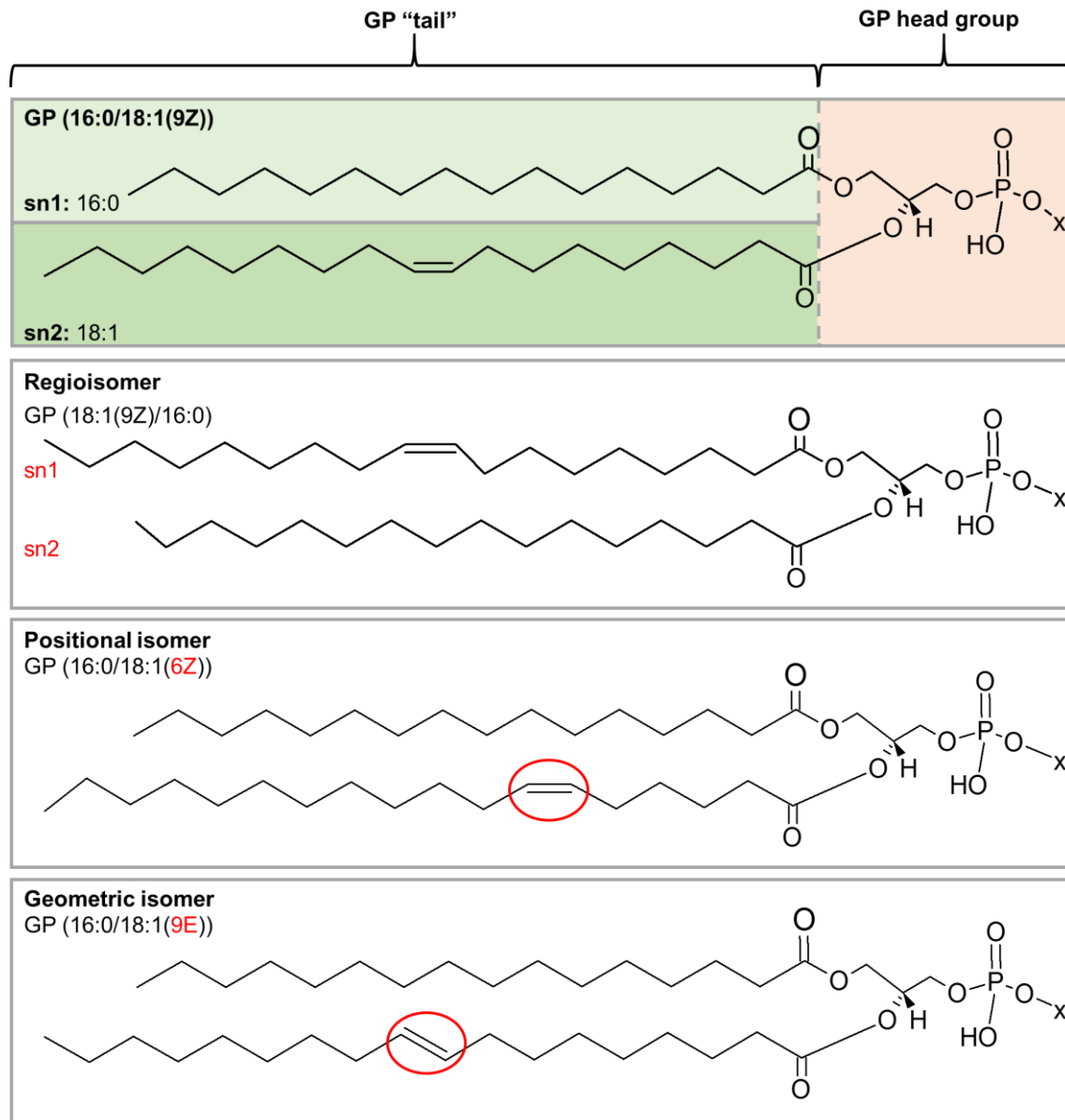
**Figure 4).** Although LC can separate some isomeric compounds, several will co-elute and exhibit identical fragmentation spectra, making their identification difficult. IM can improve separation of isobaric and isomeric lipids due to differences in their structure and shape [24,55–57]. However, all three dimensions of LC, IM and MS are needed to target isomer

separation which still may not be complete [22,49,58], although it can be enhanced by the use of different inert gases in the mobility chamber [24,55].

The conformational differences between geometrical isomers, and in particular the curled structure caused by a cis- double bond in contrast to a more open conformation conferred by trans geometry, allows separation between cis and trans isomers of GPs [22]. In addition, cis-unsaturated positional isomers of FAs could be resolved, while their equivalents containing trans-double bonds could not be distinguished due to smaller differences in shape caused by the trans geometry compared to cis [22]. Comparably, a double bond closer to the head group of GPs causes a larger structure than when positioned further down the acyl chain, allowing separation of PC positional isomers [59]. In contrast, positional isomers of mono-unsaturated FAs could only be separated when injected individually, while their mixtures could not be resolved due to only minor differences among the isomers conformations [23]. Separation of GP regioisomers was observed in targeted [48], multidimensional (IMS<sup>3</sup>-MS) [34] approaches, with a more elongated three-dimensional conformation observed when the longer FA chain is linked to the sn1 position [22]. Interestingly, the opposite holds for lysoPC (which only have a single acyl chain attached to either sn1 or sn2 position of the GP backbone), where a FA in sn2 position results in a larger cross-section for the ion [22].

The separation of isomers whose spatial conformations are too similar can be enhanced with several strategies, such as using instruments with a higher IM resolving power [50,55,60]. Indeed, on a lower resolution instrument the two structural isomers PG and bis(monoacylglycerol) phosphate (BMP) could be distinguished only following evaluation of their fragmentation pattern [61], while their separation as native ions was possible by increasing the IM resolving power [22]. However, the choice of commercially available IM-MS instruments offering a high resolving power today is limited [50]. To overcome this deficiency, derivatization can be used to increase the shape differences between isomers and was shown to successfully resolve synthetically generated steroid isoforms [62]. Similarly, the formation of adducts with alkali ions can enhance isomers separation thanks to the differences in coordination geometries, as demonstrated for steroid epimers, ceramide stereoisomers, and diastereoisomeric forms of sphingosine [22,59,63]. Furthermore, interaction of alkali ions with the double bond, in contrast to protonation which happens in the head group, can increase the separation between sodiated and potassiated adducts of PC geometrical and regioisomers [64]. Likewise, the ability of silver to bind both lone pairs of electrons from heteroatoms and  $\pi$  electrons of the double bond allowed resolution of PC as well as GL isomers [65,66]. **Another approach to assign double bond positions in complex lipids and distinguish GL isomers is ozone-induced dissociation (OzID) [67]. Although not the main focus of their study, Poad and co-workers observed mobility separation between precursor and OzID product ions, indicating**

OzID coupled with IM as a promising approach to gain further understanding into lipid structures.



**Figure 4: Isomeric structures of glycerophospholipids.** Glycerophospholipids (GPs) consist of a polar head group (glycerol, choline, ethanolamine, inositol, or serine indicated as "x") and two fatty acyl groups at sn1 (16:0) and sn2 (18:1 (9Z)) position forming the GP tail. Three types of isomers are possible: a regioisomer presenting the fatty acyl chains in inverted positions; a positional isomer, containing the double bond in different position (C6 instead of C9), and a geometric isomer, whose double bond is in trans (E) conformation.

## Conclusion

The chemical complexity of lipids makes highly sensitive and selective analytical tools crucial for their analysis in complex mixtures such as extracts of biological tissues. Recent technical developments in both hardware and software enabled researchers to implement IM-MS into their analytical workflows with four major improvements in lipidomic analysis. First, IM-MS improves separation of lipids in complex mixtures, crucial to resolving isobaric species in biological samples. Second, IM improves the spectral clarity of product ion spectra derived from fragmentation experiments. This is important in both lipid identification and structural elucidation, which are challenging tasks due to isomeric nature of many lipid species. On the other hand, analysis of product ion spectra derived from untargeted fragmentation acquisitions remains challenging due to the required powerful processing tools. Third, IM improves separation of isomeric lipids. However, to completely uncover the isomeric distribution in complex lipid mixtures, a higher resolving power than is commercially available today may be required. Last, CCS values obtained from IM-MS analysis effectively increase confidence in lipid identification. However, validation of CCS values is restricted to the limited number of commercially available lipid standards. Therefore, *in silico* CCS prediction is an important tool to improve identification efficiency in lipidomics. Concluding, IM-MS is a promising analytical tool for lipidomic studies that has the potential of providing new insights into lipid biology.

### **Acknowledgements**

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