2017

Advances and unresolved challenges in the structural characterization of isomeric lipids

Sarah Hancock
University of Wollongong, sen965@uowmail.edu.au

Berwyck L. J Poad
Queensland University of Technology, bpoad@uow.edu.au

Amani M. Batarseh
University of Wollongong, amani@uow.edu.au

Sarah K. Abbott
University of Wollongong, sarahmac@uow.edu.au

Todd W. Mitchell
University of Wollongong, toddm@uow.edu.au

Publication Details

Hancock, S. E., Poad, B. L.J., Batarseh, A., Abbott, S. K. & Mitchell, T. W. (2017). Advances and unresolved challenges in the structural characterization of isomeric lipids. Analytical Biochemistry, 524 45-55.

Advances and unresolved challenges in the structural characterization of isomeric lipids

Abstract
As the field of lipidomics grows and its application becomes wide and varied it is important that we don't forget its foundation, i.e. the identification and measurement of molecular lipids.Advances in liquid chromatography and the emergence of ion mobility as a useful tool in lipid analysis are allowing greater separation of lipid isomers than ever before. At the same time, novel ion activation techniques, such as ozone-induced dissociation, are pushing lipid structural characterization by mass spectrometry to new levels. Nevertheless, the quantitative capacity of these techniques is yet to be proven and further refinements are required to unravel the high level of lipid complexity found in biological samples.At present there is no one technique capable of providing full structural characterization of lipids from a biological sample. There are however, numerous techniques now available (as discussed in this review) that could be deployed in a targeted approach. Moving forward, the combination of advanced separation and ion activation techniques is likely to provide mass spectrometry-based lipidomics with its best opportunity to achieve complete molecular-level lipid characterization and measurement from complex mixtures.

Disciplines
Medicine and Health Sciences

Publication Details
Hancock, S. E., Poad, B. L.J., Batarseh, A., Abbott, S. K. & Mitchell, T. W. (2017). Advances and unresolved challenges in the structural characterization of isomeric lipids. Analytical Biochemistry, 524 45-55.

Advances and unresolved challenges in the structural characterization of isomeric lipids

Sarah E. Hancock[1], Berwyck J. Poad[2], Amani Batarseh[1], Sarah K. Abbott[1], Todd W. Mitchell[1*]

[1] School of Medicine, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia

[2] Central Analytical Research Facility, Queensland University of Technology, Brisbane, QLD 4001, Australia

***Corresponding Author**

Assoc. Prof. Todd Mitchell

School of Medicine

University of Wollongong

Wollongong, NSW 2522

Australia

Ph: +61 2 4221 5443

Email: toddm@uow.edu.au

Abstract

As the field of lipidomics grows and its application becomes wide and varied it is important that we don't forget its foundation, i.e. the identification and measurement of molecular lipids.

Advances in liquid chromatography and the emergence of ion mobility as a useful tool in lipid analysis are allowing greater separation of lipid isomers than ever before. At the same time, novel ion activation techniques, such as ozone-induced dissociation, are pushing lipid structural characterization by mass spectrometry to new levels. Nevertheless, the quantitative capacity of these techniques is yet to be proven and further refinements are required to unravel the high level of lipid complexity found in biological samples.

At present there is no one technique capable of providing full structural characterization of lipids from a biological sample. There are however, numerous techniques now available (as discussed in this review) that could be deployed in a targeted approach. Moving forward, the combination of advanced separation and ion activation techniques is likely to provide mass spectrometry-based lipidomics with its best opportunity to achieve complete molecular-level lipid characterization and measurement from complex mixtures.

Key words

Lipidomics, Mass Spectrometry, Ion Mobility, Chromatography, lipid isomers

Introduction

Several definitions of lipidomics have been published, yet the most comprehensive was provided back in the nascent era of the field: "the full characterization of lipid

molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" [1]. In the same editorial the authors listed three "first tasks" that the field of lipidomics needed to achieve in order to meet this definition: (i) new analytical approaches for mapping the lipidome, (ii) the application of biophysical methods to understand lipid-protein interactions, and (iii) identifying the lipid network, including lipid mediators, for metabolic and gene regulation and its integration with non-lipid signaling. Thirteen years later, lipidomics researchers are still searching for the best approaches to achieve these "first tasks". All three are still relevant and necessary if lipidomics is to provide the biological and physiological insight it promises; however, the ability to fully characterize the lipidome is required before lipid-protein interactions or pathway mapping can be fully realized.

We have previously discussed the importance of lipid structural characterization [2–4] and most recently reviewed the area in 2012 [5]. Since that time there has been significant growth in the number of researchers working on new techniques to determine lipid structure and several new and innovative approaches have been published. While our increasing armory of analytical techniques aides our endeavor to resolve and measure molecular lipid isomers, i.e. sn-position isomers (Figure 1a vs 1b), double bond position isomers (Figure 1a vs 1c), double bond stereochemical isomers (Figure 1a vs 1d), and enantiomers (Figure 1a vs 1e), much work still remains. This review will describe and evaluate the analytical techniques being developed to resolve and measure these lipid isomers with a particular focus on developments that have occurred since 2012.

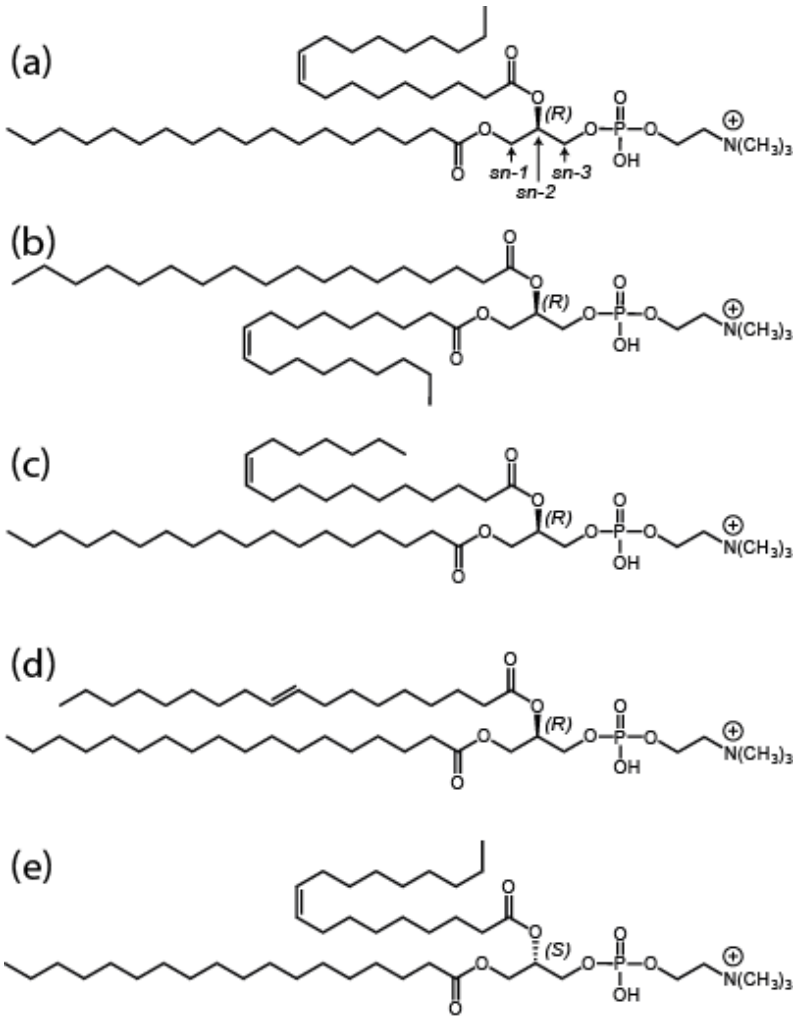

Figure 1: Examples of some of the possible isomeric forms of PC 36:1 arising from sn-positional isomerism, double bond positional isomerism, cis/trans isomerism about the double bond and possible R/S chiral variation for (a) PC 18:0/18:1(9Z), (b) PC 18:1(9*Z*)/18:0, (c) PC 18:0/18:1(7*Z*), (d) PC18:0/18:1(9E) (e) PC 18:0/18:1(9Z).

Sn regioisomers

The location of acyl chain esterification on the glycerol backbone, assigned as sn-1, sn-2 and sn-3 (Figure 1a), is often assigned in lipidomics studies (sometimes inadvertently due to inconsistencies in nomenclature) when there is insufficient data to allow this level of structural characterization. There are some current and

emerging techniques that do provide this information however, extra analysis is often required.

Nuclear magnetic resonance (NMR) spectroscopy

NMR continues to play a significant role in lipid analysis; not only is it used for fatty acid composition and lipid class-identification, the technique has been used to provide information on the regiospecific distribution of fatty acids in triacylglycerols (TAGs) and phospholipids. Different carbon atoms produce signals in different regions of the ^{13}C -NMR spectrum, i.e. carbonyl (172–174 ppm), olefinic (126–134 ppm), glycerol (74–60 ppm) and aliphatic (19–35 ppm) regions. This allows the regiospecificity (sn-distribution) of fatty acyl-chains of TAGs to be determined by ^{13}C –NMR. This technique has been used to verify the authenticity and adulteration of food products, e.g. fish oils, milk and animal fat [6, 7]. ^{13}C NMR is an excellent tool for regiospecific analysis of intact lipids however, the requirement for relative large amounts of sample precludes it from many lipidomic analyses.

Mass spectrometry (MS) based analyses

Although NMR has played a significant role in understanding lipid structure, advances in MS have driven the field of lipidomics and made it the analytical tools of choice.

Collision-induced dissociation (CID)

Initial work studying lipids on sector-based mass spectrometers using high collision energy (keV) in the 1980s and 90s [8] contributed significantly to our current knowledge regarding the fragmentation and structural elucidation of ionized lipids [2, 3]. Tandem time-of-flight (TOF/TOF) mass spectrometers provide access to similar CID energies; however, initial platforms had wide isolation windows of approximately 4 Th, hampering the ability to analyze complex lipid mixtures that require mass selection at unit resolution [9]. Recent developments in TOF/TOF technology allow higher resolution precursor ion selection, and this has been successfully utilized to determine sn-positions of TAGs in olive oil [10]. As this approach is non-targeted, i.e. almost all bonds are broken, the available charge is

spread over many ions, which can limit its ability to determine sn-position in low abundance lipids.

The use of low-energy CID for structural characterization and sn-assignment in glycerophospholipids as well as the mechanisms of fragmentation processes in both polarities have been reviewed in detail [3, 11] . While low energy CID allows for the identification of the most abundant sn-position isomer present, it is often difficult if not impossible to determine the presence of isomers [5] unless comparisons to calibration curves are made for each lipid of interest [12]. Multistage CID approaches can be used to generate MS^n spectra containing diagnostic ions that allow the assignment of the sn-position of triacylglycerols and glycerophospholipids [13, 14]. This works well for high abundance lipids but is limited in sensitivity.

Ion-molecule reactions

Ozone-induced dissociation (OzID)

In combination with CID, OzID produces unique fragment ions indicative of sn-position. In such an approach, mass-selected lipids are collisionally activated before being trapped and allowed to react with ozone within the collision cell, resulting in CID/OzID spectra that can be used to determine lipid regiospecificity (reviewed by [3]). In the initial CID step either the phospholipid head group [15] or a FA from the TAG backbone [5, 16] is lost. This creates a new double bond between carbons 1 and 2 of the "attacking" acyl chain that is subsequently cleaved by ozone, allowing determination of sn-position (see [15] and [16] for mechanisms). This process is fast enough to be performed on an LC timescale [17, 18], and has even been used to determine the distribution of sn-positional isomers directly from sheep brain tissue [19].

Odd-electron fragmentation

Another approach to determining sn-position within lipids is generation of odd-electron products by radical-driven dissociation. Unlike low energy CID, radical-driven dissociation can break carbon-carbon bonds, which in some instances is able to produce data diagnostic of sn (and double bond) position.

Electron-induced Dissociation (EID)

EID arises from the interaction of singly-charged cations at electron energies of 2-70 eV. Typically performed using Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers, this method results in excitation and fragmentation similar to that obtained by CID. While EID is able to fragment lipids along their acyl chain it produces no unique ions diagnostic for double bond position [20]; thus providing no significant gain in sn-position information beyond that obtained from low energy CID. This was highlighted by the fact that known sn-position impurities in synthetic PC 16:0/18:1 and 18:1/16:0 [12, 21] were not detected by this technique.

Electron-impact excitation of ions from organics (EIEIO)

More recently EID has been implemented as EIEIO on other more common lipidomic platforms such as ion trap [22] and quadrupole time-of-flight (QTOF) mass spectrometers [23, 24] using a novel branched ion-trap design (Figure 2). Like EID, EIEIO also induces extensive acyl chain fragmentation, but has the added advantage of producing an ion diagnostic for the sn-2 fatty acid from the fragmentation of the glycerol backbone [24]. However, like all untargeted fragmentation processes the efficiency of producing this sn-2 diagnostic fragment is low and much of the available charge is spread across a large number of ions. This means that the ability to identify low abundance sn-position isomers in complex biological mixtures is likely to be poor.

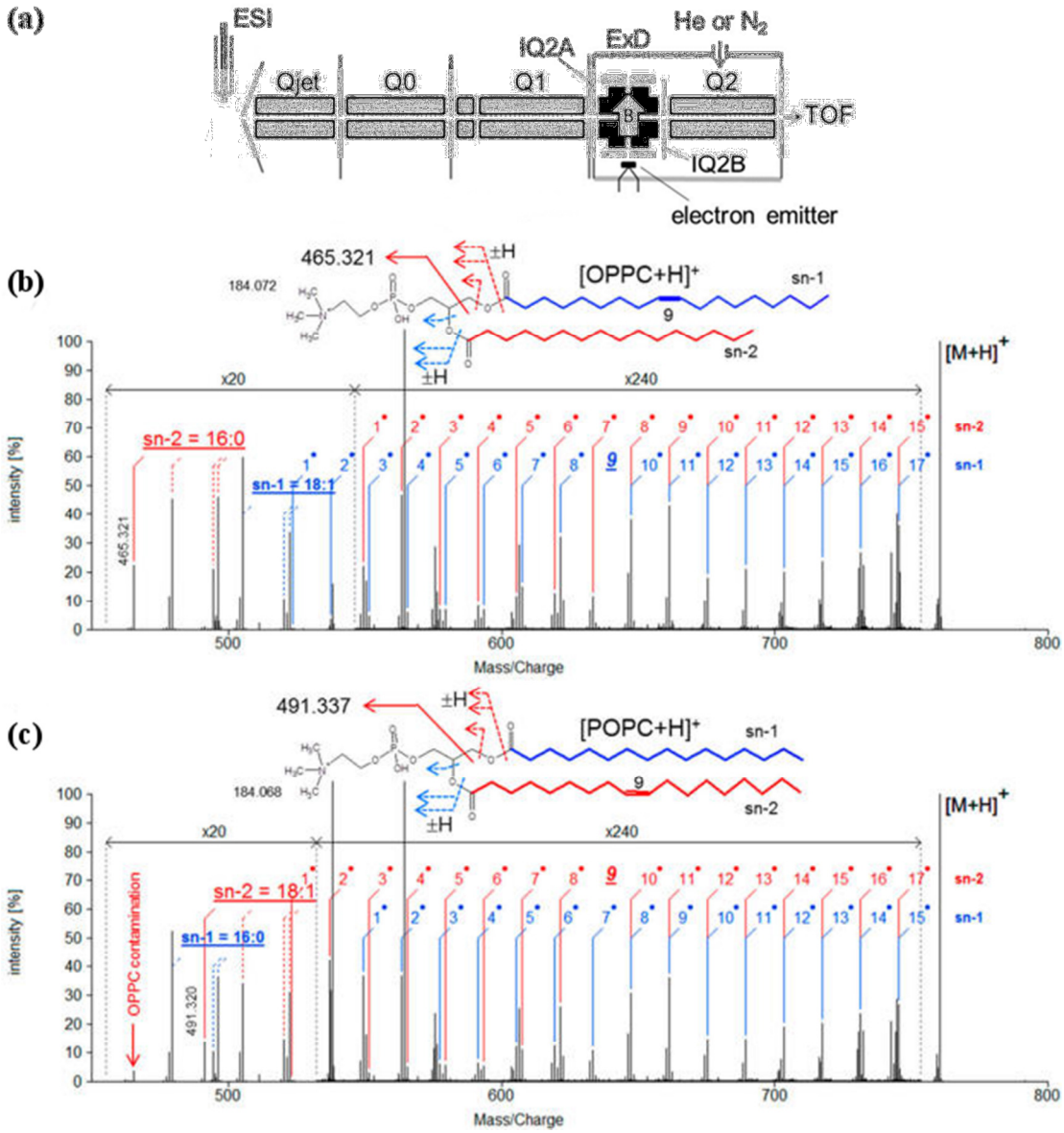

Figure 2: EIEIO allows characterisation of sn position in phospholipids by electron-induced acyl chain fragmentation. (a) shows the implementation of the electron-ion reaction device (ExD cell) on a quadrupole time-of-flight mass spectrometer. EIEIO produces unique diagnostic fragment ions for sn-1/sn-2 position, allowing unambiguous discrimination between (b) PC 18:1/16:0 and (c) PC 16:0/18:1, as well as ions indicative of double bond position. Figure adapted from [23, 24].

Separation techniques

High performance liquid chromatography (HPLC)

HPLC separation of regioisomeric lipids, particularly by C18 reverse phase chromatography, has chiefly been limited to lipids with large differences in acyl chain length [25]. More recent work has explored the use of new reverse phase columns with a high number of carbons (C28-C30) to separate regioisomeric TAGs [26, 27]. Such columns are able to resolve some sn-2 specific TAG isomers, but not sn-1 from sn-3. For example, Nagai and colleagues were able to separate TAG isomers from bovine milk fat with either 16:0 or 18:1 in the sn-2 position with a C28 column, but were unable to resolve sn-1 vs sn-3 isomeric TAGs [26].

Chiral chromatography has shown some promise for overcoming the limitations of reverse phase HPLC, allowing the detection of a wide range of sn-1, sn-2 and sn-3 regiospecific TAGs in bovine milk fat [27], hazelnut oil and human plasma [28], yeast [29], and algae [30]. Despite these improvements a number of sn-positional TAG isomers in biological extracts remain under-resolved by chiral chromatography.

Hydrophilic interaction chromatography (HILIC) has also shown some ability to separate regioisomeric lyso-phospholipids in sea bream [31], egg yolk, porcine brain and soy [32]; however, this method does not appear to have the capacity to separate regioisomeric diacyl phospholipids.

Supercritical fluid chromatography (SFC)

Similar to reverse phase HPLC, SFC utilizing a C30 reverse phase column is also able to separate TAG sn-2 vs sn-1/sn-3 positional isomers, allowing the identification of a combined eight TAG regioisomers in palm and canola oil [33]. Although this methodology provides a similar level of separation to that of reverse phase HPLC, SFC has the advantage of much shorter retention times and less generation of hazardous solvent wastes.

Ion Mobility Spectrometry (IMS)

In recent years IMS coupled to MS (IMS-MS) has emerged as an alternative technique to HPLC-MS for the separation and identification of lipids from complex

biological extracts. In particular, drift time and differential ion mobility spectrometry (DMS) have emerged as being useful techniques for studying lipids.

In drift time ion mobility, ions migrate through an inert buffer gas under a low electric field at either atmospheric or reduced pressure. The time taken for a given ion to traverse the drift tube is related to its collisional cross section (CCS); larger (or more extended) ions undergo more interactions with the buffer gas, slowing their migration. Through this method, near baseline separation of $[M+Ag]^+$ PC 16:0/18:1 and 18:1/16:0 isomers has been achieved, permitting their detection in porcine brain extract [34]. However, other adducts such as $[M+H]^+$ show only limited separation by IMS [35].

A related IMS technique, DMS, relies on the differential movement of ions when exposed to an alternating asymmetric waveform [36]. DMS has also been used to separate $[M+Ag]^+$ PC 16:0/18:1 from 18:1/16:0 [21]. Moreover, these PC sn-positional isomers were able to be quantified as confirmed by traditional phospholipase A_2 experiments, allowing the abundance of these two PC isomers in chicken egg yolk, cow kidney and cow brain to be determined. Similarly, DMS can resolve regioisomeric $[M+Ag]^+$ TAG with combinations of saturated and monounsaturated fatty acyl chains with the use of gas-phase modifiers (*n*-butanol) in porcine adipose tissue [37].

Although completely saturated lipids are not common in most biological samples, the lack of at least one double bond precludes their separation by drift time IMS and DMS as silver ion adducts. Likewise, comprehensive reference libraries of drift time and CCS for lipids generated from authentic standards are needed before the wider application of IMS to lipid isomer characterisation in biological extracts can be realised.

Combining techniques

HPLC-CID/OzID

Recent work combining reverse phase HPLC with CID/OzID demonstrated separation of some sn-position isomers [17]. As with previous work [25], HPLC was able to separate sn-position isomers with large differences in the length of the two acyl

chains (i.e. PC 16:0/20:4 from 20:4/16:0) but was unable to separate sn-position isomers where the acyl chains were of similar length. Extracted ion chromatograms using sn-specific CID/OzID ions did appear to provide quantitative areas under the curve for a one-to-one mixture of PC 16:0/18:1 and 18:1/16:0 [17]. Nevertheless, further evidence is required to determine the quantitative ability of this approach.

HPLC-IMS-MS

The combination of IMS with reverse phase HPLC has allowed the separation of regio-isomeric lyso-PC 16:0 and 16:1 from a human embryonic epithelial kidney cell line [35]. Like previous HPLC methods, this separation relies on the large differences present between the acyl pairs and requires long separation times of up to 90 minutes. However, this methodology does have some capability to also separate lyso-PC 16:1 by double bond geometry (see below).

Double bond position

The identification of double bond position within lipids, such as differentiating between PC 18:0/18:1(9Z) in Figure 1a and PC 18:0/18:1(11Z) in Figure 1c, has long been the domain of gas chromatography (GC) and silver ion HPLC; however, contemporary lipidomics has shifted primarily to analytical methods utilizing MS. One advantage of such a workflow is that it enables the identification of complex lipid structures from biological samples, but typically these methods are unable to determine double bond position on their own. Therefore, more recent work has focused on developing novel analytical methods that can be used in conjunction with conventional mass spectrometers (i.e. triple quadrupole, ion trap, and TOF) to provide information on double position within lipids.

High-energy CID

High-energy CID has been used previously to identify double bond position in phospholipids and TAGs by charge-remote fragmentation [8, 38–40]. More recently, a TOF/TOF system has been used to characterize double bond position in a triolein

standard; however, the presence of other double bond isomers was not confirmed by this method in an olive oil extract [10].

Charge-switch derivatization

Originally devised as a means to increase the sensitivity of mass spectrometric detection of eicosanoids by charge-reversal with *N*-(4-aminomethylphenyl)pyridinium (AMPP) [41], the collisional activation of fatty acids derivatized with AMPP also allows double bond position identification through charge-remote fragmentation [42, 43]. An advantage of this approach is that it is quantitative and does not appear to be affected by acyl chain length and the number of double bonds present [43]; however, this method cannot be applied to the study of intact complex lipids and produces complicated diagnostic spectra.

Ion-molecule reactions

OzID and other ozonolysis methods

OzID utilizes the reaction of ozone with carbon-carbon double bonds inside the mass spectrometer to produce fragment ions characteristic of double bond position [44]. Combining OzID with multi-stage CID (i.e. MS^n) within a linear ion trap mass spectrometer has enabled the regiospecific assignment of double bond position in lipids by "top-down" fragmentation [15]. This same approach has also been used to uncover the presence of conjugated double bonds in lipids extracted from foodstuffs [45], as well as double bond position in unusual TAGs extracted from *Drosophila* [16]. However, the application of such an approach to complex biological samples is limited due to long acquisition times and spectral complexity arising from the presence of isomeric or isobaric lipids present in directly-infused samples. The combination of online liquid chromatography with OzID and CID/OzID [17, 18] reduced spectral complexity in biological samples, and demonstrated that OzID spectra can be obtained within a chromatographic timescale.

Analysis of double bonds by ozonolysis can also be achieved prior to MS analysis by passing a lipid mixture through an ozone-permeable membrane located upstream from the source of the mass spectrometer [46]. In combination with silver ion liquid

chromatography, this approach has been utilized to identify double bond isomers in fatty acid methyl esters (FAMEs) derived from bovine adipose tissue [46], and in the detection of conjugated linoleic acid (CLA) methyl esters derived from CLA supplements, bovine milk fat and *Lactobacillus plantarum*. [47]. When used in combination with 2D-HPLC, this "inline-ozonolysis" approach can provide double bond positional information from phospholipids derived from biological extracts [48]. One advantage that this approach has over other ozone based techniques (such as performing OzID on mass-selected ions inside the mass spectrometer) is that it can be utilized on a walk-up instrument.

Paternò-Büchi reaction

A more recent application of ion/molecule reactions to the localization of double position has utilized the Paternò-Büchi reaction combined with tandem MS [49–51]. This is achieved by the in-source UV irradiation of ionized lipid solutions spiked with acetone. Alongside a fast reaction time and compatibility with shotgun lipidomics, this approach has the advantage of being able to quantify the amount of double bond isomers present in biological extracts [49]. However, this approach also suffers from spectral complexity due to isomeric/isobaric interference, as well as artefactual side-reactions that limit its usefulness. However, it is envisaged that the inclusion of a prior separation step, such as LC or IMS, will significantly reduce any interference from lipid isomers and isobars.

Odd-electron dissociation

Electron capture/transfer dissociation (ECD/ETD)

Both ECD and ETD have been successfully applied to the study of multiply-charged ions from proteins and peptides, providing additional structural information not afforded by CID. However, lipids typically only form singly charged species, making the application of ETD or ECD to lipids analysis challenging. One such attempt applied ETD to $[M+2Na]^+$ PC 18:0/20:4, but this approach yielded only limited information on double bond position [52]. The use of high energy (or 'hot') ECD on Mn^{2+}-adducted fatty acids provided greater structural information on the position of

double bonds, but suffered from spectral complexity above even that seen by high-energy CID [53].

EID

As described above, EID can be used to induce substantial acyl chain fragmentation in singly-charged lipids, allowing the characterization of double bond position [20]. However, due to low fragmentation efficiency only low amounts of diagnostic ions were produced.

EIEIO

Using their modified QTOF EIEIO instrumentation (Figure 2), Campbell and Baba [24] were able to characterize a number of double bond isomers from highly abundant egg yolk PCs with a relative short trapping time (5-40ms). However, much longer trapping times are required for less abundant PCs (up to 18 min), and the product ion spectra produced can be complicated.

Helium metastable atom-activated dissociation (He-MAD)

He-MAD is a novel ion-activation method which employs a fast atom bombardment gun to induce radical-driven fragmentation in a quadrupole ion trap [54]. This method has been used to determine double bond position in PC standards, producing spectra with similar qualities to that of EID. However, He-MAD suffers from the same spectral complexity and low fragmentation efficiency as EID/EIEIO, potentially precluding its applicability to complex biological extracts. Additionally, this technique produces a considerable ion signal background and requires long acquisition times and background subtraction to achieve a reasonable signal-to-noise ratio making the analysis of less abundant lipids in biological extracts challenging.

Radical directed dissociation

Radical direction dissociation has been used to determine double bond position and chain-branching in glycerophospholipids, SM and TAGs [55]. In this approach, lipids are adducted to bifunctional molecules containing a photocaged radical initiator and a lipid-adducting group (4-iodoaniline or 4-iodobenzoic acid). UV irradiation of these

complexes in the gas-phase cleaves the carbon-iodine bond to form a highly reactive phenyl radical, with subsequent CID generating radical-driven fragmentation of the adducted lipid. One drawback of this approach is that non-covalent interactions are not applicable to the analysis of simple lipids such as fatty acids. However, derivatization with a photocaged radical complex (4-iodobenzoic acid) can yield double bond location on mono- and polyunsaturated fatty acids [56]. Remarkably, CID of proton-bound lipid-photocaged radical complexes can also yield information of the double bond position of FAMEs, allowing discrimination between conjugated and non-conjugated fatty acids [57]. So far, these radical-directed dissociation methods have only been applied to simple biological extracts (i.e. TAG purified from lipoproteins), and like many techniques described above produce complex product ion spectra.

Separation techniques

Ultra-high performance SFC

An emerging chromatographic approach to identifying double bond position has utilized ultra-high performance SFC to separate FAME double bond isomers derived from commercial fish oils [58]. Unlike other new methods for double bond elucidation, this method can be coupled to an evaporative light-scattering detector rather than MS, and separates FAME isomers by their equivalent carbon number (ECN) using a C18 reverse phase column. This technique does, however, require comparison to known synthetic standards precluding it from *de novo* identification of double bond isomers. So far UHPSFC has only been applied to separation of double bond isomers in derivatized FAME, and it is currently unknown what scope this approach may have for other, more complex lipids.

IMS

Both drift time IMS and DMS have demonstrated applications for the separation of lipid double bond isomers [34, 35, 59]. Drift time IMS can separate double bond isomers within monounsaturated free fatty acid standards, with the “kink” caused by the double bond providing a unique CCS [35]. Likewise, PC 18:1(9Z)/18:1(9Z) and PC

18:1(6Z)/18:1(6Z) isomers can be separated by drift time IMS [35]. Application of this approach to a bovine heart identified PC 18:1(9Z)/18:1(9Z) as the sole isomer [34]. Nevertheless, PC 18:1(6Z)/18:1(6Z) is not commonly observed in biology and therefore it is yet to be determined if drift time IMS is able to separate the more subtle isomers common in biology, e.g. PC 18:1(9Z)/18:1(9Z) vs. PC 18:1(9Z)/18:1(11Z). The lack of commercially available standards also appears to be a major roadblock in developing drift time IMS methods at present.

DMS has shown capability to distinguish double bond position in 1-deoxysphingolipids, with OzID subsequently being used to verify the position of the double bonds [59]. This method unequivocally assigned the Δ14 position of the double bond in 1-deoxyceramide and 1-deoxysphingosine from a HEK293 cell line (as opposed to the more common Δ4 isomer seen in canonical ceramides and sphingosines). It is unknown at this time, however, if this method can distinguish double bond isomers with a closer spatial relationship (for example, lipids with 18:1(Δ9) vs 18:1(Δ11) fatty acids).

Combining techniques

2D-HPLC/in-line ozonolysis

The combination of in-line ozonolysis (see above) with 2D-HPLC has allowed the rapid and unambiguous characterization of double bond position in PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), SM, lyso-PE and lyso-PC derived from egg yolk [48]. This approach utilizes a heart-cut online LC workflow in which the phospholipid class of interest is first isolated by HILIC, followed by separation by ECN using reverse-phase LC (Figure 3). Nevertheless, this in-line ozonolysis technique appears to have less sensitivity to double bond isomers than OzID. For example, 2D-HPLC/in-line ozonolysis identified the presence of PC 16:0_18:1(Δ9) alone within egg yolk extract [48], while HPLC/OzID was also able to detect small amounts of the n-7 isomer as $[M+Na]^+$ ions [18].

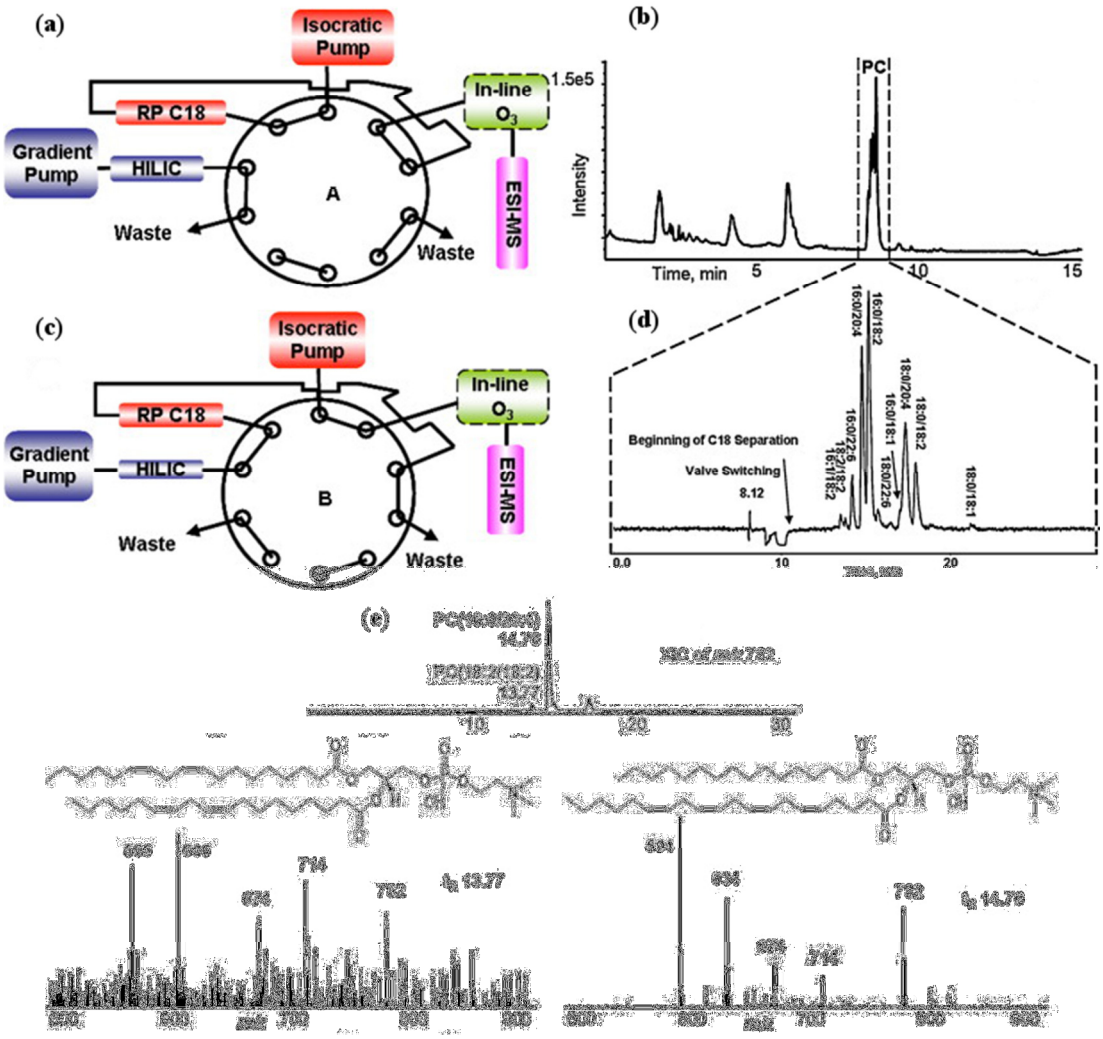

Figure 3: Heart-cut two dimensional liquid chromatography workflow combined with inline ozonolysis mass spectrometry used by Sun and colleagues to determine double bond position within PC phospholipids (adapted from [48]). In this approach, a 10-port 2 position switching valve is used to divert PC phospholipids separated by HILIC in the first dimension (a) & (b) onto a reverse phase column for separation by equivalent carbon number (c) & (d). In combination with in-line ozonolysis this workflow allows differentiation of double bond position within isomeric lipid species (e).

HPLC/acetonitrile adducts

Gas-phase acetonitrile-dervied adducts have previously been used to establish double bond position in a variety of standard FAMEs and FAMES derived from

biological extracts [60–65], as well as in synthesized TAG [66]. In this approach, an ion/molecule reaction between $[C_2H_2N]^+$ and neutral acetonitrile results in the formation of the (1-methyleneimino)-1-ethenylium ion (MIE), which forms a covalent adduct with any double bonds present. The isolation and collisional activation of this MIE adduct results in diagnostic fragments indicative of double bond position. Most recently, Háková et al. [67] combined this approach with HPLC to identify double position in TAG extracted from olive oil and vernix caseosa. The decrease in acetonitrile within the mobile phase at later retention times reduced the spectral intensity of MIE-adducted TAGs with high ECN; however, the post-column addition of acetonitrile might ameliorate this problem.

DMS/EIEIO

Recently Baba et al. [23] combined DMS with their EIEIO methodology to improve selectivity for double bond identification in SM from natural extracts. However, some SM species required background filtering and the reaction time was approximately $1/5^{th}$ slower than that seen previously for PC [24]. Ultimately this approach yielded the identification of some 200 SM species with differing double bond position in natural extracts [23].

Double bond stereochemistry

Determining the *cis*/*trans* stereochemistry of lipid double bonds in complex biological samples is problematic in modern lipidomics. Typical lipidomics analyses are achieved via MS, which as a sole technology is incapable of determining double bond geometry. However, coupling MS with separation technologies such as IMS or with ion chemistry such as ozonolysis can help us differentiate *cis*/*trans* stereoisomers (although at this stage such data is mostly limited to the identification of stereoisomers from standards).

GC is well established as the benchmark method for determining fatty acid composition in a wide range of different biological tissues, having first being described in 1952 [68]. However, this technique has a number of disadvantages: it requires comparison to known lipid standards (which are not always easily

available), sample preparation is quite time consuming as FAMEs must be formed, it requires large sample amounts (relative to MS analysis), and no information on the parent lipid is obtained unless extensive separation is carried out initially.

A comprehensive review by Chatgilialoglu et al. [69] details a number of analytical techniques used to determine lipid geometrical isomers, with an overview of GC, GC-MS, MS, argenation techniques, HPLC and HPLC-MS, NMR spectroscopy, infrared (IR) spectroscopy, Raman and ultraviolet (UV) spectroscopy. Further advances in these technologies will be described herein.

Spectroscopic techniques

A number of spectroscopy techniques are commonly used for determining total *trans* fat content of a sample, including NMR, IR, UV and Raman spectroscopy. Such approaches have gained particular popularity in the food industry.

The application of NMR spectroscopy in the determination of lipid double bond stereochemistry in edible oils and fats has been reviewed by Chatgilialoglu et al. [69]. More recently, Tsiafoulis et al. [70] used a combination of 1D ^{1}H-NMR, 2D ^{1}H - ^{1}H TOCSY and 2D ^{1}H - ^{13}C HSQC NMR to determine double bond geometric isomers of CLA in lipid fractions of lyophilized milk samples. This method overcame the common problem of sensitivity issues for low concentration metabolites seen in NMR analysis and also required no sample derivatization of the lipid extract, which can cause additional isomerization of the sample.

Although Raman spectroscopy only provides information on the total *cis/trans* content of a lipid extract, this technique still has its advantages: low cost, rapid analysis times, non-destructive and is able to analyze intact lipids (i.e. no requirement for derivatization prior to analysis). Fourier transform Raman spectroscopy has been applied to lipid extracts from human serum to determine the *cis*/*trans* conformation of cholesterol esters by comparison to an established spectral library [71]. *Cis* isomers produce spectral bands with lower frequencies compared to *trans* isomers (~ 1660 cm^{-1} and ~ 1667 cm^{-1} respectively). Double bond

stereochemistry of human sera determined via Raman spectroscopy was consistent with GC analysis and suggests that this technique has potential application to lipidomics.

Ion-molecule reactions

OzID

Poad et al. [72] demonstrated that OzID is potentially capable of distinguishing double bond stereochemistry for a number of unsaturated lipid standards (including phosphatidylglycerol, PC and fatty acid isomers). Overall, *trans* isomers were shown to be more reactive with ozone than *cis* isomers, with *trans*-isomers displaying OzID product ion abundances up to 2.5-times larger than *cis* isomers. Nevertheless, this method relies on relative abundances of identical ions and requires reference to appropriate lipid standards, which are often not commercially available.

CID of iodoaniline adducts

CID of non-covalent, proton-bound complexes between lipids and iodo-containing reagents produce diagnostic fragment ion pairs from 18:1(9Z), 18:1(9E), 18:1(11Z), 18:1(11E), 16:1(9Z), and 16:1(9E) FAMEs [57]. Importantly, the abundance ratio of these ions is inverted for geometrical isomers of the same FAME when 4-Iodoanaline was used as the adducting agent. For example, two diagnostic product ions for oleic acid were produced (*m/z* 295 and 297) with the *m/z* 295 ion the more abundant for the *cis* isomer and the *m/z* 297 more abundant for the *trans* isomer. This was found to be consistent for various double bond positions and chain lengths and was demonstrated in both linear ion trap and triple quadrupole instruments over wide ranging collision energies. Again, this technique relies on ion ratios so calibration curves created from synthetic standards for each isomer pair are required if this is to be applied to biological samples.

Separation techniques

HPLC

Reverse phase HPLC has the capability to separate synthetic di-18:1(9E) or di-18:1(9Z) stereoisomers from a range of different phospholipid classes [73–75]. This method has been applied to the study of PC 18:1/18:1 from rat serum and liver mitochondria [73], and porcine brain [74]; however, PC 18:1/18:1 stereoisomers are not completely baseline-resolved from isomeric PC 18:0_18:2 by this method [73]. At the present time, reverse phase HPLC appears unable to resolve stereoisomers with heterogeneous acyl combinations (i.e. 18:1(9Z)/18:1(9E)) from those with homogeneous acyl geometry.

Despite this limitation with phospholipids, reverse phase HPLC has had some success separating 2-hydroxy and 4-hydroxy glucosylceramide stereoisomers extracted from rice callus and orchardgrass leaves [76]. Both sphingenine and sphingadienine glucosylceramide isomers could be separated, including sphingadienines with differing *cis*/*trans* geometry along the long-chain base (i.e. d18:2(4E,8E) vs d18:2(4E,8Z)). It should be noted, however, that the structure of stereoisomers could not be confirmed by MS; rather, this identification was made *a priori* through comparison of the same extracts with GC. Improvements to the limit-of-detection and limit-of-quantification of this method were later achieved by derivatization of glucosylceramides with 4-Fluoro-7-nitrobenzofurazan [77].

IMS

Drift time IMS has been shown to distinguish between PC di-18:1(9Z) and di-18:1(9E), allowing the identification of the *cis* isomer in biological samples (including bovine heart, porcine brain and yeast) [34]. However, this technique was unable to determine any isomers with heterogeneous acyl stereochemistry (i.e. PC di-18:1(9Z) vs PC 18:1(9Z)/18:1(9E)). Extensive libraries of drift time and CCS values also need to be established if de novo identification of isomers is to be achieved by this method.

Combining techniques

HPLC-IMS-MS

Combining HPLC with IMS-MS has shown some success in differentiating synthetic fatty acids and phospholipid double bond stereoisomers [35, 74]; however, reported application of HPLC-IMS-MS to complex biological samples appears to be limited to a single lyso species, i.e. identification of lyso PC 16:1 *cis*/*trans* isomers in mouse and human embryonic epithelial kidney and hepatoma cell lines [35]. Additionally, only small differences are seen in CCS between PG di-18:1(9Z) and di-18:1(9E), providing little more orthogonal information to that of HPLC alone [74]. So while it appears that IMS-MS shows some promise for differentiating stereoisomers, to date this technology has only been successfully applied to synthetic lipids and any applications to complex biological samples is limited.

Chirality

Perhaps the most challenging level of complexity for lipid analysis is the determination of the absolute configuration about chiral centers within the lipid (Figure 4). Typically, enantiomeric analysis would be undertaken in one of four approaches: chromatographic separation, NMR spectroscopy, Circular Dichroism (CD) and other optical spectroscopies, or chemical derivatization to a diastereomer, the latter of which presents a simpler problem for identification. NMR assignment of stereochemistry often relies on the formation of diastereomers [78]. However, recent studies have shown that it is possible to distinguish enantiomers using ^{1}H NMR without the need for diastereomer formation prior to analysis [79] .

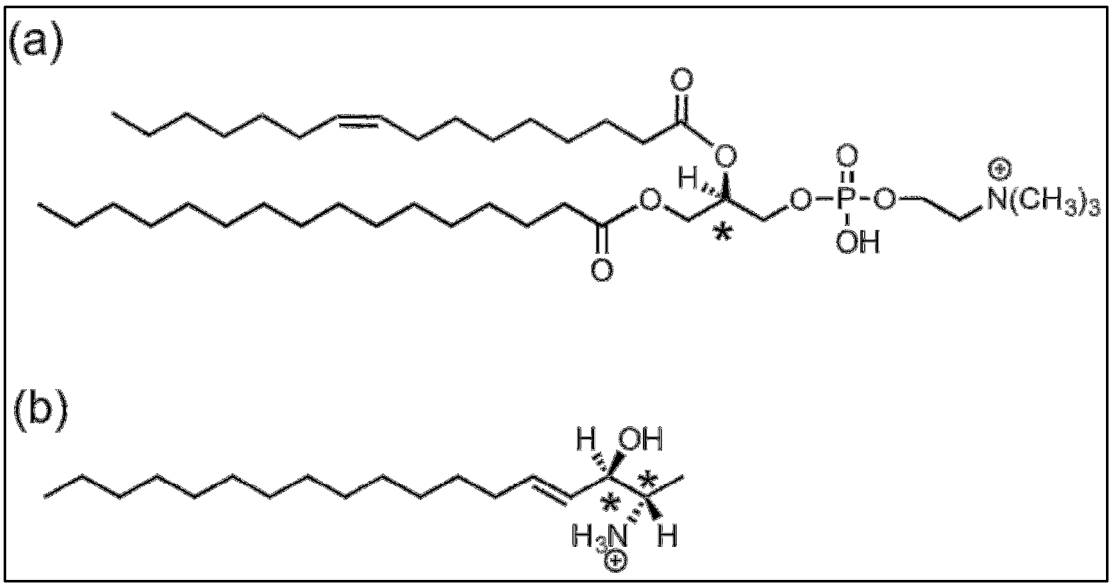

Figure 4: Two examples of chiral lipids (a) PC 16:1/18:0 and (b) 1-deoxysphingosine. The chiral centres have been marked with an asterisk.

MS based analyses

The surge in lipidomics over the past 10 years has been driven, in part, by MS based analyses. Unfortunately, many of these MS tools are by themselves 'chirally blind' and unable to distinguish between *R*- and *S*- enantiomers or determine an enatiomeric excess within a sample. Part of the problem is that both enantiomeric forms of the molecule ionize with similar efficiency, and the activation methods used for tandem MS measurements, such as collisional activation, are generally insensitive to the chirality of the precursor ion, even when using a chiral collision gas. For example, a recent study of the fragmentation of protonated amino acids using high-energy CID (with center-of-mass collision energies up to 1 keV) employing chiral collision gasses yielded no enantiomer-specific fragmentation patterns [80]. Combining MS with laser based spectroscopic approaches has shown some promise; however, most techniques that can differentiate enantiomers are focused towards small molecules. Correlated photoelectron-photoion coincidence experiments using circularly polarized laser light can distinguish mixtures of limonene and camphor [81]. Extending this technique to larger biomolecules however, may prove

problematic due to the fine vibrational structure being obscured by the additional vibrational modes of the larger biomolecule. Microwave rotational spectroscopy also provides good distinction between *R*- and *S*- enantiomers [82], but again, these studies are predominantly targeted towards small molecules and are yet to be targeted towards lipids.

Separation Techniques

GC and HPLC

One route to overcome some of the shortfalls associated with shotgun lipidomics is to employ some degree of isomer separation prior to either ionization or mass analysis. Chromatographic separation of chiral lipids has seen arguably the most widespread deployment, with separation of enantiomeric lipids by GC and HPLC using chiral columns demonstrated since the 1980s [83, 84], and these techniques can be coupled to mass spectrometry to provide enantiomer separation prior to ionization [30, 85]. Another approach that has been effective for peptide separation is an electrophoretic method providing pre-source separation on a very short time scale, however again this is yet to be applied to lipid analysis [86].

IMS

IMS-MS is quickly becoming a key tool for profiling complex lipid mixtures by mass spectrometry and can be considered to be similar to chromatographic separation, but deployed post-ionization rather than before ionization. In principle, the stereochemistry of a gas-phase ion will influence the overall geometry, thus influencing the CCS. This has been applied with good success to separate different lipid classes by their differing mobilities [34, 87–89]. By doping a chiral auxiliary (such as the enantiomerically pure isomers of 2-butanol) into the drift gas, some discrimination between enantiomers can be shown in a drift tube ion mobility device [90, 91]. Recently, IMS has been deployed to separate the ceramides d18:1/18:0(2S-OH) and d18:1/18:0(2R-OH) in positive ion mode as sodium adducts [35]. Interestingly, in this study, no separation between enantiomers was observed in

negative ion mode, indicating that the binding of the sodium counter ion influences the structure of the ions and thus their mobility.

Spectroscopic techniques

In the solution phase, the main tool used to determine enantiomeric excess is CD spectroscopy. This is a technique where the amount of light absorbed by a chiral molecule is measured as a function of the incident light polarization. A chiral sample will absorb left- and right-circularly polarized light in differing amounts, and it is the difference in absorption between these two polarizations that is used to quantify the CD response of a sample. This approach is routinely employed for probing the secondary structure of proteins, and binding of lipids to proteins [92], however the sample must be pure and solvent absorption effects must be taken into account when interpreting the resulting CD spectrum. An extension of this approach is Vibrational Circular Dichroism (VCD) which has been used to probe the chirality of glycerophospholipids, exploiting the VCD activity arising from the interaction of the two carbonyl groups on the fatty acyl chains [93].

Of the aforementioned techniques, only a few have been directly applied to enantiomeric profiling of lipids, with the principle approach employed by most researchers being either GC or HPLC separation. While chromatographic methods will continue to be a mainstay of lipid analysis, advances in the specificity of other approaches such as ion mobility and spectroscopic detection hold the promise of being able to determine enantiomeric excess of a sample on short time scales.

Conclusions

The push to characterize lipid structure has accelerated significantly in the last 4-5 years. In that time there have been significant improvements in the separation of lipid isomers and the development of several novel and innovative ion-activation techniques.

Despite these advances there are several things to keep in mind. First, many of the techniques have only been demonstrated using "hand-picked" examples of lipid isomers. These isomers are chosen to maximize structural difference and hence technical success, but they are often not common isomers found in biological samples. Therefore, further work is required to determine how successful they will be in identifying the plethora of lipid isomers found in real-world samples. Second, quantitative capacity has only been described for a small number of the techniques discussed above. Finally, further work is required if the determination of stereochemistry/chirality is to be incorporated into lipidomic workflows.

It appears unlikely that any one technique will be the panacea for complete structural analysis of lipids. Nevertheless, the use of advanced separation techniques (HPLC, IMS or both) to facilitate quantification and simplify spectral complexity followed by one of the novel ion activation techniques, e.g. EIEIO or OzID for structural characterization, shows real promise in achieving true molecular level lipidomic analyses (Figure 5).

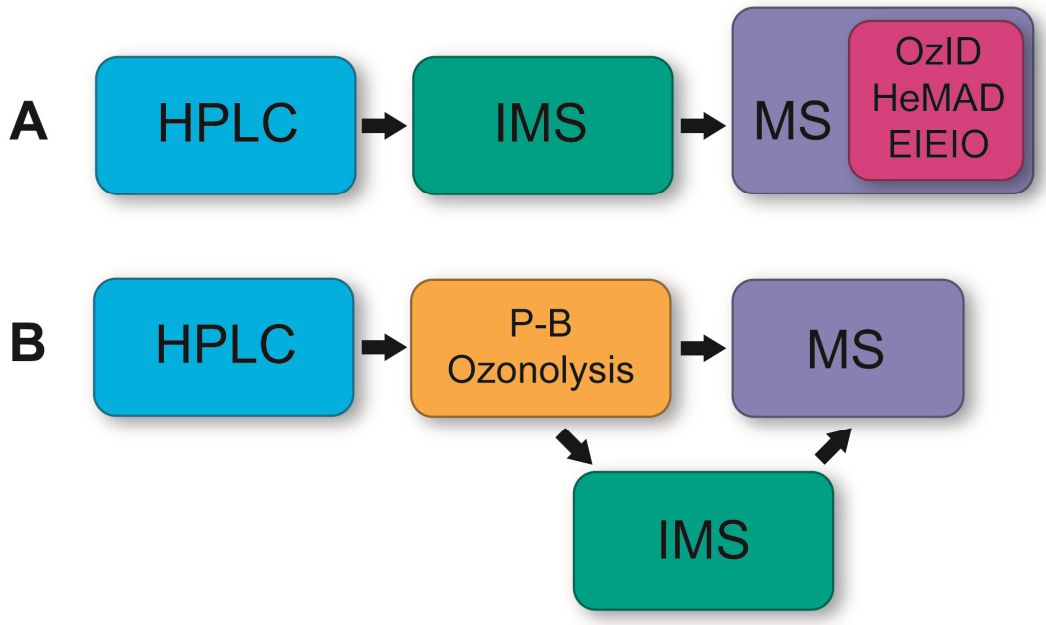

Figure 5: Two examples of possible combination approaches for the near-complete structural elucidation of lipids. (A) links two separation techniques, high performance liquid chromatography (HPLC) and ion mobility spectrometry (IMS)

with ion activation methods for lipid structural characterisation, including: electron-impact excitation of ions from organics (EIEIO), ozone-induced dissociation (OzID), or helium metastable atom-activated dissociation (HeMAD). (B) joins HPLC separation with in-line ozonolysis or the Paternò-Büchi reaction (P-B) for structural characterisation, with an optional additional separation by IMS separation before mass detection.

References

1. Spener F, Lagarde M, Géloên A, Record M (2003) Editorial: What is lipidomics? Eur J Lipid Sci Technol 105:481–482. doi: 10.1002/ejlt.200390101

2. Blanksby SJ, Mitchell TW (2010) Advances in mass spectrometry for lipidomics. Annu Rev Anal Chem Palo Alto Calif 3:433–465. doi: 10.1146/annurev.anchem.111808.073705

3. Brown SHJ, Mitchell TW, Blanksby SJ (2011) Analysis of unsaturated lipids by ozone-induced dissociation. Biochim Biophys Acta BBA - Mol Cell Biol Lipids 1811:807–817. doi: 10.1016/j.bbalip.2011.04.015

4. Brown SHJ, Mitchell TW, Oakley AJ, Pham HT, Blanksby SJ (2012) Time to Face the Fats: What Can Mass Spectrometry Reveal about the Structure of Lipids and Their Interactions with Proteins? J Am Soc Mass Spectrom 23:1441–1449. doi: 10.1007/s13361-012-0410-2

5. Mitchell TW, Brown SHJ, Blanksby SJ (2012) Structural Lipidomics. In: Ekroos K (ed) Lipidomics. Wiley-VCH Verlag GmbH & Co. KGaA, pp 99–128

6. Standal IB, Axelson DE, Aursand M (2009) Differentiation of Fish Oils According to Species by 13C-NMR Regiospecific Analyses of Triacyglycerols. J Am Oil Chem Soc 86:401–407. doi: 10.1007/s11746-009-1370-y

7. Ruiz-Lopez N, Stubhaug I, Ipharraguerre I, Rimbach G, Menoyo D (2015) Positional Distribution of Fatty Acids in Triacylglycerols and Phospholipids from Fillets of Atlantic Salmon (Salmo Salar) Fed Vegetable and Fish Oil Blends. Mar Drugs 13:4255–4269. doi: 10.3390/md13074255

8. Jensen NJ, Tomer KB, Gross ML (1987) FAB MS/MS for phosphatidylinositol, -glycerol, -ethanolamine and other complex phospholipids. Lipids 22:480–489.

9. Pittenauer E, Allmaier G (2009) The renaissance of high-energy CID for structural elucidation of complex lipids: MALDI-TOF/RTOF-MS of alkali cationized triacylglycerols. J Am Soc Mass Spectrom 20:1037–1047. doi: 10.1016/j.jasms.2009.01.009

10. Kubo A, Satoh T, Itoh Y, Hashimoto M, Tamura J, Cody RB (2012) Structural Analysis of Triacylglycerols by Using a MALDI-TOF/TOF System with Monoisotopic Precursor Selection. J Am Soc Mass Spectrom 24:684–689. doi: 10.1007/s13361-012-0513-9

11. Hsu F-F, Turk J (2009) Electrospray Ionization with Low-energy Collisionally Activated Dissociation Tandem Mass Spectrometry of Glycerophospholipids: Mechanisms of Fragmentation and Structural Characterization. J Chromatogr B Analyt Technol Biomed Life Sci 877:2673–2695. doi: 10.1016/j.jchromb.2009.02.033

12. Ekroos K, Ejsing CS, Bahr U, Karas M, Simons K, Shevchenko A (2003) Charting molecular composition of phosphatidylcholines by fatty acid scanning and ion trap MS3 fragmentation. J Lipid Res 44:2181–2192. doi: 10.1194/jlr.D300020-JLR200

13. Hsu F-F, Turk J (2010) Electrospray Ionization Multiple-stage Linear Ion-trap Mass Spectrometry for Structural Elucidation of Triacylglycerols: Assignment of Fatty Acyl Groups on the Glycerol Backbone and Location of Double Bonds. J Am Soc Mass Spectrom 21:657–669. doi: 10.1016/j.jasms.2010.01.007

14. Hsu F-F, Turk J (2008) Structural characterization of unsaturated glycerophospholipids by multiple-stage linear ion-trap mass spectrometry with electrospray ionization. J Am Soc Mass Spectrom 19:1681–1691. doi: 10.1016/j.jasms.2008.07.023

15. Pham HT, Maccarone AT, Thomas MC, Campbell JL, Mitchell TW, Blanksby SJ (2014) Structural characterization of glycerophospholipids by combinations of ozone- and collision-induced dissociation mass spectrometry: the next step towards “top-down” lipidomics. Analyst 139:204–214. doi: 10.1039/C3AN01712E

16. Marshall DL, Pham HT, Bhujel M, Chin JSR, Yew JY, Mori K, Mitchell TW, Blanksby SJ (2016) Sequential Collision- and Ozone-Induced Dissociation Enables Assignment of Relative Acyl Chain Position in Triacylglycerols. Anal Chem 88:2685–2692. doi: 10.1021/acs.analchem.5b04001

17. Kozlowski R, Mitchell T, Blanksby S (2015) Separation and identification of phosphatidylcholine regioisomers by combining liquid chromatography with a fusion of collision-and ozone-induced dissociation. Eur J Mass Spectrom 21:191. doi: 10.1255/ejms.1300

18. Kozlowski RL, Campbell JL, Mitchell TW, Blanksby SJ (2015) Combining liquid chromatography with ozone-induced dissociation for the separation and identification of phosphatidylcholine double bond isomers. Anal Bioanal Chem 1–12. doi: 10.1007/s00216-014-8430-3

19. Kozlowski RL, Mitchell TW, Blanksby SJ (2015) A rapid ambient ionization-mass spectrometry approach to monitoring the relative abundance of isomeric glycerophospholipids. Sci Rep. doi: 10.1038/srep09243

20. Jones JW, Thompson CJ, Carter CL, Kane MA (2015) Electron-induced dissociation (EID) for structure characterization of glycerophosphatidylcholine: determination of double-bond positions and localization of acyl chains. J Mass Spectrom 50:1327–1339. doi: 10.1002/jms.3698

21. Maccarone AT, Duldig J, Mitchell TW, Blanksby SJ, Duchoslav E, Campbell JL (2014) Characterization of acyl chain position in unsaturated phosphatidylcholines using differential mobility-mass spectrometry. J Lipid Res 55:1668–1677. doi: 10.1194/jlr.M046995

22. Baba T, Campbell JL, Le Blanc JCY, Hager JW, Thomson BA (2015) Electron Capture Dissociation in a Branched Radio-Frequency Ion Trap. Anal Chem 87:785–792. doi: 10.1021/ac503773y

23. Baba T, Campbell JL, LeBlanc JCY, Baker PRS (2016) In-depth Sphingomyelin Characterization using Electron Impact Excitation of Ions from Organics (EIEIO) and Mass Spectrometry. J Lipid Res jlr.M067199. doi: 10.1194/jlr.M067199

24. Campbell JL, Baba T (2015) Near-Complete Structural Characterization of Phosphatidylcholines Using Electron Impact Excitation of Ions from Organics. Anal Chem 87:5837–5845. doi: 10.1021/acs.analchem.5b01460

25. Nakanishi H, Iida Y, Shimizu T, Taguchi R (2010) Separation and quantification of sn-1 and sn-2 fatty acid positional isomers in phosphatidylcholine by RPLC-ESIMS/MS. J Biochem (Tokyo) 147:245–256. doi: 10.1093/jb/mvp171

26. Nagai T, Gotoh N, Mizobe H, Yoshinaga K, Kojima K, Matsumoto Y, Wada S (2011) Rapid separation of triacylglycerol positional isomers binding two saturated Fatty acids using octacocyl silylation column. J Oleo Sci 60:345–350.

27. Nagai T, Watanabe N, Yoshinaga K, Mizobe H, Kojima K, Kuroda I, Odanaka Y, Saito T, Beppu F, Gotoh N (2015) Abundances of Triacylglycerol Positional Isomers and Enantiomers Comprised of a Dipalmitoylglycerol Backbone and Short- or Medium-chain Fatty Acids in Bovine Milk Fat. J Oleo Sci 64:943–952. doi: 10.5650/jos.ess15040

28. Lísa M, Holčapek M (2013) Characterization of Triacylglycerol Enantiomers Using Chiral HPLC/APCI-MS and Synthesis of Enantiomeric Triacylglycerols. Anal Chem 85:1852–1859. doi: 10.1021/ac303237a

29. Rezanka T, Kolouchová I, Cejková A, Cajthaml T, Sigler K (2013) Identification of regioisomers and enantiomers of triacylglycerols in different yeasts using reversed- and chiral-phase LC-MS. J Sep Sci 36:3310–3320. doi: 10.1002/jssc.201300657

30. Řezanka T, Sigler K (2014) Separation of Enantiomeric Triacylglycerols by Chiral-Phase HPLC. Lipids 49:1251–1260. doi: 10.1007/s11745-014-3959-7

31. Granafei S, Losito I, Palmisano F, Cataldi TR, I (2015) Identification of isobaric lyso-phosphatidylcholines in lipid extracts of gilthead sea bream (Sparus aurata) fillets by hydrophilic interaction liquid chromatography coupled to high-resolution Fourier-transform mass spectrometry. Anal Bioanal Chem 407:6391–6404. doi: http://dx.doi.org.ezproxy.uow.edu.au/10.1007/s00216-015-8671-9

32. Lísa M, Cífková E, Holčapek M (2011) Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography–mass spectrometry. J Chromatogr A 1218:5146–5156. doi: 10.1016/j.chroma.2011.05.081

33. Lee JW, Nagai T, Gotoh N, Fukusaki E, Bamba T (2014) Profiling of regioisomeric triacylglycerols in edible oils by supercritical fluid chromatography/tandem mass spectrometry. J Chromatogr B 966:193–199. doi: 10.1016/j.jchromb.2014.01.040

34. Groessl M, Graf S, Knochenmuss R (2015) High resolution ion mobility-mass spectrometry for separation and identification of isomeric lipids. Analyst. doi: 10.1039/C5AN00838G

35. Kyle JE, Zhang X, Weitz KK, Monroe ME, Ibrahim YM, Moore RJ, Cha J, Sun X, Lovelace ES, Wagoner J, Polyak SJ, Metz TO, Dey SK, Smith RD, Burnum-Johnson KE, Baker ES (2016) Uncovering biologically significant lipid isomers with liquid chromatography, ion mobility spectrometry and mass spectrometry. Analyst. doi: 10.1039/C5AN02062J

36. Krylov EV, Nazarov EG, Miller RA (2007) Differential mobility spectrometer: Model of operation. Int J Mass Spectrom 266:76–85. doi: 10.1016/j.ijms.2007.07.003

37. Šala M, Lísa M, Campbell JL, Holčapek M (2016) Determination of triacylglycerol regioisomers using differential mobility spectrometry. Rapid Commun Mass Spectrom 30:256–264. doi: 10.1002/rcm.7430

38. Cheng C, Gross ML, Pittenauer E (1998) Complete Structural Elucidation of Triacylglycerols by Tandem Sector Mass Spectrometry. Anal Chem 70:4417–4426. doi: 10.1021/ac9805192

39. Jensen NJ, Tomer KB, Gross ML (1986) Fast atom bombardment and tandem mass spectrometry of phosphatidylserine and phosphatidylcholine. Lipids 21:580–588.

40. Tomer KB, Crow FW, Gross ML (1983) Location of double-bond position in unsaturated fatty acids by negative ion MS/MS. J Am Chem Soc 105:5487–5488. doi: 10.1021/ja00354a055

41. Bollinger JG, Thompson W, Lai Y, Oslund RC, Hallstrand TS, Sadilek M, Turecek F, Gelb MH (2010) Improved Sensitivity Mass Spectrometric Detection of Eicosanoids by Charge Reversal Derivatization. Anal Chem 82:6790–6796. doi: 10.1021/ac100720p

42. Yang K, Dilthey BG, Gross RW (2013) Identification and Quantitation of Fatty Acid Double Bond Positional Isomers: A Shotgun Lipidomics Approach Using Charge-Switch Derivatization. Anal Chem 85:9742–9750. doi: 10.1021/ac402104u

43. Wang M, Han RH, Han X (2013) Fatty Acidomics: Global Analysis of Lipid Species Containing a Carboxyl Group with a Charge-Remote Fragmentation-Assisted Approach. Anal Chem 85:9312–9320. doi: 10.1021/ac402078p

44. Thomas MC, Mitchell TW, Harman DG, Deeley JM, Nealon JR, Blanksby SJ (2008) Ozone-Induced Dissociation: Elucidation of Double Bond Position within Mass-Selected Lipid Ions. Anal Chem 80:303–311. doi: 10.1021/ac7017684

45. Pham HT, Maccarone AT, Campbell JL, Mitchell TW, Blanksby SJ (2013) Ozone-Induced Dissociation of Conjugated Lipids Reveals Significant Reaction Rate Enhancements and Characteristic Odd-Electron Product Ions. J Am Soc Mass Spectrom 24:286–296. doi: 10.1007/s13361-012-0521-9

46. Sun C, Zhao Y-Y, Curtis JM (2013) The direct determination of double bond positions in lipid mixtures by liquid chromatography/in-line ozonolysis/mass spectrometry. Anal Chim Acta 762:68–75. doi: 10.1016/j.aca.2012.12.012

47. Sun C, Black BA, Zhao Y-Y, Gänzle MG, Curtis JM (2013) Identification of Conjugated Linoleic Acid (CLA) Isomers by Silver Ion-Liquid Chromatography/In-line Ozonolysis/Mass Spectrometry (Ag+-LC/O3-MS). Anal Chem 85:7345–7352. doi: 10.1021/ac401242z

48. Sun C, Zhao Y-Y, Curtis JM (2015) Characterization of Phospholipids by Two-Dimensional Liquid Chromatography Coupled to In-line Ozonolysis–Mass Spectrometry. J Agric Food Chem 63:1442–1451. doi: 10.1021/jf5049595

49. Ma X, Chong L, Tian R, Shi R, Hu TY, Ouyang Z, Xia Y (2016) Identification and quantitation of lipid C=C location isomers: A shotgun lipidomics approach enabled by photochemical reaction. Proc Natl Acad Sci 113:2573–2578. doi: 10.1073/pnas.1523356113

50. Ma X, Xia Y (2014) Pinpointing Double Bonds in Lipids by Paternò-Büchi Reactions and Mass Spectrometry. Angew Chem Int Ed 53:2592–2596. doi: 10.1002/anie.201310699

51. Stinson CA, Xia Y (2016) A method of coupling the Paternò–Büchi reaction with direct infusion ESI-MS/MS for locating the CC bond in glycerophospholipids. Analyst. doi: 10.1039/C6AN00015K

52. Liang X, Liu J, LeBlanc Y, Covey T, Ptak AC, Brenna JT, McLuckey SA (2007) Electron transfer dissociation of doubly sodiated glycerophosphocholine lipids. J Am Soc Mass Spectrom 18:1783–1788. doi: 10.1016/j.jasms.2007.07.013

53. Yoo HJ, Håkansson K (2010) Determination of Double Bond Location in Fatty Acids by Manganese Adduction and Electron Induced Dissociation. Anal Chem 82:6940–6946. doi: 10.1021/ac101217x

54. Deimler RE, Sander M, Jackson GP (2015) Radical-induced fragmentation of phospholipid cations using metastable atom-activated dissociation mass spectrometry (MAD-MS). Int J Mass Spectrom 390:178–186. doi: 10.1016/j.ijms.2015.08.009

55. Pham HT, Ly T, Trevitt AJ, Mitchell TW, Blanksby SJ (2012) Differentiation of Complex Lipid Isomers by Radical-Directed Dissociation Mass Spectrometry. Anal Chem 84:7525–7532. doi: 10.1021/ac301652a

56. Pham HT, Trevitt AJ, Mitchell TW, Blanksby SJ (2013) Rapid differentiation of isomeric lipids by photodissociation mass spectrometry of fatty acid derivatives. Rapid Commun Mass Spectrom 27:805–815. doi: 10.1002/rcm.6503

57. Pham HT, Prendergast MB, Dunstan CW, Trevitt AJ, Mitchell TW, Julian RR, Blanksby SJ (2015) Dissociation of proton-bound complexes reveals geometry and arrangement of double bonds in unsaturated lipids. Int J Mass Spectrom 390:170–177. doi: 10.1016/j.ijms.2015.07.006

58. Ashraf-Khorassani M, Isaac G, Rainville P, Fountain K, Taylor LT (2015) Study of UltraHigh Performance Supercritical Fluid Chromatography to measure free fatty acids with out fatty acid ester preparation. J Chromatogr B 997:45–55. doi: 10.1016/j.jchromb.2015.05.031

59. Steiner R, Mostafa E, Othman A, Arenz C, Maccarone AT, Poad BLJ, Blanksby SJ, Eckardstein A von, Hornemann T (2016) Elucidating the chemical structure of native 1-deoxysphingosine. J Lipid Res jlr.M067033. doi: 10.1194/jlr.M067033

60. Alves SP, Tyburczy C, Lawrence P, Bessa RJB, Brenna JT (2011) Acetonitrile covalent adduct chemical ionization tandem mass spectrometry of non-methylene-interrupted pentaene fatty acid methyl esters. Rapid Commun Mass Spectrom RCM 25:1933–1941. doi: 10.1002/rcm.5065

61. Lawrence P, Brenna JT (2006) Acetonitrile Covalent Adduct Chemical Ionization Mass Spectrometry for Double Bond Localization in Non-Methylene-Interrupted Polyene Fatty Acid Methyl Esters. Anal Chem 78:1312–1317. doi: 10.1021/ac0516584

62. Michaud AL, Yurawecz MP, Delmonte P, Corl BA, Bauman DE, Brenna JT (2003) Identification and Characterization of Conjugated Fatty Acid Methyl Esters of Mixed Double Bond Geometry by Acetonitrile Chemical Ionization Tandem Mass Spectrometry. Anal Chem 75:4925–4930. doi: 10.1021/ac034221+

63. Michaud AL, Diau G-Y, Abril R, Brenna JT (2002) Double bond localization in minor homoallylic fatty acid methyl esters using acetonitrile chemical ionization tandem mass spectrometry. Anal Biochem 307:348–360. doi: 10.1016/S0003-2697(02)00037-4

64. Van Pelt CK, Carpenter BK, Brenna JT (1999) Studies of structure and mechanism in acetonitrile chemical ionization tandem mass spectrometry of polyunsaturated fatty acid methyl esters. J Am Soc Mass Spectrom 10:1253–1262. doi: 10.1016/S1044-0305(99)00109-9

65. Van Pelt CK, Brenna JT (1999) Acetonitrile Chemical Ionization Tandem Mass Spectrometry To Locate Double Bonds in Polyunsaturated Fatty Acid Methyl Esters. Anal Chem 71:1981–1989. doi: 10.1021/ac981387f

66. Xu Y, Brenna JT (2007) Atmospheric Pressure Covalent Adduct Chemical Ionization Tandem Mass Spectrometry for Double Bond Localization in Monoene- and Diene-Containing Triacylglycerols. Anal Chem 79:2525–2536. doi: 10.1021/ac062055a

67. Háková E, Vrkoslav V, Míková R, Schwarzová-Pecková K, Bosáková Z, Cvačka J (2015) Localization of double bonds in triacylglycerols using high-performance liquid chromatography/atmospheric pressure chemical ionization ion-trap mass spectrometry. Anal Bioanal Chem 407:5175–5188. doi: 10.1007/s00216-015-8537-1

68. James AT, Martin AJP (1952) Gas-liquid partition chromatography: the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. Biochem J 50:679–690.

69. Chatgilialoglu C, Ferreri C, Melchiorre M, Sansone A, Torreggiani A (2014) Lipid geometrical isomerism: from chemistry to biology and diagnostics. Chem Rev 114:255–284. doi: 10.1021/cr4002287

70. Tsiafoulis CG, Skarlas T, Tzamaloukas O, Miltiadou D, Gerothanassis IP (2014) Direct nuclear magnetic resonance identification and quantification of geometric isomers of conjugated linoleic acid in milk lipid fraction without derivatization steps: Overcoming sensitivity and resolution barriers. Anal Chim Acta 821:62–71. doi: 10.1016/j.aca.2014.03.010

71. Melchiorre M, Ferreri C, Tinti A, Chatgilialoglu C, Torreggiani A (2015) A Promising Raman Spectroscopy Technique for the Investigation of trans and cis Cholesteryl Ester Isomers in Biological Samples. Appl Spectrosc 69:613–622. doi: 10.1366/14-07706

72. Poad BLJ, Pham HT, Thomas MC, Nealon JR, Campbell JL, Mitchell TW, Blanksby SJ (2011) Ozone-induced dissociation on a modified tandem linear ion-trap: Observations of different reactivity for isomeric lipids. J Am Soc Mass Spectrom 21:1989–1999. doi: 10.1016/j.jasms.2010.08.011

73. Bird SS, Marur VR, Stavrovskaya IG, Kristal BS (2012) Separation of Cis–Trans Phospholipid Isomers Using Reversed Phase LC with High Resolution MS Detection. Anal Chem 84:5509–5517. doi: 10.1021/ac300953j

74. Damen CWN, Isaac G, Langridge J, Hankemeier T, Vreeken RJ (2014) Enhanced lipid isomer separation in human plasma using reversed-phase UPLC with ion-mobility / high-resolution MS detection. J Lipid Res jlr.D047795. doi: 10.1194/jlr.D047795

75. Narváez-Rivas M, Zhang Q (2016) Comprehensive untargeted lipidomic analysis using core–shell C30 particle column and high field orbitrap mass spectrometer. J Chromatogr A 1440:123–134. doi: 10.1016/j.chroma.2016.02.054

76. Imai H, Hattori H, Watanabe M (2012) An Improved Method for Analysis of Glucosylceramide Species Having cis-8 and trans-8 Isomers of Sphingoid Bases by LC–MS/MS. Lipids 47:1221–1229. doi: 10.1007/s11745-012-3725-7

77. Ishikawa T, Imai H, Maki K-Y (2013) Development of an LC–MS/MS Method for the Analysis of Free Sphingoid Bases Using 4-Fluoro-7-nitrobenzofurazan (NBD-F). Lipids 49:295–304. doi: 10.1007/s11745-013-3871-6

78. Tan H-H, Makino A, Sudesh K, Greimel P, Kobayashi T (2012) Spectroscopic evidence for the unusual stereochemical configuration of an endosome-specific lipid. Angew Chem Int Ed Engl 51:533–535. doi: 10.1002/anie.201106470

79. Labuta J, Ishihara S, Šikorský T, Futera Z, Shundo A, Hanyková L, Burda JV, Ariga K, Hill JP (2013) NMR spectroscopic detection of chirality and enantiopurity in referenced systems without formation of diastereomers. Nat Commun 4:2188. doi: 10.1038/ncomms3188

80. Kulyk K, Rebrov O, Stockett MH, Alexander JD, Zettergren H, Schmidt HT, Thomas RD, Cederquist H, Larsson M (2015) High-energy collisions of protonated enantiopure amino acids with a chiral target gas. Int J Mass Spectrom 388:59–64. doi: 10.1016/j.ijms.2015.08.010

81. Fanood MMR, Ram NB, Lehmann CS, Powis I, Janssen MHM (2015) Enantiomer-specific analysis of multi-component mixtures by correlated electron imaging-ion mass spectrometry. Nat Commun 6:7511. doi: 10.1038/ncomms8511

82. Shubert VA, Schmitz D, Pérez C, Medcraft C, Krin A, Domingos SR, Patterson D, Schnell M (2016) Chiral Analysis Using Broadband Rotational Spectroscopy. J Phys Chem Lett 7:341–350. doi: 10.1021/acs.jpclett.5b02443

83. Christie, W W (1992) The Chromatographic Resolution of Chiral Lipids. The Oily Press

84. Laakso P, Christie WW (1990) Chromatographic resolution of chiral diacylglycerol derivatives: potential in the stereospecific analysis of triacyl-sn-glycerols. Lipids 25:349–353.

85. Ito J, Nakagawa K, Kato S, Hirokawa T, Kuwahara S, Nagai T, Miyazawa T (2015) Direct separation of the diastereomers of phosphatidylcholine hydroperoxide bearing 13-hydroperoxy-9Z,11E-octadecadienoic acid using chiral stationary phase high-performance liquid chromatography. J Chromatogr A 1386:53–61. doi: 10.1016/j.chroma.2015.01.080

86. Moini M, Rollman CM (2016) Portable, Battery Operated Capillary Electrophoresis with Optical Isomer Resolution Integrated with Ionization

Source for Mass Spectrometry. J Am Soc Mass Spectrom 27:388–393. doi: 10.1007/s13361-015-1314-8

87. Fenn LS, McLean JA (2008) Biomolecular structural separations by ion mobility-mass spectrometry. Anal Bioanal Chem 391:905–909. doi: 10.1007/s00216-008-1951-x

88. Kliman M, May JC, McLean JA (2011) Lipid Analysis and Lipidomics by Structurally Selective Ion Mobility-Mass Spectrometry. Biochim Biophys Acta 1811:935–945. doi: 10.1016/j.bbalip.2011.05.016

89. Shvartsburg AA, Isaac G, Leveque N, Smith RD, Metz TO (2011) Separation and Classification of Lipids Using Differential Ion Mobility Spectrometry. J Am Soc Mass Spectrom 22:1146–1155. doi: 10.1007/s13361-011-0114-z

90. Dwivedi P, Wu C, Matz LM, Clowers BH, Siems WF, Hill HH (2006) Gas-phase chiral separations by ion mobility spectrometry. Anal Chem 78:8200–8206. doi: 10.1021/ac0608772

91. Enders JR, McLean JA (2009) Chiral and structural analysis of biomolecules using mass spectrometry and ion mobility-mass spectrometry. Chirality 21 Suppl 1:E253-264. doi: 10.1002/chir.20806

92. Sommer LAM, Schaad M, Dames SA (2013) NMR- and Circular Dichroism-monitored Lipid Binding Studies Suggest a General Role for the FATC Domain as Membrane Anchor of Phosphatidylinositol 3-Kinase-related Kinases (PIKK). J Biol Chem 288:20046–20063. doi: 10.1074/jbc.M113.467233

93. Taniguchi T, Manai D, Shibata M, Itabashi Y, Monde K (2015) Stereochemical analysis of glycerophospholipids by vibrational circular dichroism. J Am Chem Soc 137:12191–12194. doi: 10.1021/jacs.5b05832

Acknowledgements

The authors acknowledge financial support from the Australian Research Council (DP150101715 and LP140100711). TWM is supported by an Australia Research Council Future Fellowship (FT110100249).