UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Enhancing the Characterisation of the Phosphatidylinositols

by David Gertner

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Under the supervision of:
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University of Technology Sydney
Faculty of Science

10 / 2023

Certificate of Original Authorship:

I, *David Gertner*, declare that this thesis is submitted in fulfilment of the requirements for the award of *Doctor of Philosophy*, in the *Faculty of Science* at the *University of Technology Sydney.*

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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Abstract:

The phosphatidylinositols are an interesting and diverse class of phospholipid. These molecules are ubiquitous throughout known life and are involved in almost every aspect of a cell's life and death. The roles they play are understood to be varied, and certain species are even recognised as some of the most multifunctional molecules throughout biology. Stemming from their many precise activities, these lipids are of significant interest when attempting to understand the status and internal processes of biological systems. Consequently, they are also of significant interest as biomarkers, as deviations in their lipidomic profiles are associated with the development of many serious pathologies. The phosphatidylinositols and their derivatives are additionally recognised for their nutritional and therapeutic properties and ability to induce physiological or pathological responses once consumed; thus, the interests in characterising their profile are as diverse as their function. However, despite this broad interest, the methods and tools used to characterise these lipids are limited, as they provide only a shallow glimpse at the distribution of the molecular species present. To enhance the future characterisation of these molecules, a re-evaluation of current lipidomic protocols may be necessary to favour their analysis. This thesis outlines the of steps taken to this end, where a series of advancements were made. These include the publication of an *in-silico* fragmentation spectra generator, which was developed to accurately predict the fragmentation patterns for thousands of hypothetical lyso-phosphatidylinositol, phosphatidylinositol and phosphatidylinositol phosphate species, in addition to 84 other lipid classes, covering 136 adducts. These predicted spectra are currently the most comprehensive and accurate of their type and enabled the identification of species of interest in the following chapters. Various novel analysis conditions for the phosphatidylinositols were also evaluated and compared against literature methods. This experimentation revealed that current lipidomic methods inadvertently suppress the detection of phosphatidylinositols and that a 0.25 mM ammonium fluoride buffer comparatively enhanced the signal-to-noise ratio for these species by 38-fold on average. Evaluation of chromatographic conditions culminated in the production of a set of analysis conditions based on an ammonium carbonate buffer, which was suited for the simultaneous analysis of the lyso-phosphatidylinositols, phopshatidylinositols and phosphatidylinositol phosphates, in addition to an unanticipated class of oxidised phosphatidylinositol species. In total 148 unique molecular species were identified, and principal component analysis of these species enabled the differentiation of four cultivars of canola based exclusively on their phosphatidylinositol-lipidome. To date, this represents the most comprehensive characterisation of phosphatidylinositol species in any matrix.

List of papers/publications included:

Lipid Spectra Generator: a simple script for the generation of accurate in-silico lipid fragmentation spectra

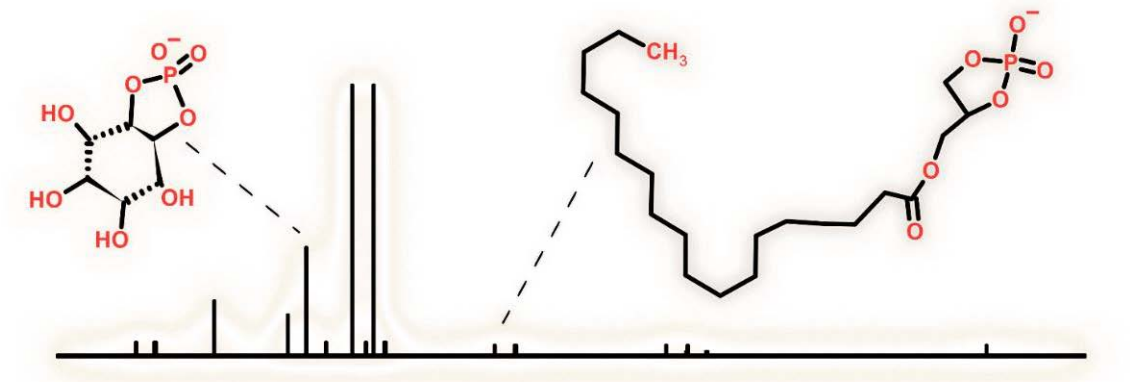
Gertner, D. S., et al. (2023). "Lipid Spectrum Generator: A Simple Script for the Generation of Accurate In Silico Lipid Fragmentation Spectra." Analytical Chemistry **95**(5): 2909-2916. https://doi.org/10.1021/acs.analchem.2c04518

Enhancing Coverage of Phosphatidylinositol Species in Canola Through Specialised Liquid Chromatography-Mass Spectrometry Buffer Conditions

Gertner, D. S., et al. (2021). "Enhancing Coverage of Phosphatidylinositol Species in Canola Through Specialised Liquid Chromatography-Mass Spectrometry Buffer Conditions." Journal of Chromatography A **1637**: 461860. https://doi.org/10.1016/j.chroma.2020.461860

Optimisation of Chromatographic Buffer Conditions for the Simultaneous Analysis of Phosphatidylinositol and Phosphatidylinositol Phosphate Species in Canola

Gertner, D. S., et al. (2023). "Optimization of chromatographic buffer conditions for the simultaneous analysis of phosphatidylinositol and phosphatidylinositol phosphate species in canola." Journal of Separation Science: 2300165. https://doi.org/10.1002/jssc.202300165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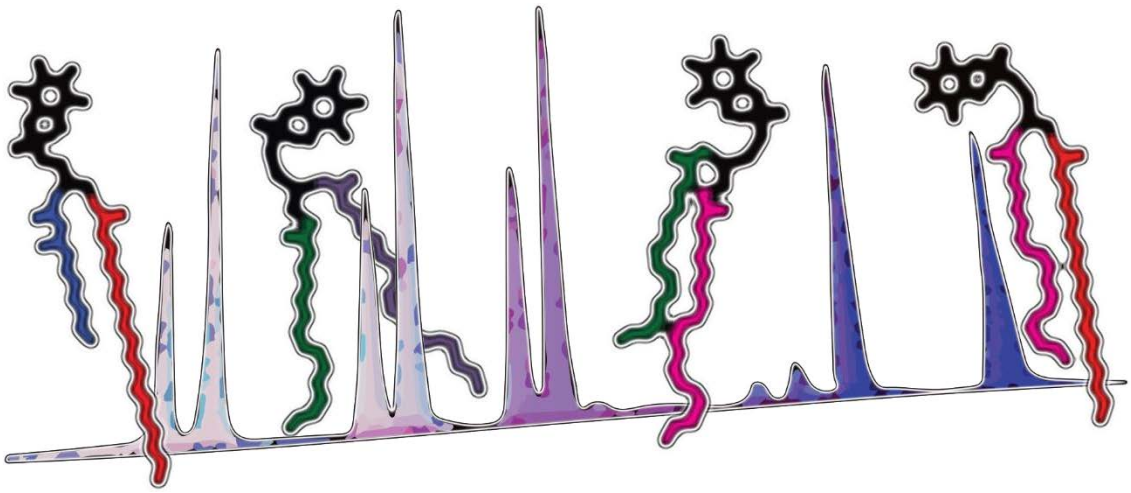

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List of Abbreviations:

PA – Phosphatidic acid
LPA – Lyso Phosphatidic acid
PE – Phosphatidylethanolamine
LPE – Lyso Phosphatidylethanolamine
PC – Phosphatidylcholine
LPC – Lyso Phosphatidylcholine
PG – Phosphatidylglycerol
LPG – Lyso Phosphatidylglycerol
PI – Phosphatidylinositol
LPI – Lyso Phosphatidylinositol
PIP – Phosphatidylinositol phosphate
PS – Phosphatidylserine
LPS – Lyso Phosphatidylserine
SM – Sphingomyelin
MAG – Monoacylglycerol
DAG – Diacylglycerol
TAG – Triacylglycerol
CL – Cardiolipin
MLCL – Monolyso Cardiolipin
CE – Cholesterol Ester

FA – Fatty Acid
SN – Stereospecific numbering

LLE – Liquid-liquid Extraction
SPE – Solid Phase Extraction
TLC – Thin Layer Chromatography

DDA – Data Dependant Analysis
EIC – Extracted Ion Chromatogram
ESI – Electrospray Ionisation
IMS – Ion Mobility Spectrometry
MS – Mass Spectrometry
TIC – Total Ion Chromatogram
LC – Liquid Chromatography
HILIC – Hydrophilic Interaction Liquid Chromatography
RPLC – Reversed Phase Liquid Chromatography

ACN – Acetonitrile
C – Chloroform
EDTA – Ethylenediamine tetraacetic Acid
GAA – Glacial Acetic Acid
IPA – Isopropyl alcohol
MeOH – Methanol

AmAc – Ammonium Acetate
AmbiC – Ammonium Bicarbonate
AmC – Ammonium Carbonate
AmF – Ammonium Fluoride
AmOH – Ammonium Hydroxide

LOD – Limit of Detection
PCA – Principal Component Analysis
RSD – Relative Standard Deviation

LSG – Lipid Spectrum Generator

HM – Human Milk

Chapter One:

From Lipids to Phosphatidylinositols

Lipids

Lipids are biomolecules that are ubiquitous throughout biology, loosely categorised together due to their hydrophobicity and solubility in organic solvents.[22, 23] These molecules are immense in their diversity and vary greatly in structure and function, which perhaps suggests hydrophobicity is too broad a singular concept to classify molecules. Current estimates suggest that the number of lipid species present in a single matrix range from the tens to hundreds of thousands,[23-26] although a distinction between diversity and randomness should be stressed. The physicochemical properties of lipids, and thus their biological activities, rely on their precise chemical structures, which, therefore, necessitate biological regulation.[27] Hence, rather than resembling a random assortment of molecules, the diversity amongst lipids appears to manifest systematically through the production of highly specific chemical moieties and certain structural arrangements. Examples of lipid structures are provided in Figure 1 and Figure 2. These patterns may, to some extent, distinguish these lipids into individual classes based on the presence of certain chemical groups. Interestingly, many of these classes appear to be conserved across biology, and the cross-over between lipid structures from bacteria to those in mammals is immediately recognisable, demonstrating the crucial nature of certain structures to life in general. Yet, unlike proteins, lipids have no obvious function in isolation, and understanding the reasons behind certain arrangements is a fundamental challenge in biology.[22, 27]

The phospholipids are one such class that have a characteristic structure. They are derived in cells from either the glycerolipids or ceramides to form the glycerophospholipids or phosphosphingolipids, respectively; and these classes are easily distinguishable from their precursors through the presence of a phosphate moiety and polar headgroup, as shown in Figure 2. Due to the presence of this polar headgroup, these lipids simultaneously exhibit some hydrophilic capacity, in addition to their hydrophobic characteristics, which enable them to partake in numerous complex interactions. Most notably, in the context of a cell, these lipids can aggregate together to form a composite structure known as a lipid membrane, which constitute the many barriers that encapsulate and compartmentalise cells. On its own, these membranes are quite convenient for life, as they permit a distinction between interior and exterior environments, not only by location but also in terms of the chemical conditions across the membrane. However, cell membranes are far removed from a set of static walls, and they are, in actuality, fluid structures that are active participants in biological processes, which exhibit physical and chemical properties that arise from the mosaic of their individual constituents.[27-29]

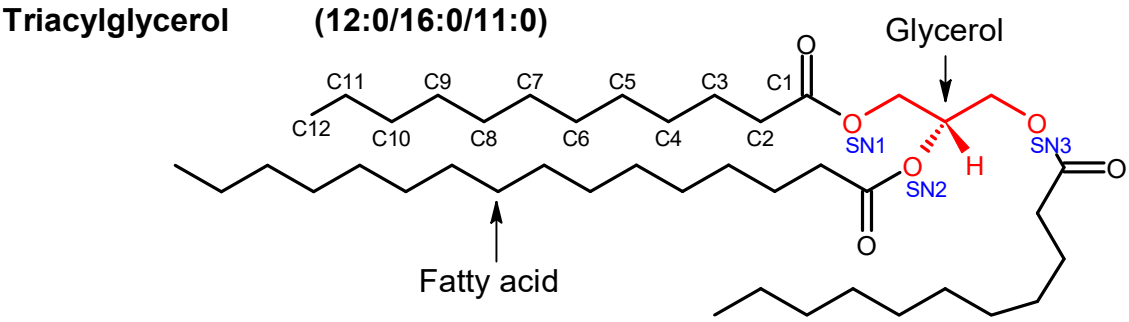

Figure 1 – An example lipid structure from the triacylglycerol class. The triacylglycerols are a sub-class of glycerolipids, which are defined by the presence of a glycerol backbone. Lipids of this class vary through the length, type and positioning of their fatty acid constituents, and the systematic nature of these structures permits a systematic naming convention. The above lipid, Triacylglycerol (12:0/16:0/11:0), is named as such as it contains three fatty acids of length 12, 16 and 11, esterified to the glycerol backbone in order through positions *SN1* to *SN3*. These positions are named in relation to the chiral centre at the middle of the glycerol. The numbers following the carbon chain length indicate that the chains lack any desaturation. Loss of any single fatty acid would produce a lipid of the class diacylglycerol, whereas loss of any two fatty acid constituents would produce a lipid of class monoacylglycerol.

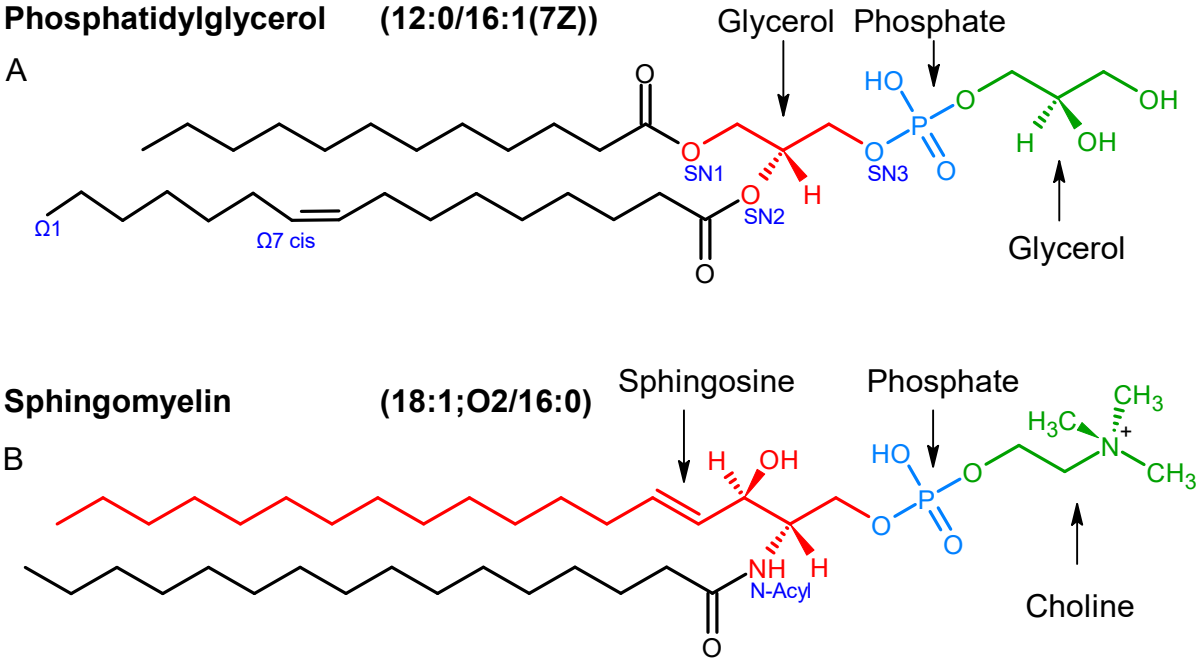

Figure 2 – Example structures of a glycerophospholipid, of sub-class phosphatidylglycerol (A), and a phosphosphingolipid, of sub-class sphingomyelin (B). The similarities between the phosphatidylglycerol and the triacylglycerol shown in Figure 1 are evident. Notice, the *SN2* fatty acid on the phosphatidylglycerol is desaturated. The ceramides are constructed with a sphingoid base, rather than glycerol. Numerous unique sub-classes emerge beyond these shown here, and some additional headgroups are provided in Figure 3.

Membrane curvature is an example of one such physical characteristic, emerging from the geometric arrangement of these lipid constituents. This curvature arises as the relative size difference between the polar headgroup of a phospholipid, in relation to its hydrophobic constituents, endows it with a specific shape, which contributes to the supramolecular structure of the membrane.[28] A simple rendition of this curvature is illustrated in Figure 3 and Figure 4, along with a range of common glycerophospholipid headgroups. Although

conceptually, this may seem like a rather simplistic feature of the membrane, precise control over membrane shape is strictly necessary for cellular function as it directly influences the activities of integral and associated proteins.[27, 28, 30, 31] This relationship arises as many proteins express particular structural motifs that can sense membrane properties, including curvature and thickness,[30] and they often necessitate specific membrane environments for folding, stabilisation and function.[28] Despite this, a living lipid membrane's molecular profile is highly dynamic, and individual lipids are constantly being remodelled through biological processes that change their headgroups and hydrophobic constituents. Naturally, this alters membrane properties such as shape, fluidity and permeability; hence, associated proteins and other membrane features continually adapt and respond by adopting different conformations and arrangements.[28] Thus, through these interactions, the membrane may be understood to be a vibrant place, where the lipid aggregate continuously evolves and cooperates with embedded proteins over vast and variable periods.

However, interactions based on membrane curvature are perhaps more so emergent properties of lipids when compared to the individual interactions that can occur between lipid molecules and proteins. As a result of the aqueous environment that is the cell cytoplasm, or extracellular matrix, phospholipids are oriented such that their hydrophobic moieties are clustered together, whilst their hydrophilic headgroups are exposed above the surface of the membrane. This conformation allows the lipid headgroups to directly interact with their surroundings and extend their chemical influence into these environments, whereupon they may induce highly specific lipid-protein interactions. Lipid-binding domains allow proteins to recognise these chemical moieties, enabling them to behave as signalling molecules with influence over various biological processes, although lipid headgroups may also anchor or recruit proteins to specific compartments and membrane subdomains.[27, 28, 30, 31] This is not to say that lipid-protein interactions are at all deficient of a lipid’s hydrophobic moieties; in fact, the hydrophobic constituents of signalling lipids are known to be crucial in correctly positioning the headgroup above the membrane so it may adequately interact with its associated proteins.[32] Even so, beyond this, particular proteins may interact exclusively with lipid species based on their hydrophobic domain, whether through activation of membrane integral proteins or binding to specific lipid-binding pockets. For these reasons, it is understood that all portions of a phospholipid are crucial to their function as membrane constituents, which justifies the stringent regulation biological systems express over their structure. An illustration summarising these various forms of lipid-protein interactions is provided in Figure 4.

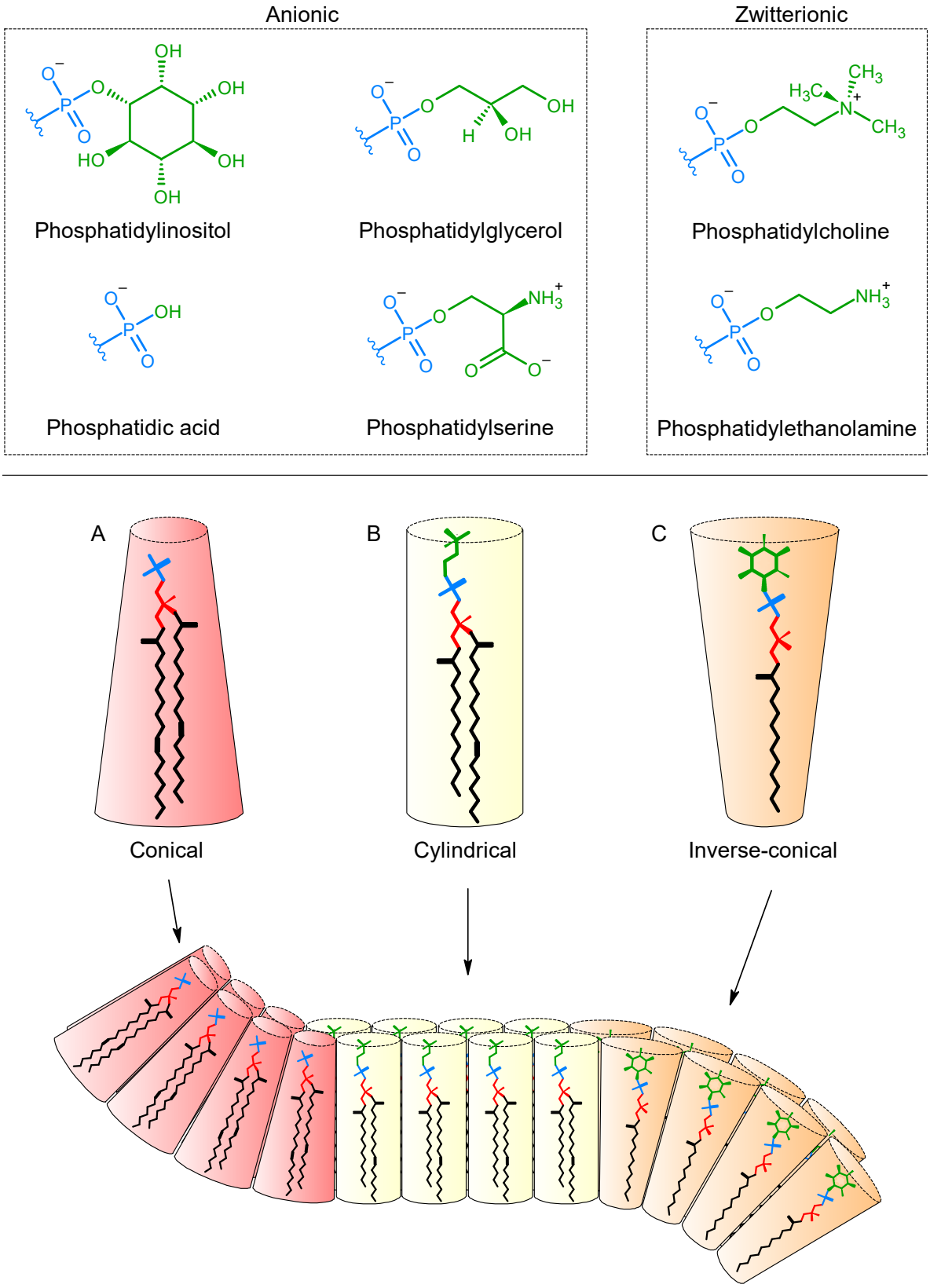

Figure 3 – TOP: Six of the most frequently observed headgroup moieties amongst the glycerophospholipids, along with the associated name for each sub-class. BOTTOM: A demonstration of how membrane curvature may emerge due to the influence of these headgroup moieties, in conjunction with their hydrophobic constituents. The geometry demonstrated here include the conical (A), cylindrical (B) and inverse-conical (C). Notice, the inverse-conical lipid contains a single fatty acid constituent. Lipids of this type are known as lyso-lipids. In the case shown here, the arrangement of the individual lipid constituents causes significant curvature. However, lipid arrangements in nature are far more heterogenous and dense than what is depicted here.

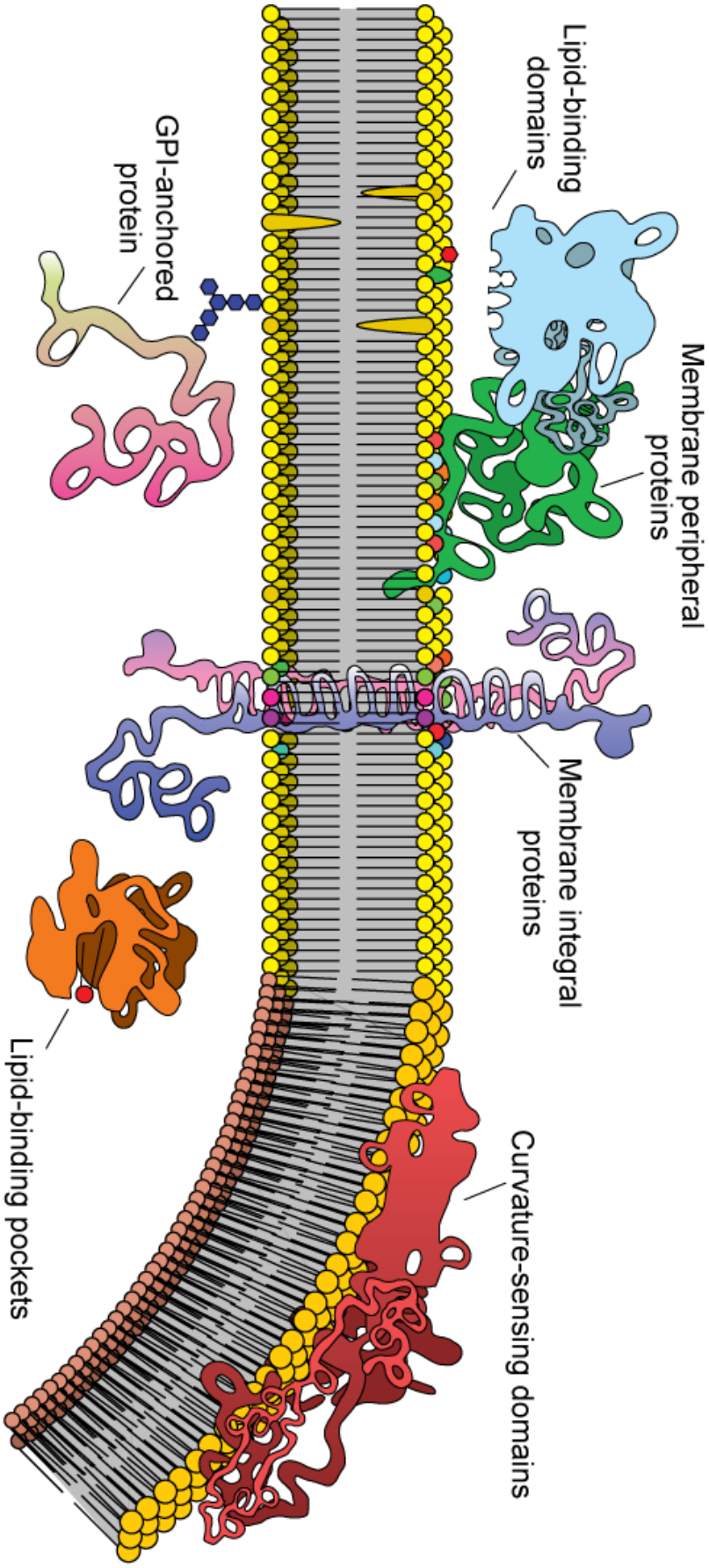

Figure 4 – An illustration of a lipid membrane depicting numerous mechanisms through which associated and integral proteins may interact with the various lipid constituents.

As different parts of the cell are associated with different functions, the membranes of individual organelles may require unique environments and, thus, express specialised phospholipid distributions. Indeed, the compositional breakdown of lipid membranes varies greatly, not only between the specific organelles of cells, but also in the type of tissue they comprise.[27, 28] This can provide an immediate indication of where and how specific lipids may be significant. In eukaryotic cells, including those that comprise mammals, anionic phospholipid species and sphingomyelins are considerably minor constituents, and their proportions vary extensively between specific cell compartments. Many of these species are enriched only on the inner leaflet of the plasma membrane and are rarely expressed elsewhere except under specific circumstances;[27, 28] thus, these molecular species may be significant in modulating certain localised processes. Conversely, the zwitterionic phospholipids, such as the phosphatidylcholines (PC) and phosphatidylethanolamines (PE), are disproportionally abundant, comprising 41-61% and 17-38% of membrane lipids, respectively, and thus contribute significantly to overall membrane shape and structure.[28] Interestingly, the PCs are notably enriched on the exterior face of the plasma membrane, whereas the PEs are predominant on the interior side.[28] This may relate to the geometry of the PEs, as their structures are primarily conical and are thus well suited to comprise a surface of negative curvature (i.e., like the interior face of a sphere).[27, 28] However, one should not conclude from this that the PCs and PEs are otherwise insignificant in biological processes.

Whilst the activities of these membrane constituents are crucial to the function of cells, it does not represent the be-all and end-all of the phospholipid repertoire; rather, the influence of these molecules is deeply entwined with various other cellular processes. For instance, through their interactions with proteins, phospholipids may also be metabolised to release multiple bioactive compounds, which are known to independently continue exerting influence far beyond the membrane. Examples of particular significance include any of the lyso-phospholipid species, which are produced following the hydrolysis of either fatty acid constituent. As these hydrolysis proteins are highly specific, they are selective to either the *SN-1* or *SN-2* position and thus can produce two unique lyso-phospholipid species per precursor phospholipid, with potentially independent ramifications. Typically, these molecules are short-lived intermediates that serve as the precursors of other lipid species during acyl chain re-arrangement, although they appear to be abundantly secreted in the interstitial fluid where they can behave as signalling molecules in their own right.[33] Their influence in this manner is incredibly broad and highly dependent upon the precise structure of both their hydrophobic and hydrophilic moieties. In some instances, particular species may induce pro-inflammatory responses, whereas others are anti-inflammatory; some are suspected to preferentially transfer essential fatty acids to the brain, whilst others may specifically up-

regulate genes or activate cannabinoid receptors.[33] In addition to the lyso-phospholipids, the hydrolysis of phospholipid species also liberates free fatty acids, which simultaneously may share some capacity as signalling molecules.[34] The activities of these molecules are likewise very broad and highly dependent on their precise structures, which accordingly determine how they interact with particular receptors. Certain polyunsaturated fatty acids are themselves precursors to additional classes of signalling molecules with even broader implications.[34] These include arachidonic acid derivatives, such as anandamide, or the eicosanoids and prostanoids, which are significant in modulating inflammation, blood pressure, blood flow, immune function and even pain perception.[34]

As a result of the potent bioactive interactions exhibited by cellular phospholipids and their metabolites, these molecules are also often appreciated for their potential as therapeutic agents and are of considerable interest from the perspective of human health and nutrition. Whilst there is experimental evidence to demonstrate that the dietary intake of these compounds may improve health, such as by reducing cholesterol uptake [35, 36] or influencing hepatic lipid metabolism and wellness,[37-39] their implications in this context are perhaps best illustrated by the roles they play in infant nutrition, as facilitated by breastfeeding; specifically, as the phospholipid distribution of breast milk has evolved to cater especially for the nutritional requirements of infants.[40] For instance, in addition to serving as a significant source of energy, numerous lipid species may provide essential nutrients due to their particular fatty-acid constituents. As indicated in Figure 5, triacylglycerides are quantitatively the most significant source of these fatty acids due to the volumes in which they are consumed. Yet despite this, the phospholipids have demonstrated a unique ability to be preferentially uptaken over other lipid classes, whilst they are also prioritised in their ability to deliver these essential fatty acids directly to the brain and other organs where they may be required.[41-44] These compounds thus may be crucial for ensuring the proper development of the brain, nervous and cardiovascular systems in infants [45, 46], notwithstanding their proportionally minuscule intake. However, the activities of these molecules are also believed to extend well beyond their nutritional role, and many are also suggested to influence the long-term health outcomes of infants. In this capacity, they are implicated in protecting against the development of non-communicable diseases whilst also maintaining and promoting infant health into childhood and later life.[47, 48] Specifically, milk phospholipids have been associated with a litany of health benefits for developing infants and are known to be combative against obesity and adverse adiposity [48-51] whilst also reducing oxidative stress [48, 49, 52, 53] and inflammation,[48, 49] and promoting gut [47, 48, 54] and cardiovascular health,[47-49] in addition to facilitating neurological and central nervous system development.[47] On these accounts, phospholipids have tentatively been labelled as a “miracle drug” at the risk of overstating their significance.[49] These varied functions seemingly

arise from the immense diversity of these compounds present in breast milk, where a lipid's specific biological function or nutritional capacity relies extensively on a combination of its class, fatty acid constituents and stereo-isomerism.[4, 55] In contrast, breastmilk substitutes have often been associated with detrimental effects,[56, 57] some of which have been connected to a mismatch between the specific structure of plant-derived lipids and those provided in breastmilk. [58, 59]

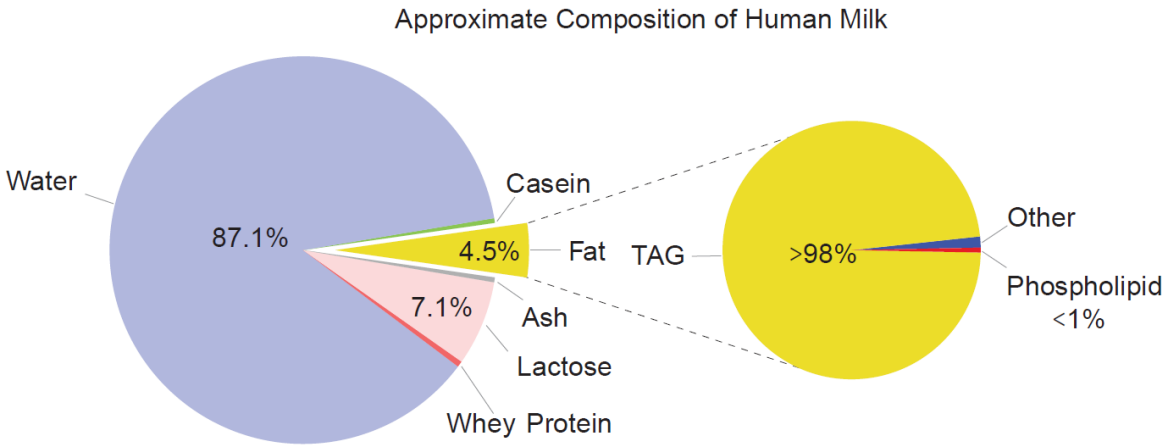

Figure 5 – Approximate composition of human milk fats, collated from various sources [1-4]. The exact composition of the phospholipid fraction is highly variable and is prone to differ between milks. The average amount of phospholipid is 0.6%, although it ranges from 0.39 to 0.97%. HM also contains other complex lipids not elaborated here, including acidic glycosphingolipids, ceramides, glycosylceramides, and cholesterols.

The phospholipids are also regarded as compounds of interest in the development and proliferation of various pathologies, although this is not limited only to their involvement in regulating inflammatory processes and immune signalling.[60] Understandably, the incredibly diverse roles lipid molecules play in biological processes necessitate their stringent regulation, not only in terms of their precise structures but also where and when these molecules are expressed. Dysregulation of these molecules may consequently produce perturbations in their distribution, which interfere with the homeostatic progression of these processes and are hence associated with various diseases.[60] The pathologies associated with lipid dysregulation have been linked to numerous types of cancers,[61-66] along with bipolar disorder,[62, 67] Alzheimer's disease,[60, 62, 64, 67] chronic inflammatory diseases,[60] cardiovascular disease and diabetes, amongst numerous other forms of pathology.[60] As a result, these perturbations are also of significant interest, as the abnormal distributions of lipid molecules that develop may represent diagnostic features in developing pathologies.

Lipidomics

Undoubtedly, the lipids are molecules fundamental to the very structure and function of cells. They play pivotal and diverse roles closely tied to their precise molecular structures and distributions. Accordingly, these molecules necessitate constant supervision, and the manners in which cells produce, circulate, store and metabolise lipids are tightly regulated. Yet, unlike proteins, lipids and lipid profiles are not genetically encoded; but rather, they emerge dynamically as the products of metabolism, and their composition can fluidly transition in response to various environmental factors, such as diet and temperature.[24] Consequently, the distribution of lipid molecular species present within a cell at any particular time, collectively referred to as the lipidome, can provide a direct indication of its current state and internal affairs.[25] Therefore, analysis of these features may reveal how cells respond to and develop with their changing internal and external environments. Thus, measured alterations of the lipid profile, in response to these factors, are of direct significance in advancing the understanding of cell metabolism, nutrition, and disease.[23-25] However, the challenges associated with lipid analysis are considerable in scale, and there is a perpetual need to develop both innovative and alternative analysis protocols to aid in answering the questions underlying lipid profiles and their roles in biology.[24] To this end, the field of Lipidomics has arisen with the aim of addressing the challenges associated with lipid analysis and enable an understanding of the information encoded by the lipidome.

In general, lipidomic analyses are made considerably complex due to the diversity of the lipidome,[68] which may comprise up to tens to hundreds of thousands of unique molecular species distributed across a plethora of chemically distinct classes.[23-26] The distributions of these classes are also closely related to one another, as represented in Figure 6, and it is near impossible to predict how the ramifications of contributing factors may cascade through this network. In this way, it is unclear where and how changes in the lipid profile may manifest, and even localising these changes represents a considerable challenge.[24] For this reason, the characterisation of these compounds is often approached through untargeted and exploratory surveys that maximise the likelihood of observing particular changes of significance.[24] Hence, analysis methods typically require the capacity to speciate numerous lipid classes at once.

These ‘comprehensive’ methods are a valid approach to lipid analysis, as they are likely to reveal changes in the lipid profile without necessitating an understanding of the relationships between different metabolic networks. However, their application has considerable limitations, especially due to the impossibility of comprehensively characterising an entire lipidome within a single experiment.[24] As a result, in their attempt to analyse numerous chemically distinct molecules simultaneously, these methods inherently make compromises to achieve their

breadth. Often, these compromises result in reduced chromatographic performance or sensitivity for particular classes, and thus, despite their utility, these broad analysis protocols may, in reality, prove unsuitable for characterising particular lipids. Nevertheless, these generalised methods are used routinely to analyse lipid profiles and characterise specific lipid classes, where they may achieve only a shallow depth of characterisation.

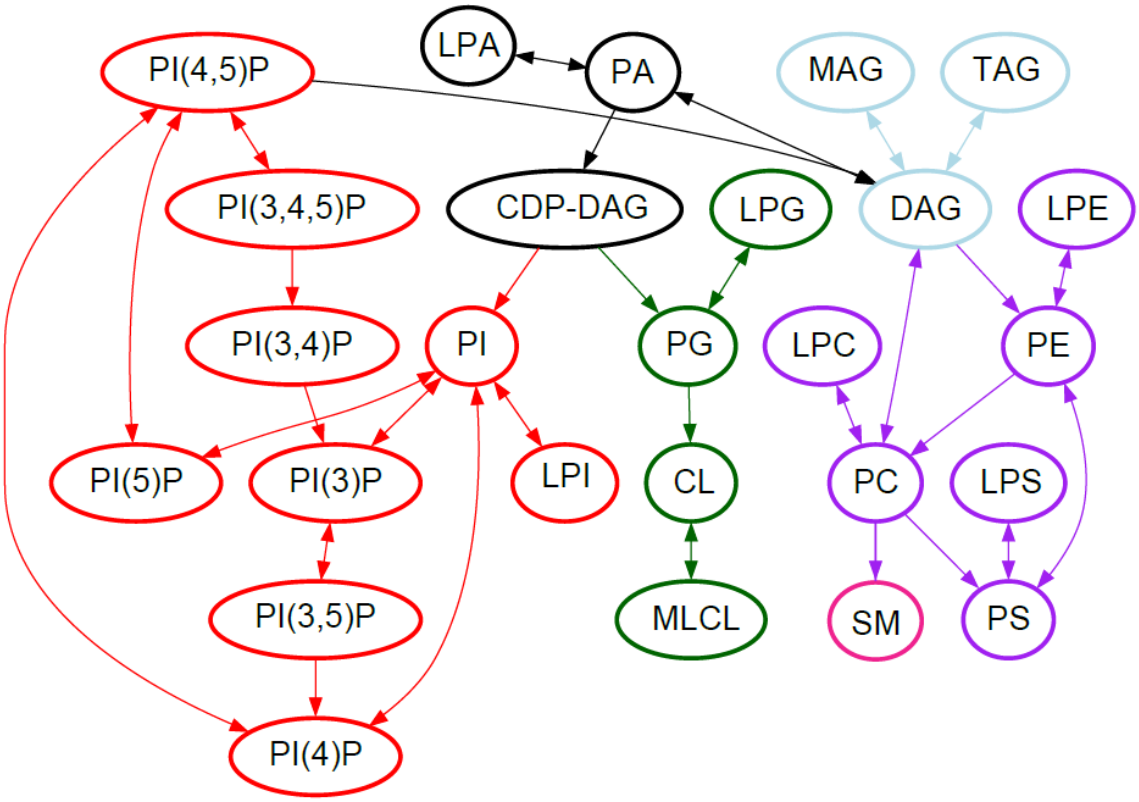

Figure 6 – A simplified representation of the interconnected nature of the glycerolipid classes, and sphingomyelin, in mammals [21]. The lipid classes are coloured arbitrarially based on the closeness of their relationship to one another. The proteins and metabolites responsible for converting one lipid species into another are excluded for the purpose of simplifying and compressing the network. In red are those lipids derived from the phosphatidylinositols (PI), including lyso-phosphatidylinositol (LPI) and the various phosphatidylinositol phosphates (PIPs). In black are those lipids derived from phosphatidic acid (PA) including cytidine diphosphate diacylglycerol (CDP-DAG) and lyso-phosphatidic acid (LPA). In green are those lipids derived from phosphatidylglycerol (PG), including lyso-phosphatidylglycerol (LPG), cardiolipin (CL) and monolyso-cardiolipin (MLCL). In cyan are the glycerolipids monoacylglycerol (MAG), diacylglycerol (DAG) and triacylglycerol (TAG). In purple are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), along with their respective lyso-derivitives (LPC, LPE and LPS). In pink is sphingomyelin (SM).

Consequently, these broad methods are generally only suited for the characterisation of the major lipid components of cells, such as the glycerolipids, and particular phospholipids, such as the phosphatidylcholines and phosphatidylethanolamines, whilst they are often severely limited for the analysis of the low-abundance signalling lipids which often display inferior separation and detectability.[69, 70] Thus, to observe these species under generalised conditions, lipidomic experiments often require considerably concentrated samples, and these species are thus generally underrepresented in characterisations. This bias is clearly shown in the distribution of phospholipid species observed in human milk, shown in Figure 7, as these

characterisations depend on general lipidomic methods due to the broad interest in lipid constituents.[5-17] In an effort to maximise the depth of these broad analysis conditions, optimisations have been performed with consideration for the analysis of particular classes;[71] however, there is often a contradiction as to which conditions are indeed optimal.[71-74] Hence, there remains the need to fine-tune analysis conditions to permit the characterisation of these low-abundance signalling lipids,[23] especially when considering the significance of these minor lipid classes due to their deeply interwoven relationships with membrane-associated proteins and, hence, their considerably bioactive influence.

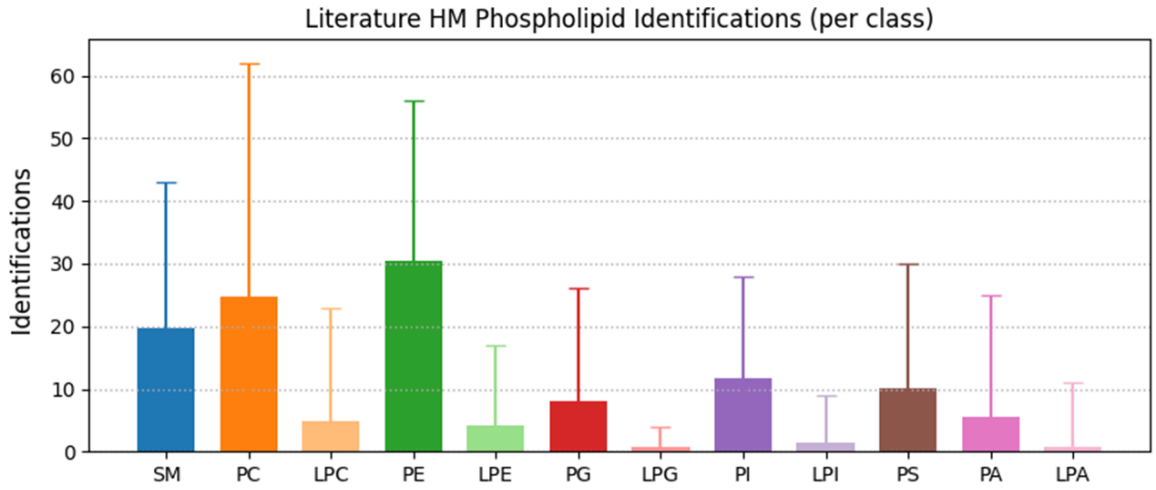

Figure 7 – A breakdown of the number of phospholipid identifications achieved by 13 contemporary human milk (HM) characterisations [5-17]. The number of species observed mainly constitute SM, PC or PE species. On average, only 12 PI species and 10 PS species are observed in HM characterisations despite the anticipation of an immense chemical diversity. SM: Sphingomyelin, PC: Phosphatidylcholine, LPC: Lyso-Phosphatidylcholine, PE: Phosphatidylethanolamine, LPE: Lyso-phosphatidylethanolamine, PG: Phosphatidylglycerol, LPG: Lyso-Phosphatidylglycerol, PI: Phosphatidylinositol, LPI: Lyso-phosphatidylinositol, PS: Phosphatidylserine, PA: Phosphatidic acid, LPA: Lyso-phosphatidic acid.

The Phosphatidylinositols

Whilst there are numerous lipid classes for which characterisation would necessitate further method development, the phosphatidylinositols (PIs) are perhaps one of the most significant. Although these lipids are particularly sparse, constituting, on average, only about 6% of cellular phospholipids in mammals,[28] they are understood to exhibit far-reaching biological influence through their interactions with various membrane-associated proteins. Yet, beyond these direct functionalities, these molecules also express a unique capacity for modification.[64, 75-78] Consequentially, in addition to their essential roles in cells, they are also recognised to produce a diversity of biologically significant derivatives critical to life, from plants to yeast to mammals.[66] In some instances, these modifications arise through the addition of a sugar or a fatty acid species to a specific position on its myo-inositol headgroup; yet, in mammals, these additional lipids typically originate through phosphorylation. Modification in this regard enables

the production of up to seven distinct phosphorylated-PI sub-classes,[32, 64] or phosphatidylinositol phosphates (PIPs), as shown in Figure 8 and Figure 9. These molecules are considered immensely important, and they are assigned responsibility for maintaining numerous cellular functions, to the extent that they are involved in almost every aspect of a cell's life and death.[32, 64, 66, 79-81] However, the activities of these molecules also extend beyond the single-cell level, and previous evaluations of these molecular species indicate they are deeply involved in promoting and maintaining tissue growth, brain development, blood vessel development, and immune function.[64] Thus, certain species are even recognised as some of the most multifunctional molecules throughout biology.[82] Unsurprisingly, there is considerable interest in evaluating the activities of these molecules when considering the function of both healthy cells and tissue, especially due to their involvement in almost all aspects of cellular function.

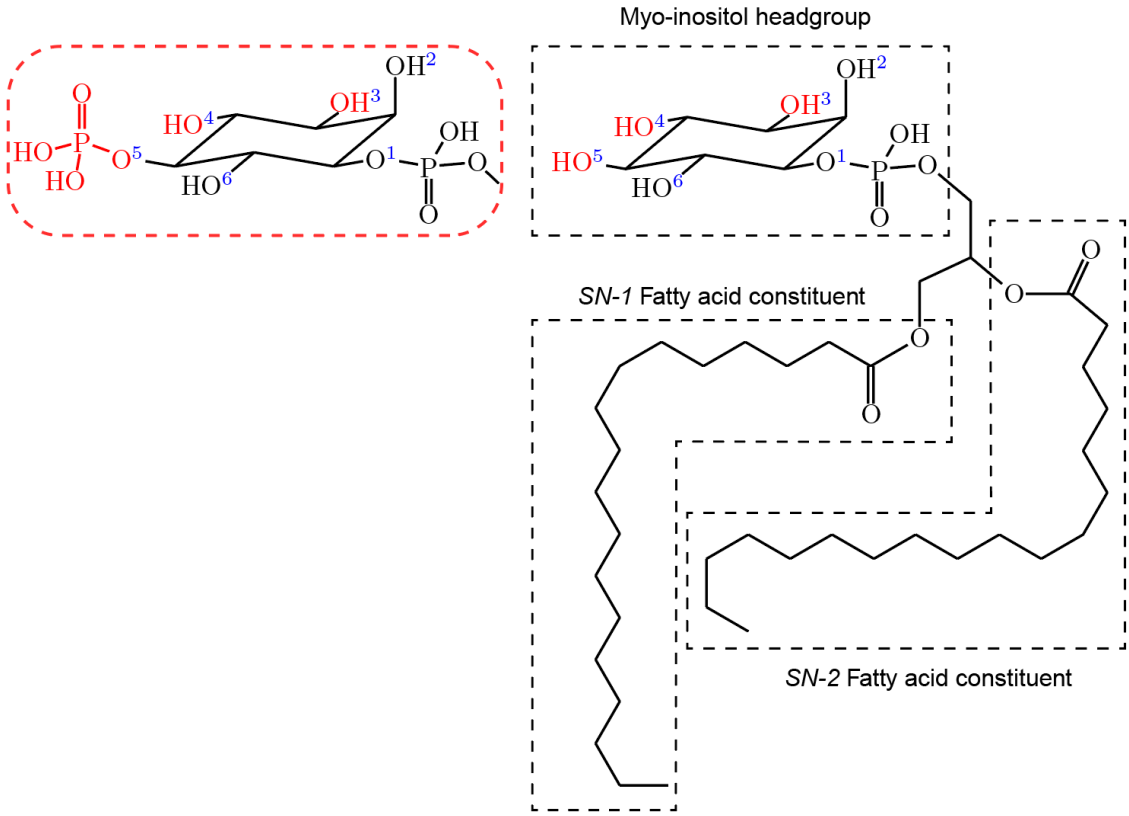

Figure 8 – The general structure of phosphatidylinositol (PI). The myo-inositol headgroup is attached to the *sn-3* position of the glycerol backbone through a phosphor-diester bond. Three additional sites on the myo-inositol ring may also be phosphorylated, highlighted in red. Phosphorylation of these sites produces either mono, di or tri-phosphorylated PI (PIP, PI2P, PI3P). A phosphorylated headgroup corresponding to PI(5)P is provided. These lipid classes are shown in Figure 9. Loss of *sn-1* or *sn-2* fatty acid constituents produces either an *sn-1* or *sn-2* Lyso-PI (LPI).

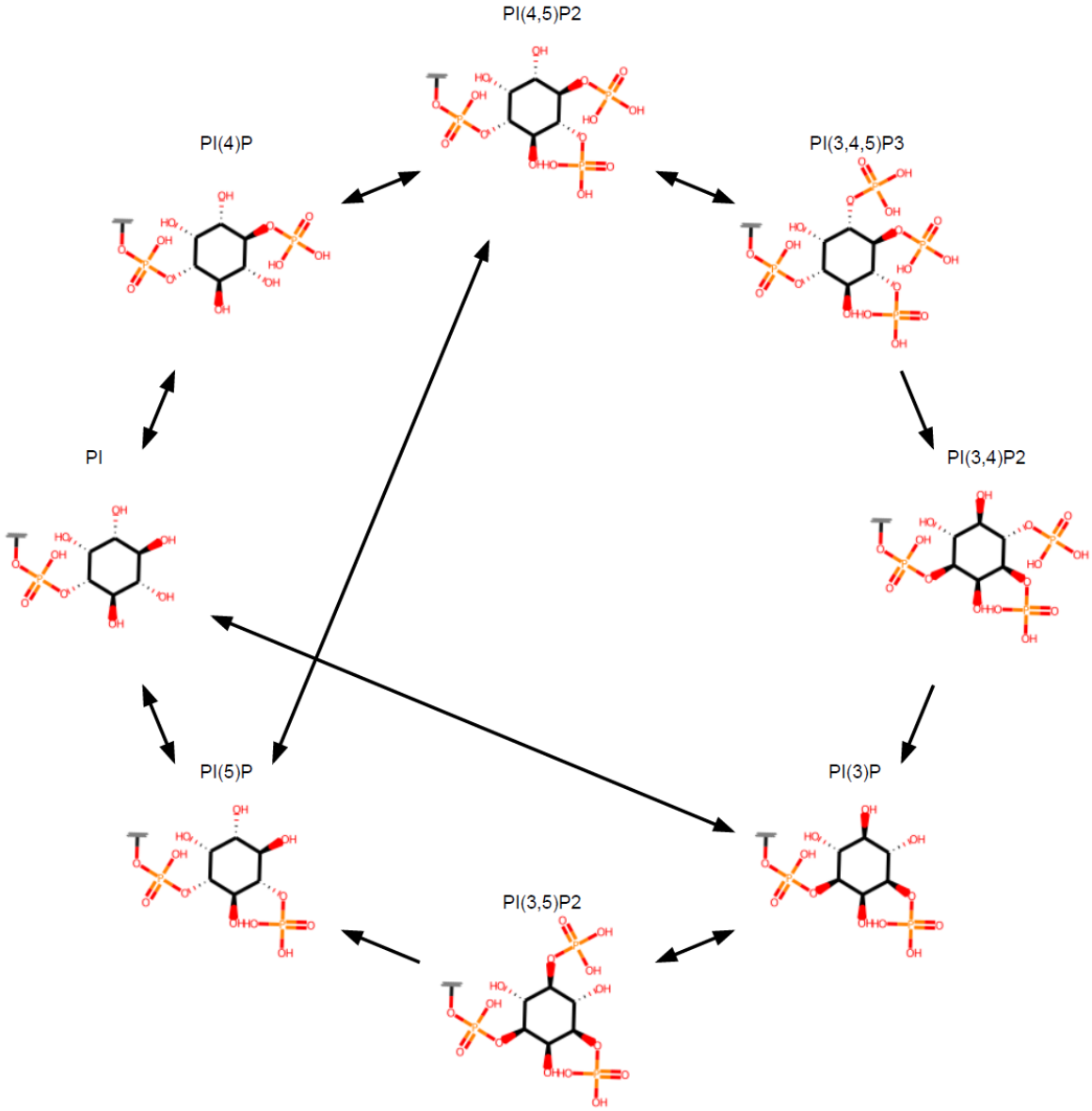

Figure 9 – The inter-relations between PI and its phosphorylated counterparts. Only the lipid headgroups are shown. The arrows represent the manner in which these modifications may occur, due to specific protein interactions, although the identifications of the proteins involved are omitted for clarity.

Conversely, the remarkable bioactivity of PIPs also implicates them as molecules of considerable interest from the perspective of developing pathologies [32, 64, 66, 79-81], even beyond their associations with immune function and their activities in the progression of infections, allergies and auto-immune diseases.[64] In healthy tissue, cells necessitate not only specific structural qualities in terms of both their headgroup and fatty acid constituents, but also strict control over where in the membrane these lipids are expressed.[64] Hence, any irregularity in the distributions of these molecules that arise due to their dysregulation is associated with the development of diseases such as diabetes, obesity and cardiovascular diseases,[64, 66] but also several neurological diseases [62-64] and cancers.[62-66] Thus, these molecules are also compelling targets when considered as biomarkers in the progression of numerous diseases.

In addition to the PI derivatives that arise through headgroup modifications, the lyso-PI species are also of considerable interest due to their alternative bioactivities. The distribution of these molecules likewise notably varies with the development and progression of various diseases such as cancer and obesity, and they may serve as valuable biomarkers;[33, 83] however, these lipids seemingly also exhibit various therapeutic properties and can induce both physiological and pathological responses. These include stimulating insulin release [33, 84] and adipogenesis [33] whilst influencing bone development, gut motility, the central nervous and cardiovascular systems, pain perception and inflammation.[85] Hence, collectively, the PIs and their associated derivatives are fascinating targets from numerous perspectives due to the insight they may provide into almost every aspect of cells and the development of pathologies.[75, 86-89]

Unfortunately, due to a combination of their unique chemical properties, exceptionally low natural abundance, and rapid decomposition, the analysis of these molecules represents a considerable challenge. Thus, lipidomic experiments seem to only shallowly characterise these molecules if they observe them at all.[90, 91] Considering the broad and influential associations of these molecules in numerous areas, including growth, health, disease and general cellular function, there is reasonable justification for further developing lipidomic analysis protocols to permit an in-depth evaluation of their profile. However, due to the simultaneously broad distribution of structures and chemical characteristics exhibited by the PIs and their derivatives, it is perhaps preferable that a similarly broad set of analysis conditions is developed to evaluate these molecules as a group, that is, rather than producing a series of individual analysis protocols suited for their separate characterisation. Whilst a broad method may result in a similar set of complications as experienced with the generalised lipidomic analysis protocols, and thus be un-optimised for the analysis of certain classes, it enables a wider survey of the lipid profile, and it thus compounds the utility of analysis by permitting the characterisation of numerous classes at once. Conversely, individual protocols only compound the cost of analysis and blindsight characterisations to those classes specifically targeted.

In addition to those conditions that may be suited for their analysis, the extraction and isolation of these molecular species present further complications and are primarily a major contributing factor to the previous insufficiency in their characterisation. Thus, in pursuit of enhancing the analysis of PIs and their related compounds, specific consideration must first be given to these additional aspects.

The Extraction of PIs

Lipidomic characterisations generally use a bi-phasic liquid-liquid extraction (LLE) technique to recover targeted analytes, where the suitability of these systems depends on their ability to solvate and partition the targeted molecules. Therefore, selecting an appropriate solvent system is essential for analysing specific classes, as the degree to which these compounds are included or excluded from the extraction solvents will consequently determine the extent to which they may be characterised. However, the suitability of any extraction conditions may also be subject to which matrix they are applied, requiring specific consideration of sample type. Unfortunately, the literature regarding lipid extraction efficiencies is generally limited to those few matrices where these compounds are predominantly considered such as from cells or plasma. In these particular matrices there appears to be a consensus regarding the extraction of PIs; solvent systems based on chloroform and methanol, such as those used in the Folch or Bligh & Dyer protocols, appear most effective in ensuring their recovery.[92-94] Yet, due to the polarity exhibited by certain PI species, many of these compounds are excluded from non-polar extraction solvents and are poorly recovered, if at all.[64]

In the case of the LPIs, these mixtures may partially exclude their extraction, and butanol [95] or methyl tert-butyl ether [96] may be better suited. However, these conditions may also enhance the extraction of various hydrophilic metabolites and inorganic salts, which could decompose the lipids and be challenging to remove whilst potentially further interfering with analysis.[97, 98] Alternatively, modifications to the Bligh & Dyer protocol have also been shown to provide greater extraction affinity [99]; yet, these conditions may also artificially inflate their concentration through the degradation of PIs. Similarly, chloroform-methanol methods provide extremely poor recoveries for PIP species and require modification through additives.[91, 97, 100, 101] Typically, these modifications would involve the addition of an acid such as HCl, an ion-pairing agent such as tetrabutylammonium, or an excess of salt such as KCl to improve solvation, as the highly acidic phosphate groups are prohibitive to their extraction when ionised.[97, 100, 101] Nevertheless, as precise conditions vary greatly between methods, their comparative benefits are ambiguous; hence, further evaluation is necessary to compare their effectiveness.

The insolubility of PIPs in unmodified chloroform-methanol systems may enable their purification, as a preliminary extraction with these solvents can deplete the matrix from interfering lipid compounds prior to a modified protocol. These modified conditions may also enhance the recovery of the PIs and LPIs due to their similar acidic nature and intermediate polarity,[99, 101] and it may be worthwhile to extract these compounds simultaneously with the modified conditions.

Beyond the suitability of extraction solvents, additional considerations are necessary regarding sample treatment, mainly due to the ability of these lipids to rapidly decompose.[91, 99, 101] For example, under particularly acidic or alkaline conditions, extracted lipids may be susceptible to degradation through hydrolysis, headgroup substitution and fatty acid oxidation;[97, 101-104] hence, if such conditions are used, exposure is recommended to be brief and conducted under colder temperatures.[91, 97, 103-105] Additionally, it should be noted that various lipid species, such as the plasmenyl-lipids, are very susceptible to even traces of acid and will readily decompose;[103, 106] thus, the acidic conditions necessary for the recovery of PIPs are prohibitive for their analysis. Furthermore, in cases where trace quantities of salts or acids are extracted alongside the compounds of interest due to solvents used, they may be concentrated during solvent evaporation and induce further degradation.[97] In such cases, the observation of disproportionate amounts of free fatty acids, lyso-lipids, and phosphatidic acid indicates that some degradation has occurred either during or post-extraction.[107]

Short storage periods at room temperature within methanolic solutions alongside co-extracted sodium carbonate may induce transesterification,[100, 108] whereas other residual salts and acids can result in oxidation, hydrolysis or dephosphorylation.[97, 100] Similar effects may occur in solutions containing both chloroform and methanol when exposed to ultra-violet light, where acylating agents can arise to modify the lipids.[101] Other detriments arise specifically in the case of PIPs, as their recovery can be hindered considerably due to their propensity to chelate or strongly adsorb to various surfaces, such as glass, and silanisation of all glassware used throughout the extraction is necessary to prevent significant loss,[97] though this process is laborious. Conversely, polypropylene equipment has proved suitable and can provide intermediate recoveries approximating that of silanised glassware without requiring additional preparation.[97] However, it should be noted that solvents such as chloroform may extract additional unwanted materials such as plasticisers, which can further interfere with analysis.

The Isolation of PIs

Whilst ensuring adequate recovery of these compounds is an essential aspect to consider in permitting their analysis, numerous other experimental factors may simultaneously influence characterisation. This is evident as several recent milk characterisations appear to observe only 12 PI species on average, as shown in Figure 7, despite exclusively using chloroform-methanol solvent systems, which should be well suited for their analysis.[5-17] Their sparse observation suggests that additional limitations hinder their characterisation, potentially arising due to the influence of matrix suppression.[109, 110]

The effects of matrix suppression arise during mass spectrometric analyses, where the presence of observed or unobserved analytes may co-elute with targeted compounds and subtract from their ionisation efficiency, thus inhibiting their detection. As a result, certain compounds, often those with lower abundance, are hidden due to the presence of interferents. In lipidomic analysis protocols, this effect is rampant in crude phospholipid extracts, where particular lipids such as phosphatidylcholine appear to inhibit the analysis of other classes.[111] This necessitates the use of post-extraction cleanup protocols to permit the isolation of the compounds of interest and the elimination of potential suppressants.

Due to the particularly low abundance of PIs and their derivitives in cells, especially compared to the greater abundance of phosphatidylcholines and phosphatidylethanolamines, the detection of these species is likely being obscured. Thus, the simplification of the sample matrix may be necessary to avoid these consequences and enhance their characterisation. Numerous preparative separation techniques have been published specifically for the purification of lipidomic extracts,[112] with a few specialised for the various PI and PIP classes.[75, 113-119]

Matrix purification techniques utilising thin-layer chromatography (TLC) are one such format frequently used for purification, often used specifically for the isolation of PIPs from complex matrices. Amongst other techniques, TLC has the benefit of being simple, rapid, and cost-effective, whereby several separations may be conducted in parallel.[112, 120, 121] An illustration demonstrating the technique is provided in Figure 10. TLC separations targeting lipid extracts predominantly use silica plates and hydrophobic mobile phase solvents, as this combination enables the separation of compounds based on polarity. Since the polar interactions of a phospholipid class are almost exclusively determined through headgroup interactions, a lipid mixture may be resolved into bands representing the major lipid classes present. Hence, targeted phospholipid classes may be separated into individual bands and selectively removed from the silica plate for further analysis.

As outlined by the protocols in Table 1, the TLC fractionation methods used for isolating PI and PIP generally rely on simple alkaline mixtures of chloroform and methanol, along with various additives.[75, 113-116, 122] However, these mixtures are often insufficient on their own and lipid classes such as the phosphatidic acids and PIPs may exhibit some chelation to the stationary phase.[91] Additionally, the distribution of phospholipid counter-ions encountered in a sample can also interfere with separation, as the specific salts present may alter the solubility of the targeted compounds in the mobile phase. The combined influence of these two factors generally results in the forming of large smears rather than discrete bands, preventing the isolation of these compounds.[112, 123] Hence, to achieve resolution, these obstacles would

necessitate both the conversion of the phospholipid counter-ions into a single state and the pre-treatment of the stationary phase with an agent capable of preventing chelation. To this end, agents such as potassium oxalate, boric acid, and ethylenediaminetetraacetic acid (EDTA) are generally impregnated into the stationary phase through a pre-treatment protocol, as they are capable of simultaneously counteracting chelation and may convert salt forms.[112, 123]

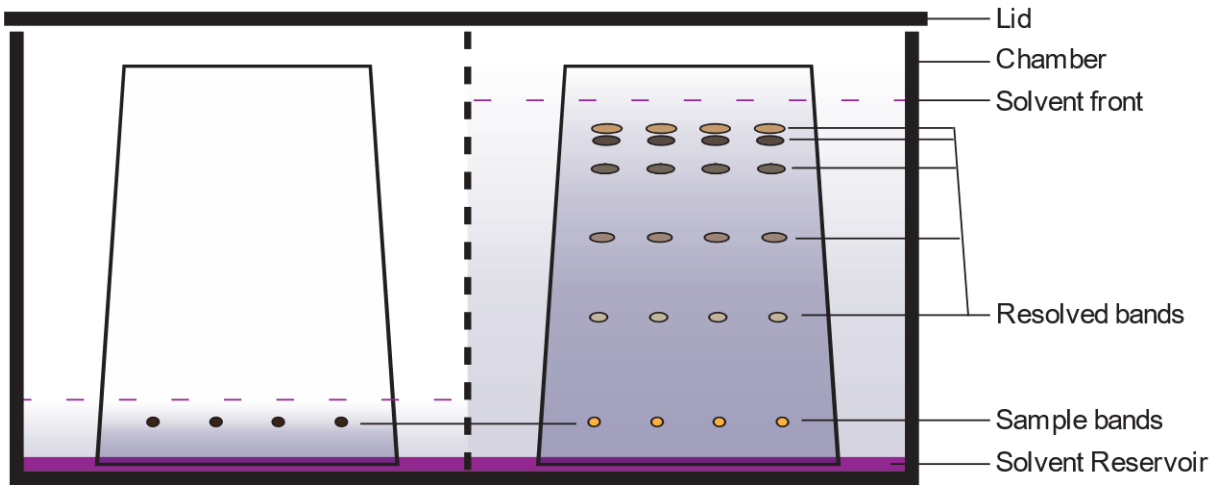

Figure 10 – An illustration of separation using thin-layer chromatography. Although several formats exist, a TLC separation is generally initiated after a silica plate is spotted with samples, dried, and then placed upright in a small volume of an appropriate mobile phase solvent. The mobile phase ascends the plate due to a combination of capillary action and the solvent vapour pressure, and the competing interactions between a compound's adsorption to the silica plate and its affinity with the rising solvent front results in the chromatographic separation of a sample into discrete bands. The images on the left and right represent the TLC plate pre- and post-separation, respectively.

Table 1 – Commonly used TLC solvents and pre-treatments for separating PI and PIPs. Solvent acronyms: C, Chloroform; M, Methanol; W, Water; IPA, Isopropanol; EtOH, Ethanol.

Pre-treatment	Solvent System	Comments	Ref.
First and second systems treated with MeOH: H_2O (2:3) + 1% K-oxalate The third system treated plates with 1% K-oxalate and 2mM NaEDTA in MeOH: H_2O (2:3) Plate activation at 110˚ C for 15 to 30 minutes	C: acetone: M: CH_3COOH: W (45: 15: 13: 12: 8 or 7) Or (80: 30: 26: 24: 14) Or (40: 15: 13: 12: 8)	▪ The first system resolved bands clearly with no coelution, high resolution between PA, PI, PIP and PIP_2 ▪ The second system also achieved clear resolution between PI, PIP, PIP_2, PA and PC ▪ The third system applied separation applied to IPCs and PLs. Enabled clear resolution between IPCs, mannosylated-IPCs, and PLs, including PI	75, 113-116
None	C: M: 25% NH_4OH: W (90: 70: 4: 16)	• Separation between PG, PE, PI, PA, PIP, PI2P	117
Sprayed with 1% K-oxalate, activated at 110˚ C for 1 hour	C: M: W: Conc. NH_4OH (45: 35: 8: 2)	• Clear resolution between PI, PIP, PIP_2 and a proposed Inositolglycan. • Observed bands appeared to have high R_f, lipids further hydrophobic than PI expected to receive no resolution if applied.	118

Plates dipped in 5% boric acid in MeOH, dried	1-propyl acetate: IPA: EtOH: 6% aq. NH_3 (3: 9: 3: 9)	• Clear resolution between PI, PIP, PIP_2, PIP_3, and other PLs, no streaking observed • Some resolution achieved between positional isomers of PIP headgroups	[119]

Yet, beyond the complications regarding separation, the visualisation of the resolved bands also presents an additional challenge due to the invisibility of these compounds on the silica plate. Hence, chemical stains are often necessary to localise the bands.[100] Many of these stains operate by chemically modifying or decomposing the targeted molecules, and they are thus not conducive to subsequent molecular characterisation, which would necessitate non-destructive dyes, such as primuline reagent.[124-127] The use of non-destructive dyes in this manner is potentially convenient for rapid analysis, as a modern approach to isolation via TLC might combine the use of these non-destructive dyes with matrix-assisted laser desorption/ionisation to conduct a rapid, in-depth characterisation of a lipidomic extract.[128, 129] However, this approach would sacrifice the benefits of a chromatographic separation in enabling the resolution of isomeric species.

Despite the benefits of TLC-based purification protocols, these methods can be challenging to implement correctly, and numerous external factors may contribute to inadequate development of chromatographic separation.[112] Environmental factors such as temperature and humidity must be controlled to ensure proper resolution and reproducibility. Additionally, initiating separation may be unwieldy, and incorrect placement of the plate may cause the rising solvent front to ascend at an angle, potentially interfering with the separation. Beyond this, as the sample must be dried onto the plate and the separation conducted exposed to the atmosphere, development and storage risks oxidation of the targeted compounds.[112]

Alternatively, lipidomic matrix purification may be conducted using solid-phase extraction (SPE). While relying on the same chromatographic principles as TLC, the alternate form factor of the stationary phase in SPE introduces an additional set of constraints. As depicted in Figure 11, a typical SPE separation is performed in a cartridge uniformly packed with a stationary phase, whereby fractions are eluted and collected following the addition of mobile phase. However, retention in SPE not only necessitates the pre-conditioning of the stationary phase to ensure that the surface has been wetted and primed to permit adequate interaction with the sample, but also requires that the sample is loaded into the cartridge with a solvent that will not interfere with the retention mechanism. Furthermore, as opposed to TLC, sample fractions may only be collected once they have eluted the cartridge; hence, specific bands corresponding to particular classes cannot be selectively removed. Instead, multiple classes are often collected together. In addition, matrix purification conducted in this manner does not

permit the simultaneous fractionation of multiple samples on the same stationary phase and requires significantly greater volumes of solvent.

Despite these constraints, SPE purification protocols provide many advantages over those that use TLC. For example, samples are not required to be dried or pre-concentrated before separation, and large volumes of lipid extracts may be applied directly following liquid-liquid extraction. Furthermore, as fractions are eluted based on the added mobile phase, their contents are predictable, and the recovery of particular classes does not require staining and manual resolvation. Additionally, the decreased resolution achieved between lipid classes may be advantageous as similar analytes may be recovered together, rather than necessitating individual identification and recovery. In comparison, as many of the aforementioned TLC protocols that enable the resolution of PI and PIP do not identify the LPIs, it may be difficult to recover these species. Moreover, following isolation via TLC, similar lipid classes and related compounds of interest may be inadvertently discarded in the case that their corresponding band lacks identification and are not intentionally recovered.

Silica-based stationary phases are infrequently used for the isolation of PIPs via SPE due to their propensity to chelate, although these methods are often used to isolate the other phospholipids from the neutral lipids.[130-133] Rather, polymer-based ion exchange resins are preferred as they enable the fractionation of various charged species whilst limiting the extent of non-specific interactions; some of these methods are provided in Table 2. Both anion and cation exchange resins have been used, where either anionic or cationic species are retained, respectively. In the case of the former, as anions are retained, they are separated from both the neutral and cationic lipids, such as the TAGs and PCs, which elute during the wash, whilst under a cationic exchange resin, the anionic lipids would elute along with the neutral lipids.

The use of an anion exchange SPE protocol is, therefore, an effective solution for the purification of PIs and PIPs from an extract, as it would enable the elimination of not only neutral lipid species, such as cholesterols, triacylglycerides and diacylglicerides, but the non-anionic species, such as the SMs, PCs, and PEs. Hence, the purified eluent would contain only anionic lipids such as the PIs, PIPs, PAs, PGs, and PSs, along with all their lyso counterparts. A protocol of this sort may also be conveniently implemented directly after LLE due to the compatibility of the extraction solvents with the stationary phase. However, current ion-exchange protocols of this type generally initiate elution of PIPs through the use of a strong acid, which competes for retention of the stationary phase, and thus forces the anionic lipids out.[70, 100, 134] As a result, a secondary LLE is required post-fractionation to eliminate the added salts and transfer the analytes back into an organic solvent, although this could potentially influence recovery.[70, 134] There is some potential for method development here, as substitution

of the hydrogen in the acid for counterions such as sodium, potassium, or ammonium may alter the solubility of the targeted lipids in organic solvents whilst simultaneously aiding recovery by 'salting out' polar lipids from the aqueous phase. Alternatively, elution could be initiated using a highly alkaline solvent to remove the charge on the stationary phase;[91, 135] however, this would necessitate using a weak anion exchange resin.

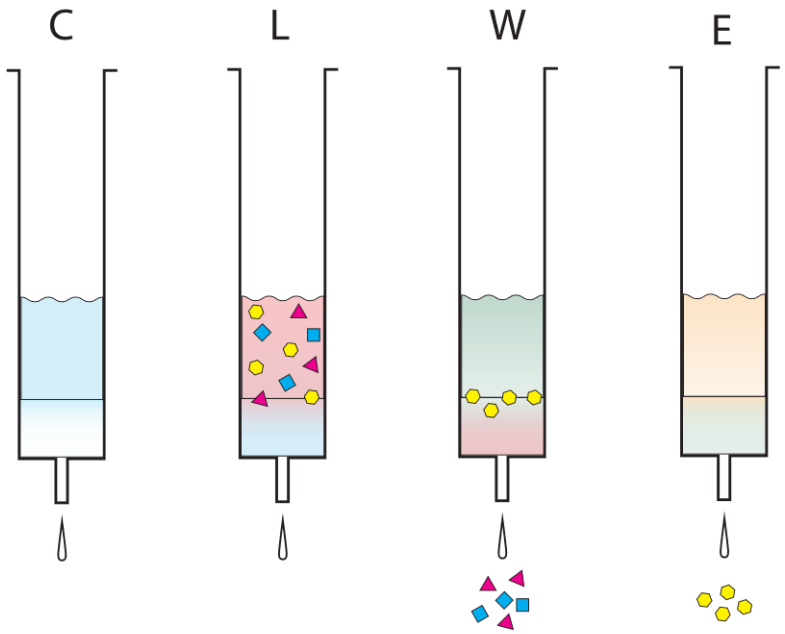

Figure 11 – A simplified illustration of an SPE protocol. These protocols generally consist of four steps, labelled above as C (Condition), L (Load), W (Wash) and E (Elute). Conditioning necessitates cleaning the stationary phase, usually by passing through a volume of the eluent solution, followed by a volume of mobile phase matching the sample solvent. During the load step, the sample is applied to the stationary phase and allowed to pass through. During the wash step, additional mobile phase is passed through to initiate the elution of interfering compounds. Finally, the targeted compounds may be eluted.

Table 2 – Examples of SPE protocols for fractionating PI and PIPs from lipidomic extracts. Acronyms WAX, Weak anion exchanger; SCX, Strong cation exchanger.

Solvent	Fraction	Comments	Ref.
1. 150mM AmAc in C:M:W (3: 6: 1) **2. 600mM AmAc in C:M:W (3: 6: 1)** **3. C:M:15N NH_4OH (3: 6: 1)**	1. All but PIPs 2. PIP 3. PIP_2	▪ Immobilized neomycin stationary phase. ▪ Not recommended due to a lack of accessibility to neomycin-functionalised stationary phases; however, it provided good resolution between PIP classes. ▪ Perhaps aminopropyl WAX is a suitable substitute, such as used in ref. [135] ▪ Only PIP and PIP_2 are considered.	91
1. C:M (1: 1) **2. C:M: HCl: W (12: 12: 1: 1)**	1. Simple lipids 2. Anionic lipids	▪ DEAE-cellulose WAX resin ▪ Sample applied directly after extraction after addition of 2 mL C:M (1: 1) ▪ PI and PIPs elute in acidic fraction	70, 134
1. Flow-through fraction collected	1. Neutral and anionic lipids	▪ Teledyne ISCO SCX column ▪ Prior to use, column is washed with ACN:HCl (1M) ▪ Equilibrated with C:M:W (20:9:1)	136

The Analysis of PIs

Multiple chromatographic formats have been used for lipidomic analysis, including those based on normal-phase, HILIC and reversed-phase stationary phases, although reversed-phase protocols are by far the most abundant.[137] Whilst chromatographic methods based on normal-phase or HILIC may be beneficial for the analysis and identification of polar lipid classes, in that they retain and cluster phospholipids together based on their headgroup moieties, they are in many ways inadequate for the in-depth characterisation of PIs. Under these conditions, the hydrophobic fatty acid moieties of the lipids would enable virtually no resolution between the individual phospholipid species. Thus, these methods are ineffective in resolving the isomeric species that arise due to precise composition and structure, and all species of a class would approximately elute together. As a consequence of this co-elution, these species may also suppress each other, further preventing the detection and characterisation of certain species. In addition to these factors, analysis methods based on HILIC typically require high concentrations of mobile-phase buffer, which itself can cause significant suppression of molecular species. Hence, methods based upon this chemistry may require immensely concentrated lipid samples, which can range from 400-800,000 ppm total lipid.[138] Conversely, analysis methods based on reversed-phase chemistries are far more conducive to the analysis of lipids, as these methods retain and resolve molecules through a hydrophobic retention mechanism. Under these conditions, the polar moieties of these lipids influence their retention, and thus, significant resolution may be achieved between all molecular species, as depicted in Figure 12.

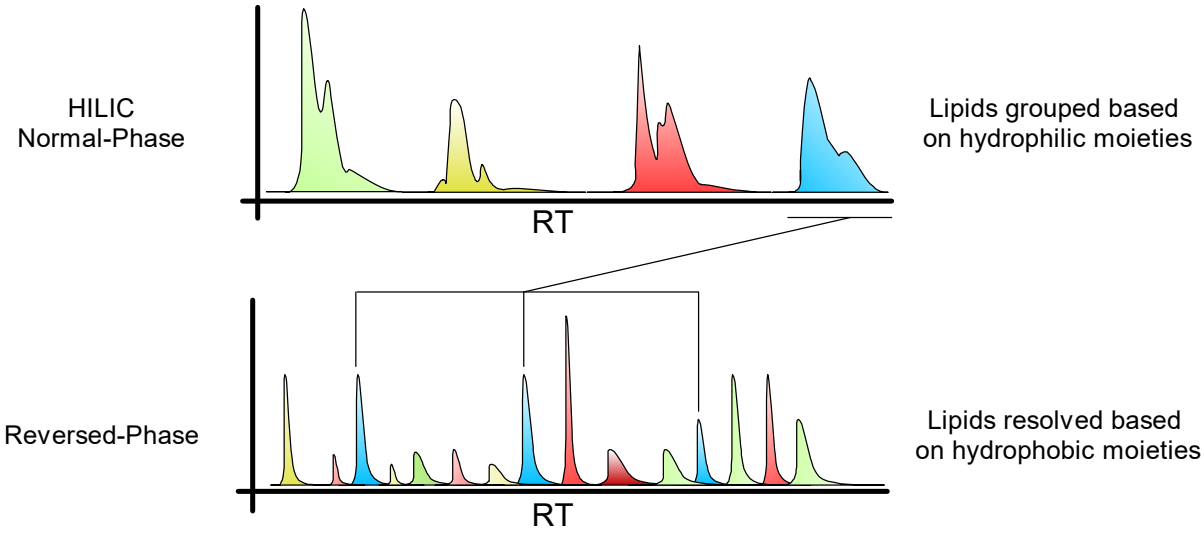

Figure 12 – A hypothetical set of chromatograms depicting the types of resolution afforded by either HILIC, normal-phase or reversed-phase. Those methods based on HILIC or normal-phase isolate compounds based on polar interactions are mediated by phospholipid headgroups. Alternatively, reversed-phase methods resolve based on non-polar interactions mediated by their tail moieties.

However, although phospholipids are hydrophobic molecules, they are also particularly polar due to their capacity for charge, and they are typically not retained to the same extent as neutral lipids such as the triacylglycerides. Consequently, a limited extent of resolution is afforded to these molecules in generalised protocols due to their rapid throughput and generally short retention times. Extending the analysis duration to permit sufficient resolution between all molecular species is not conducive to the rapid characterisation of multiple samples. However, considering the anticipated gains in characterisation achieved through the removal of interferents, eliminating the neutral classes, such as the triacylglycerides, may also permit a re-evaluation of the chromatographic gradient used to resolve the species of interest. Previously, as the analysis methods applied in the characterisation of lipid extracts have also attempted to characterise these neutral compounds, they required mobile phase compositions and chromatographic gradients with the capacity to ensure their elution. As these compounds are considerably more hydrophobic, reversed-phase elution conditions ultimately must also increase in strength. Yet, due to the considerably hydrophilic nature of the phospholipids, and hence the PIs and PIPs, the strength of these elution conditions is non-conducive for the chromatography of their isomeric species, as these compounds may elute before achieving any appreciable resolution. Thus, eliminating these neutral species may allow for a weakening of mobile phase elution strength and a shallowing of the chromatographic gradient, permitting the increased retention of the species of interest and potentially enabling the resolution of isomers without increasing the analysis duration, as illustrated in Figure 13.

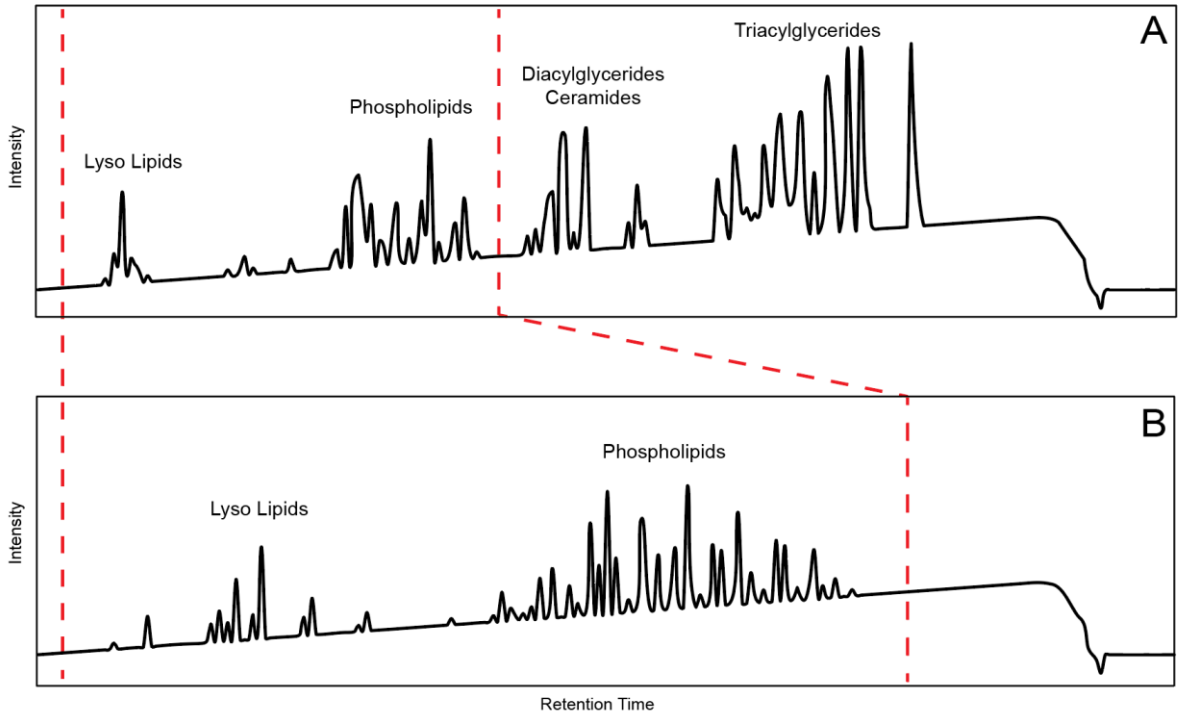

Figure 13 – A hypothetical reversed-phase chromatogram of a lipid extract demonstrating a general order of elution (A). Removing the non-polar fraction of the sample (B) would permit analysis conditions more suited to the resolution of the phospholipids and lyso lipids.

The high polarity of these lipids may also interfere with their chromatography under reversed-phase conditions, as their phosphate moieties and polyol headgroups are liable to non-specific polar interactions with the stationary phase and the instrumentation. This can result in chelation and rapid diffusion, especially in the case of multiply phosphorylated compounds such as the PIPs.[139, 140] An illustration of this mechanism is shown in Figure 14. As a consequence of this diffusion, generalised reversed-phase analysis methods typically produce broad peaks for the polar lipids, which rapidly increase in width the longer they are retained, thus counteracting any potential gains in resolution. Furthermore, these generalised analysis methods are physically unable to analyse the PIPs as they typically diffuse to the extent that they do not form peaks at all.[141] Alternatively, using a stationary phase and solvent system more suited to retaining polar analytes, such as those used in HILIC, might prove sufficient to disrupt the aforementioned non-specific interactions. Thus, it is evident that some compromises will be necessary to permit the in-depth characterisation of these compounds.

Ideally, an enhanced characterisation of the PIs would utilise a chromatographic separation technique capable of providing resolution between isomeric species, similar to what is achieved with reversed-phase chromatography, whilst limiting any non-specific interactions. This process is complicated by the fact that the analysis of intact PIPs is generally conducted through direct infusion mass spectrometry,[90, 142] and there is little precedent for how they may be chromatographically retained and resolved. In cases that do demonstrate the effective chromatographic retention of PIPs,[70, 143] these methods necessitate harsh conditions with high temperatures and highly alkaline pH, whilst also requiring ion-pairing agents and additives such as ethylamine, triethylamine and EDTA. Thus, despite their success, they may also rapidly degrade the stationary phase and are generally considered incompatible with mass spectrometric analysis due to the formation of non-volatile salts, which can contaminate the ion optics and result in signal suppression. Furthermore, due to the inclusion of the ion-pairing agents, the increased hydrophobicity of phospholipid-ion pairing agent complexes may significantly increase the retention of certain species beyond the elution capacity of a weaker mobile phase and limit the applicability of these methods in analysing the PIs. Hence, due to their particular chemical characteristics, there is a significant barrier to overcome in enabling the analysis of these molecular species. Consequently, no chromatographic methods are published outlining analysis conditions suitable for the simultaneous characterisation of the LPIs, PIs and PIPs, and this represents an obstacle to their characterisation in biological extracts.

An alternative approach for the characterisation of these compounds requires chemical modification prior to their analysis, whereby the detrimental effects imparted by the multiple

phosphate moieties on the PIPs can be nullified. The methylation of these groups has previously been demonstrated, for both PIPs and other phospholipids, enhancing their chromatography and ionisation efficiencies whilst also providing alternate and perhaps more descriptive fragmentation patterns.[144] Hence, a protocol of this type may be necessary to permit their analysis and could be incorporated directly after fractionation via SPE. However, this solution is not as straightforward as it might seem and has the potential to complicate the characterisation. In terms of chromatography, whilst methylation may prevent non-specific interaction, it may also greatly increase the hydrophobicity of certain PI species beyond the elution capacity of a weaker mobile phase, similar to the ion-pairing agents. Furthermore, multiple methylation states may arise, both in terms of the degree and sites of methylation, giving rise to numerous unnatural isomers that will interfere with the chromatography and the identification of these species. Finally, the chemical modifications achieved will induce a mass shift in the observed ions and alter their fragmentation mechanisms, thus causing current spectral libraries to become inapplicable for their characterisation. Hence, both the chromatography and identification of these compounds may present additional challenges that exceed the utility of derivatisation. Additional challenges arise around the development of conditions suitable for the analysis of the PIs, as not only must conditions be suited for their chromatography, but they are also constrained by their suitability for analysis via mass spectrometry. This necessarily excludes the aforementioned non-volatile compounds due to their incompatibility with mass spectrometric analysis despite their apparent necessity for chromatography in lieu of modifying the PIPs. Furthermore, it may also exclude certain additives necessary for mass spectrometry due to any potentially detrimental influences on chromatography. Thus, there is a need to balance both chromatographic performance and mass spectrometric sensitivity, whilst these criteria are not necessarily complementary. Attempting to develop a set of analysis conditions that adhere to all these criteria whilst permitting the characterisation of these compounds is challenging. Thus, taking into consideration the current method parameters used for the analysis of these compounds and iteratively biasing them to favour their analysis is perhaps a more efficient approach.

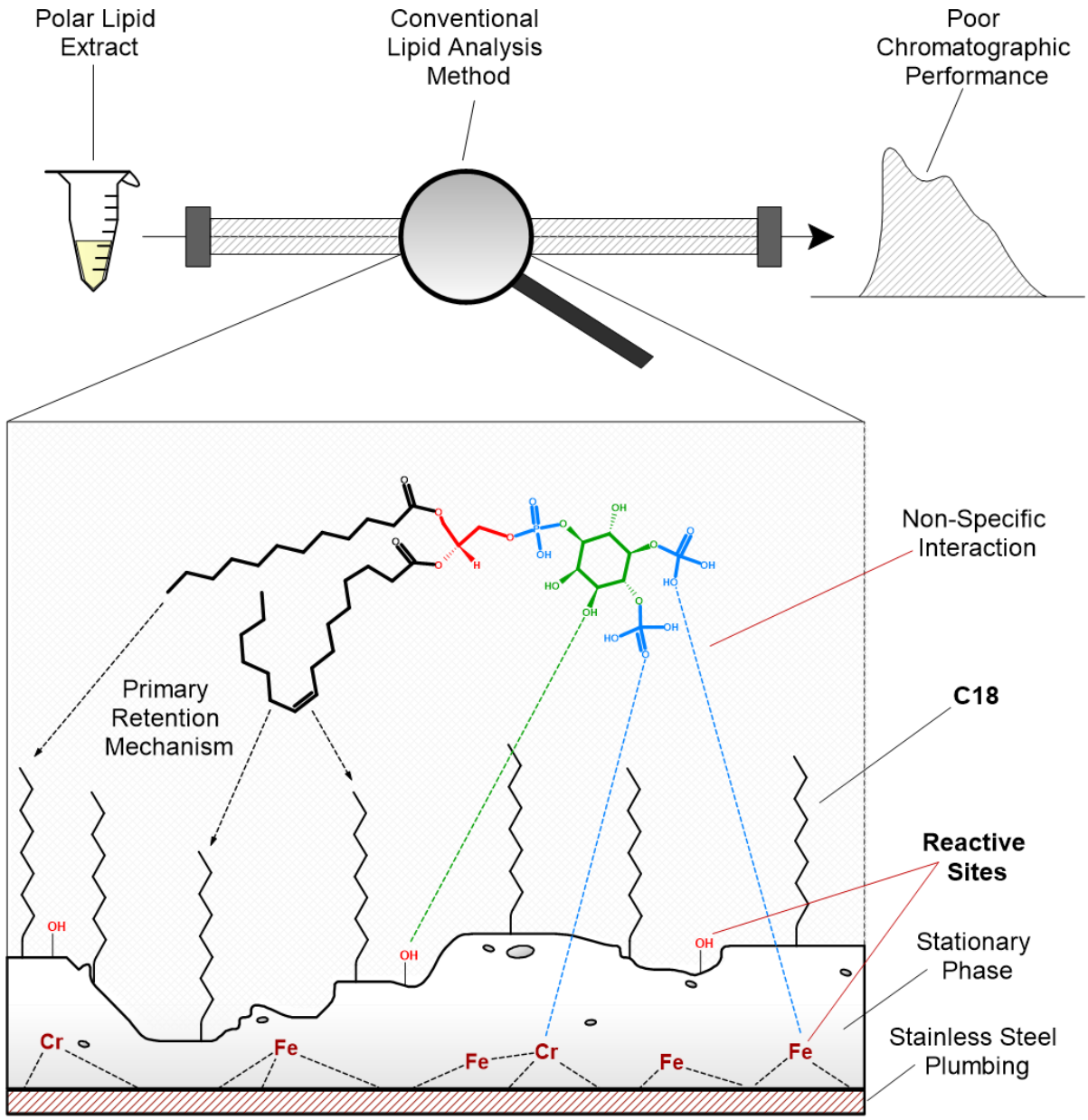

Figure 14 – An illustration depicting the mechanism of certain non-specific interactions that arise between polar lipids and a C18 (reversed-phase) stationary phase or instrumentation. Active silanols on the surface of the stationary phase or exposed metallic surfaces may serve as reactive sites. In the above illustration, these non-specific interactions compete and interfere with the primary retention mechanism and thus influence the chromatographic performance of these compounds. This may prevent the formation and resolution of peaks and thus result in poor chromatographic performance.

It is well-established that PIs ionise most readily under negative electrospray ionisation (ESI) conditions, for which ionisation may be facilitated through the use of alkaline additives,[74, 145] although there is contradictory evidence suggesting alkaline additives may suppress ionisation, and that either neutral [71] or even slightly acidic [72] modifiers may perform better. As these neutral and acidic conditions are frequently used in generalised lipidomic characterisations,[5-17] it may, to some extent, explain the often sparse number of PI species observed in these characterisations, especially as all of the aforementioned intact PIP

characterisations have instead required highly alkaline conditions,[70, 90, 142, 143] which facilitates ionisation and may disrupt the non-specific interactions.[146] Hence, a simple re-evaluation of these mobile phase additives may correspond to rapid gains in improving chromatography and ionisation.

However, further attention is necessary when considering the interpretation of mass spectrometric data generated through the analysis of these compounds, specifically as the PIPs are liable to form multiple adduct states once ionised and present several ions of unique mass-to-charge ratio (*m/z*), illustrated in Figure 15. Although this may aid in their identification, as observing multiple adducts is characteristic of these species, it may also hinder their detection as it effectively divides the signal corresponding to their concentration amongst these ions. It is possible that, to some extent, the relative ratios of these ions may be influenceable through the modification of ESI parameters or by adding mobile phase modifiers, and the influence of these parameters should be considered during method development. Furthermore, despite presenting these multiple adduct states, there is a noticeable lack of available spectra for identifying PIPs and other species, such as the Lyso PIs, where libraries such as LipidBlast [147] lack both these classes. While some experimental spectra have been gathered for a select few in repositories such as LipidMaps or the Human Metabolome Data Base, these are often of poor quality and may indicate that the characterisation of these species is predominantly a manual affair. Although it may be the case that these libraries have yet to be developed, due to the infrequent observation of these compounds, current lipidomic libraries are generally *in-silico* generated, and thus their fragmentation patterns are predicted. Ideally, to streamline the characterisation process, an in-silico generated library for identifying Lyso PIs and PIPs would be required.

There are multiple challenges to overcome in the pursuit of characterising the distribution of LPI, PI and PIP species in biological samples, from both the perspective of chromatographic analysis and mass spectrometric detection.

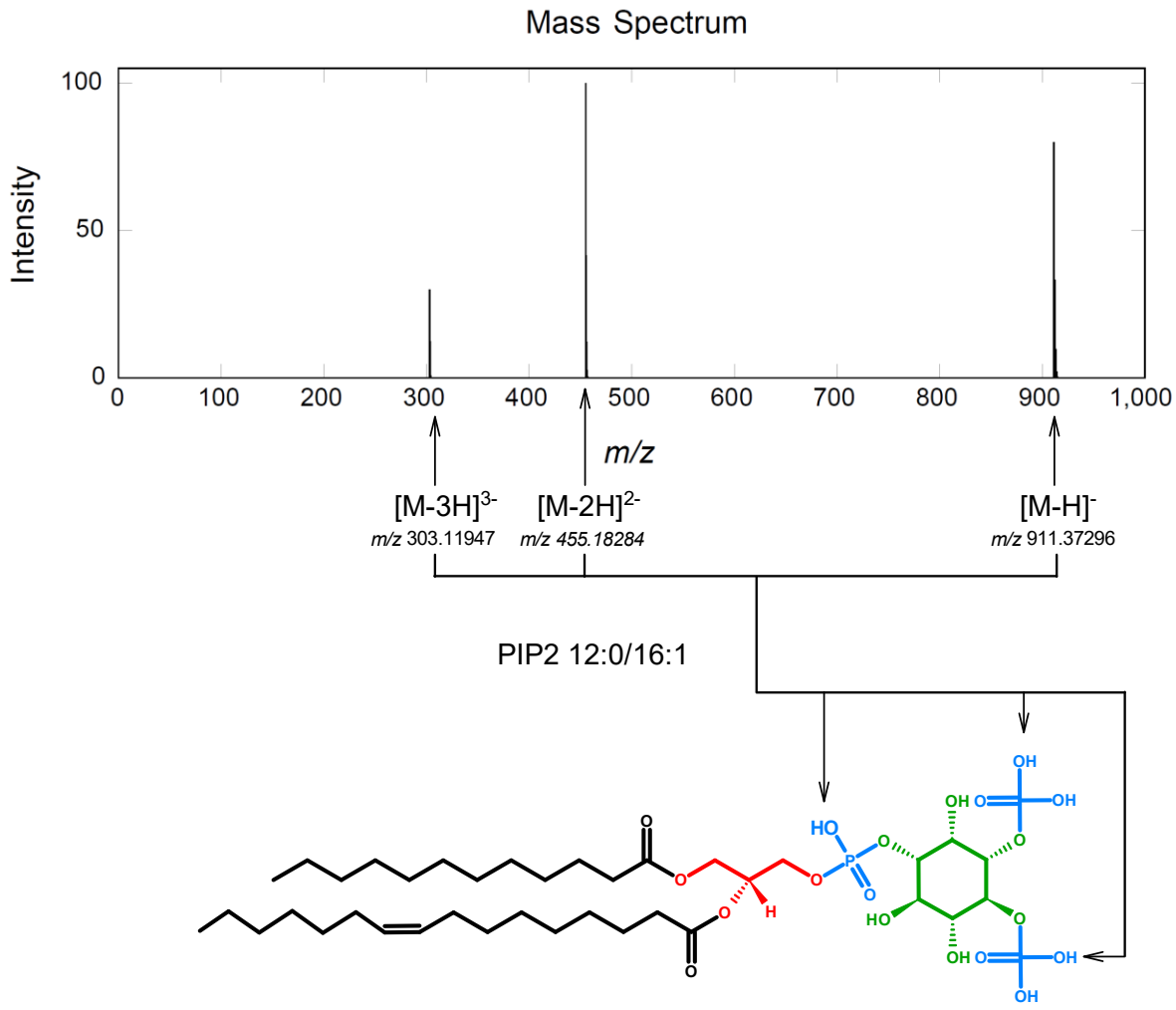

Figure 15 – A hypothetical mass spectrum consisting only of the adducts corresponding to a singular PIP2 species, i.e. PIP2 12:0/16:1. Here, three adducts are present, each corresponding to a hydrogen loss from each of the acidic phosphate groups as indicated. As a singular species creates three adducts, the signal intensity associated with the species is divided amongst the adducts. This division of signal may be particularly significant in the less abundant species, as it may prevent their detection.

Canola

Canola is a crop with large market value; it is the 3rd largest consumed oilseed, is cultivated in the order of 70 million metric tonnes globally p/a, and its production share is increasing at a rate faster than any other oilseed crop.[19] In terms of its lipid profile, canola fats are immensely valuable, nutritionally speaking, and they have been described as perhaps the only edible vegetable oil by today's standard due to their unique composition.[19] As a bi-product of oil production, canola lecithin and defatted canola meal are inserted into foods as emulsifiers, gelling agents, absorbants, stabilisers, thickeners, oleogelators, texturisers,[19] and may displace soy as the basis for infant formulae as it is non-allergenic [148] and presents a unique distribution of fatty acids compared to soy and other oil crops. To this end, the distribution of PI species and its derivitives in canola lecithins may be of particular interest in infant milk supplements due to its nutritional value and potential to induce physiological responses.

Simultaneously, as indicated in Figure 16, the quantity of PIs present in canola are expected to be relatively abundant; thus, canola extracts may serve as a valuable matrix on which to perform method development.

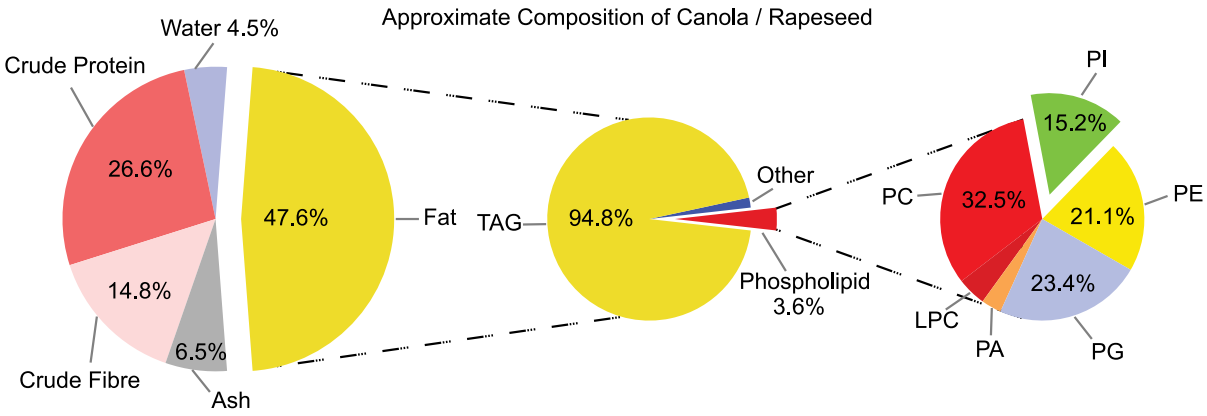

Figure 16 - An approximate compositional breakdown for canola concerning its phospholipid content, collated from various sources. [19, 148, 149]

Aims

- Develop chromatographic conditions for the simultaneous characterisation of the LPIs, PIs and PIPs,
- Enhance the detection of these compounds through either the modification of extraction protocols or analysis methods,
- Develop a mass spectral library for the characterisation of LPIs, PIs and PIPs,
- Characterise the LPI, PI and PIP species in canola.

Chapter Two:

Generation of *in-silico* Tandem Mass Spectral Database for the Identification of Phosphatidylinositols

PREAMBLE

Mass spectrometry (MS) forms the proverbial cornerstone of the modern analytical toolbox. By virtue of this technique, numerical data regarding a molecule's formula, structure, and concentration may be generated, enabling the specific identification of a sample's composition. Thus, for the purpose of matrix characterisation, MS is second to none and would be an invaluable tool in achieving the goals of this thesis. Therefore, it is unsurprising that this technique currently represents the standard approach for lipidomic characterisations due to its merits; however, one should not assume that this fact indicates a lack of complications. As this technique underpins the means of lipid identification and elucidation, it is perhaps prudent to first ensure that it is appropriately utilised for the characterisation of PIs and their derivatives.

Due to the immense quantities of data generated in a typical untargeted MS analysis, interpreting the results of an MS experiment is an overwhelming ordeal if attempted manually. Various software tools have streamlined this process by automatically conducting peak-picking, spectra-matching and enabling the simultaneous comparison of multiple samples. Therefore, the requirements for processing MS data are more or less reduced to the quality of the spectral library used for the targeted compounds. These spectral libraries essentially comprise repositories of molecules and adducts that record the distribution and ratios of product ions produced upon the fragmentation of a particular precursor structure. These ions, therefore, serve as a characteristic fingerprint for the parent structure and thus provide evidence for identification.

The fragmentation pattern of a particular compound can be very difficult to predict. In most cases, an authentic standard of the targeted compound is necessary so that the experimentally observed fragmentation pattern may be recorded in a spectral library for later use. However, the realm of molecular structures is incredibly diverse, especially in the case of lipidomic analysis, where tens to hundreds of thousands of distinct lipid species may simultaneously be present within a single matrix.[23-26] Hence, the number of standards required to annotate a sample in this way is prohibitive, even for the most well-resourced laboratories.

Certain approaches in spectral library curation can ease this burden, specifically through *in-silico* generation. This technique utilises available experimental spectral libraries, in conjunction with an understanding of fragmentation mechanisms, to extrapolate and interpolate between known structures and predict what the fragmentation pattern for a hypothetical structure may look like. In the case of lipids, structural modifications are generally systematic, and therefore, most lipid fragmentation patterns are systematically predictable.

Currently, LipidBlast [147] is the most extensive *in-silico*-generated spectral library available for lipidomic analysis, providing a total of 212,685 MS/MS spectra, covering 29 lipid classes and

57 different adducts. However, despite this immense breadth, the library contains no fragmentation spectra for the LPIs or any of the PIPs. Hence, if this library were applied in lipidomic analysis, no LPI or PIP candidates would be identified; thus, this library is inapplicable for their analysis. Very few other libraries have been curated for lipidomic analysis, and none are as comprehensive. Additional complications also arise when inspecting the fragmentation spectra provided for the PIs themselves. Although LipidBlast provides 5476 fragmentation spectra for the PIs, the number of unique fragmentation spectra represented is far less, as many of the generated spectra represent isomers of one another. LipidBlast does not differentiate these isomers by their fragmentation patterns; hence, in actuality, the LipidBlast library contains only 1176 unique fragmentation spectra for the PIs.

Whilst this remaining number of fragmentation spectra is perhaps sufficient for extensive characterisation of PIs, the spectra themselves deviate considerably from the experimentally observed fragmentation patterns for these compounds, as demonstrated in Figure 17, and are, as a result, inadequate for their identification. This deviation is not necessarily an error with the LipidBlast spectral library, as numerous external influences can contribute to the precise distribution of fragment ions observed; however, it does represent an obstacle, as initial attempts at characterisation would result in the identification of 0 LPIs, PIs and PIPs, even though one might be able to observe them with manual inspection using an intuition of their fragmentation mechanism.

These circumstances, therefore, necessitate a new and expanded spectral library to permit the identification of these species. Additionally, this library should preferentially be dynamic and modifiable based on experimental evidence to avoid potential spectra mismatching in the future. Previous work has been published concerning the fragmentation mechanisms of the PIs and PIPs,[18] summarised in Figure 18, and thus, a series of updated templates could be developed for these compounds by calculating the mass of fragment ions according to a pre-defined table of rules and transitions.

Initial attempts to recreate the fragmentation rules observed for the PIs began with a simple Python script and proved extremely successful in accurately reproducing the experimentally observed fragmentation spectra. Due to this success, the script was further developed to incorporate templates for the LPIs and PIPs, along with the fragmentation patterns for the methylated PIPs and the mannosylated PIs. A user interface was included that enabled fragmentation rules to be added or subtracted from templates and fragment intensities to be changed so these spectra could be dynamically updated. These final additions would ensure the versatility of the script, preventing the generation of static spectra, which may eventually prove invalid, so long as the experimentally acquired spectrum of a valid standard is available.

The inclusion of these additional fragmentation templates revealed the ease with which the spectral library generator could be updated and its scope expanded, and thus, the evaluation of the literature concerning lipid fragmentation continued. This work culminated in the following chapter, published in the journal *Analytical Chemistry*, titled 'Lipid Spectra Generator: a simple script for the generation of accurate in-silico lipid fragmentation spectra', at which point the script expanded to contain 136 lipid templates covering 87 lipid classes.

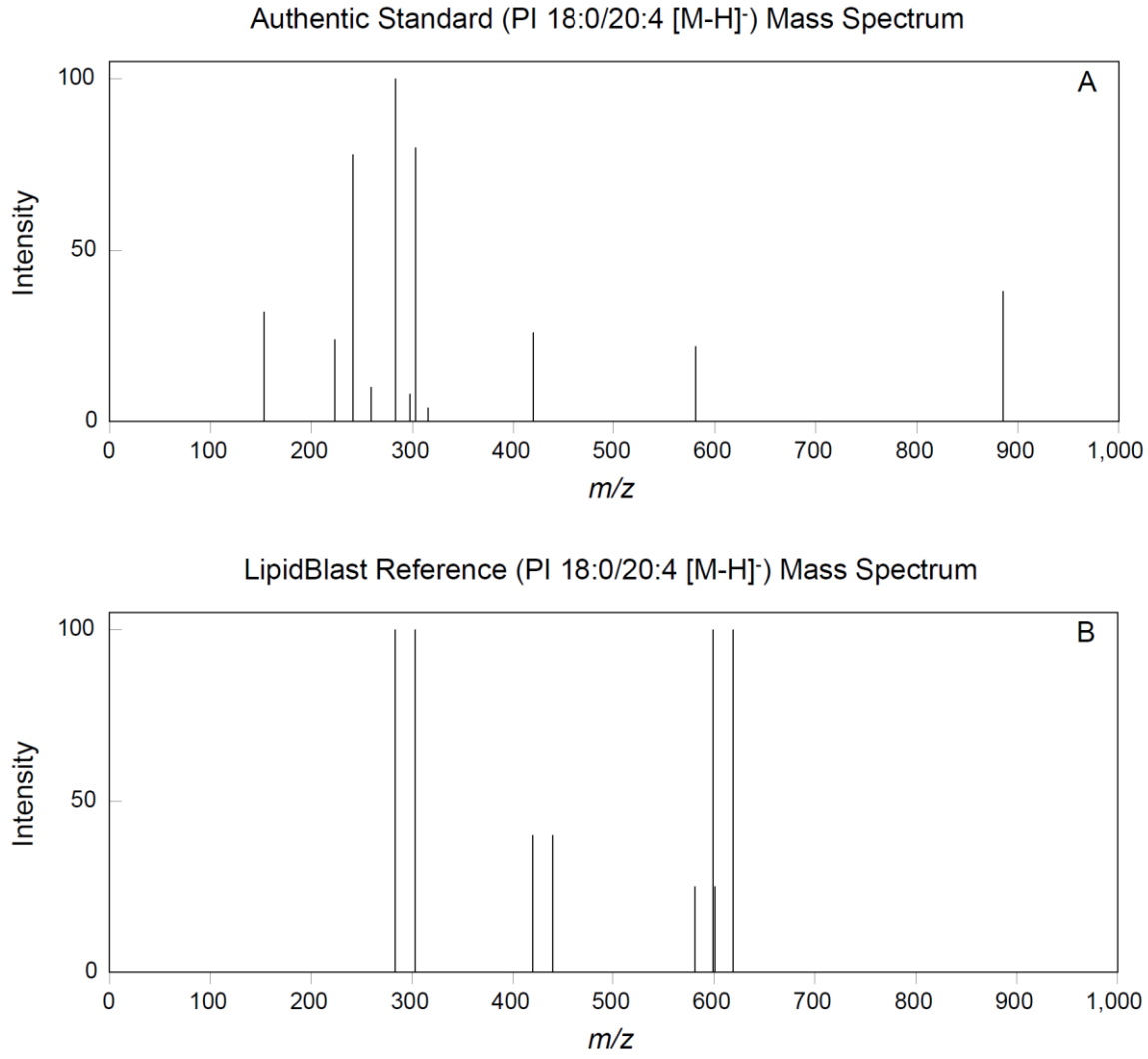

Figure 17 – A comparison between an experimentally obtained fragmentation spectra for the authentic molecular standard PI 18:0/20:4 (A), and the LipidBlast *in-silico* reference spectra for the same molecule (B). There is a stark contrast between the distribution of fragment ions observed in either case, and data processing workflows would be unable to identify the standard's spectra utilising the LipidBlast reference.

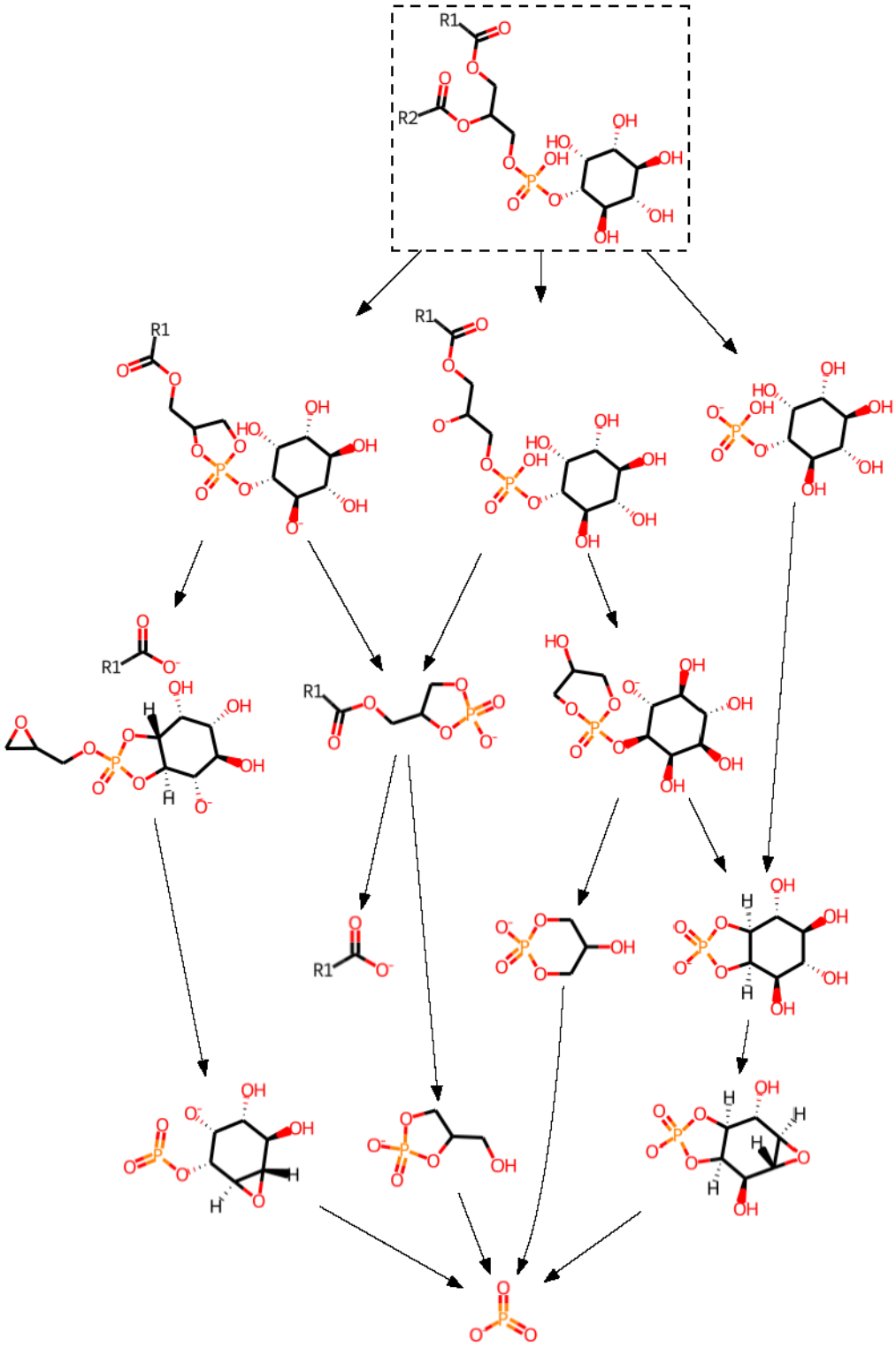

Figure 18 – A simplified representation of the generalised fragmentation pathway for a precursor [M-H]$^-$ phosphatidylinositol species (boxed), proposed by Fong-Fu Hsu and John Turk in their mechanistic study [18-20]. The inter-related nature of these fragments demonstrates the manner in which a precursor ion may generate a characteristic fingerprint of daughter ions, and thus provide evidence for its structure. Several additional fragmentation pathways are excluded for brevity. Specifically, the molecular structures provided represent only the major fragments produced after initial loss of the *sn-2* fatty acid. Additionally, the phosphatidic acid and diacylglycerol fragments produced after loss of the lipid headgroup are here excluded. These fragments are, however, equally valued for identification.

CERTIFICATE OF AUTHORSHIP

The following chapter is an accepted manuscript published in *Analytical Chemistry (Anal. Chem.)*. I, David Gertner, certify that the work in the following chapter has not been submitted as part of any other documents required for a degree.

AUTHORSHIP CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
DAVID S. GERTNER	CONCEPTUALISATION, EXPERIMENTAL DESIGN, SOFTWARE DEVELOPMENT, PERFORMED EXPERIMENTS, ANALYSED DATA, WROTE ORIGINAL DRAFT AND EDITED MANUSCRIPT	Production Note: Signature removed prior to publication.
JAKE P. VIOLI	SAMPLE PREPARATION, PERFORMED EXPERIMENTS	Production Note: Signature removed prior to publication.
DAVID P. BISHOP	REVIEWED AND EDITED MANUSCRIPT	Production Note: Signature removed prior to publication.
MATTHEW P. PADULA	REVIEWED AND EDITED MANUSCRIPT	Production Note: Signature removed prior to publication.

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Lipid Spectra Generator: a simple script for the generation of accurate in-silico lipid fragmentation spectra

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ABSTRACT: Due to the complexity of lipids in nature, the use of *in-silico* generated spectral libraries to identify lipid species from mass spectral data has become an integral part of many lipidomic workflows. However, many *in-silico* libraries are either limited in usability or their capacity to represent lipid species. Here we introduce Lipid Spectrum Generator, an open-source *in-silico* spectral library generator specifically designed to aid in the identification of lipids in LC-MS/MS analysis.

BACKGROUND

The complexity of lipids throughout nature is immense, where tens to hundreds of thousands of distinct lipid species may simultaneously be present within a single matrix.[23-26] The individual species which constitute this diversity are not themselves genetically encoded but arise in response to the dietary, physiological, and environmental factors which influence a given biological system.[24-26] Their dynamic nature in response to these factors, in conjunction with their biological importance, has led to their analysis being used to monitor cellular metabolic processes or even the development of pathologies.[23, 25, 26, 150-153] The discipline of Lipidomics, which concerns the identification and characterisation of these compounds, must therefore contend with this immense molecular diversity to reveal biomarkers of relevance. As a result, analytical technologies such as tandem mass spectrometric (MS/MS) analysis have championed the field due to their potential to structurally elucidate virtually all sample constituents.[23-25, 137, 154]

Lipid identification employing MS/MS analysis follows the fragmentation of a precursor compound and the interpretation of its product ions (fragmentation spectra).[23, 24] In place of manual interpretation, identification requires a reference against which fragmentation patterns can be compared. In ideal cases, molecular standards may be used to determine the fragmentation patterns for lipids of interest. However, the number of standards required to

annotate a sample in this way is logistically infeasible for even the most well-resourced laboratories. As a result, the interpretation of fragmentation spectra, and thus the characterisation of lipid species present in a matrix, represents a severe bottleneck in lipidomic analyses.

In the absence of standards, lipidomic workflows have relied on computationally generated (*in-silico*) fragmentation spectra, as they facilitate rapid sample annotation and characterisation where it is otherwise impossible.[137, 154-157] The advent of such spectra has resulted from an increased understanding of lipid fragmentation pathways [158, 159] and the often predictable manner in which lipids fragment. Hence, spectra of this kind are regularly produced through various rules-based,[147, 155, 156] algorithmic,[160-162] or machine learning [163] approaches, enabling an accurate representation of authentic lipid fragmentation patterns.

However, despite their broad adoption, many *in-silico* libraries are limited in their capacity to represent lipid species. Complications of this sort arise due to (i) limited consideration of lipid classes; (ii) shallow representation of lipid molecular species; (iii) incomplete fragmentation patterns which exclude characteristic ions or misrepresent the relative intensities for each fragment; (iv) incorrect annotations which assume structure not apparent within the spectra; (v) and the presence of duplicate fragmentation patterns which can obscure identifications. Whilst some recent publications have sought to remedy these shortcomings,[155, 156, 163-165] many of these focus on replacing pre-existing lipidomic workflows and provide libraries that are otherwise externally incompatible, preventing their use elsewhere. Hence despite their utility, such cases represent a deviation from the convenience and universality of previous *in-silico* libraries.[147]

In response to these limitations, we have developed an *in-silico* library generator for lipidomic analysis, Lipid Spectra Generator (LSG). The software is open-source, Python-based, and implements the cross-platform Qt5 graphical user interface to guide the user through the steps necessary to generate a highly customisable *in-silico* library for lipidomic analysis. To date, LSG contains the most comprehensive set of templates available for a software of its type, enabling spectra generation for 87 lipid classes, using over 1000 potential fatty acid chains, allowing the generation of well over 1.2 million lipid species. Users may export MSP *in-silico* generated spectral libraries from these templates, enabling incorporation into any third-party or vendor-specific mass spectral library application and CSV precursor and transition lists that permit targeted lipidomic analysis and compatibility with the Skyline ecosystem.[166]

EXPERIMENTAL

SOFTWARE DESIGN

Lipid Spectra Generator is an open-source, Python-based script that is freely available for download from GitHub (https://github.com/98104781/LSG/releases/tag/v1.0.0). A cross-

platform Qt5-based GUI is implemented using PySide2, and it is designed to guide the user through the steps necessary to produce an MSP formatted spectral library or CSV formatted inclusion and transition lists. Lipid species are generated in the software through the specification of fatty acid tails and the selection of lipid classes. Fatty acids may be individually specified or generated as a range, permitting the creation of both highly specific and comprehensive spectral libraries. Lipid fragmentation spectra are generated from a set of pre-defined templates which can be customised in software, allowing for user modification of lipid fragmentation patterns and fragment intensities. The lipid fragmentation templates used have been collated from a set of peer-reviewed fragmentation studies of lipid classes, annotated mass spectra of lipid standards, and previously validated *in-silico* templates. The literature sources for these templates are provided in Supplementary Table 1.

MATERIALS

Methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA) used were of LC/MS grade, Honeywell B&J Brand, purchased from ChemSupply Australia.

Ultra-pure water (UP) (18.2 MΩ-cm) was sourced from an in-lab water purification system (Sartorius).

PBS tablets, Avanti EquiSPLASH mix, HPLC grade chloroform, analytical reagent grade ammonium acetate and ammonium formate, and LC/MS grate (98% - 100% purity) Lichropur brand formic acid were all purchased from Sigma-Aldrich.

Glutamax, fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and eagle's minimal essential medium (EMEM) were purchased from Thermofisher scientific (Thermo Fisher Scientific, VIC, Scoresby).

SH-SY5Y SAMPLE PREPARATION

SH-SY5Y (human neuroblastoma) cells (passage 23) were cultured in a T175 flask using DMEM supplemented with 5% glutamax and 10% FBS. The cells were then seeded in a t75 flask at 50% confluency, using EMEM with 5% glutamax and 10% FBS. The cells were then left to adhere overnight. Following this, fresh media was applied, and cells were left for 48hrs. They were then harvested according to the following protocol:

First, media was removed and replaced with 5 mL of TrypLE. Flasks were then left in an incubator at 37 °C for 5 minutes. Cells suspended in the TrypLE were moved to a 15 mL tube and centrifuged at 300 g at room temperature for 5 minutes. The supernatant was then discarded, and 5 mL of PBS was added. The pellet was then gently broken up and again centrifuged under the same conditions. The previous two steps were conducted twice to ensure adequate washing. The remaining cell pellets were snap-frozen with liquid nitrogen and stored at -80 °C until extraction. Three replicate extracts were prepared according to the following protocol:

In a 15 mL falcon tube, 500 mL of chloroform was added to approximately 25 mg of cell mass, followed by 500 mL of methanol containing a mix of deuterium-labelled internal standards (50 ppb; EquiSPLASH Mix, Avanti Polar Lipids). Next, the samples were vortex-mixed for 30 seconds before adding 500 mL of ultra-pure water. Following this, the samples were then agitated for a period of 30 minutes at 4 °C on a rotary mixer before centrifugation at 3000 rpm for 10 minutes. After phase separation had occurred, 200 mL of the organic layer (bottom) was removed and dried under a continuous flow of dry N_2. Dry extracts were then reconstituted in ACN: IPA (100 mL; 1:1) and stored at –80 °C until analysis.

LC-MS/MS ANALYSIS

To demonstrate the utility of LSG, a set of LC-MS/MS lipidomic characterisations were conducted and processed against an LSG-generated spectral library. The number of identifications observed, along with their identification scores, were then compared to those achieved with a library generated using the LipiDex[155] Library Generator, and the previously published LipidBlast *in*-silico spectral library.[147] The LSG spectral library was generated using the default specified-tails list and contained only those classes expected to ionise under the selected ESI conditions. The LipiDex spectral library was generated using the LipiDex_HCD_Acetate library, with all default available fatty acids. Results were then batch processed and identified through use of MzMine 2, version 2.53.[167, 168] For each library, the MzMine peak lists were first filtered using a precursor search, and then identified using the local spectral database search option. Identifications were accepted with a precursor tolerance of 5 ppm, spectral tolerance of 25 ppm, and minimum weighted dot-product cosine of 0.5, using two minimum matched signals. The 'crop spectra to *m/z* overlap' option was disabled to permit comparison, and results were weighted using the NIST11 (LC) weights.

During analysis, SH-SY5Y extracts were chromatographically separated on a Waters Acquity CSH column (1.7 µm, 135 Å, 150 mm x 2.1 mm) using an Agilent Infinity II 1290 UHPLC. The flow rate and column temperature were maintained at 0.5 mL/min and 65 °C, respectively. The mobile phases used differed in buffer composition between positive (10mM ammonium formate + 0.1% (v/v) formic acid to both A and B) and negative (10mM ammonium acetate to both A and B) ESI modes, although the solvent composition of mobile phases A (ACN: H_2O; 60: 40) and B (IPA: ACN; 90: 10) remained the same. The same chromatographic gradient was used for both analysis modes. The mobile phase was held initially at 30% B for 2 minutes and then raised linearly to 50% B from 2 to 2.5 minutes. Additional linear increases up to 85% B and 99% B occurred between 2.5 to 13 and 13 to 13.5 minutes, respectively. The final mix of 99% B was held until 15 minutes. The column was then re-equilibrated with an immediate drop to 30% B, which was held until 18 minutes. Mass spectrometric analysis was conducted using a Thermo Scientific Q Exactive Plus in Top-10 mode. MS1 spectra were accumulated

between masses 200 to 1200 *m/z* for a max IT of 100 ms and AGC of 3e6 and analysed with a mass resolution of 70,000. MS2 spectra were accumulated for a max IT of 60 ms with an AGC of 1e5 and mass resolution of 17,500, and fragmented with a stepped collision energy at 10, 20 and 30 units.

ASSESSMENT OF TRUE-POSITIVE RATE

Validation followed the LC-MS/MS characterisations to determine the extent of true-positive identifications that could be achieved with each library. To conduct these tests, a previously evaluated reference matrix was required for comparison. For this purpose, the publicly available AdipoAtlas [169] samples were used, which consisted of a database of 1,636 lipid identifications. 9 lipid classes from subcutaneous and visceral fat extracts were assessed in positive ion mode only. These classes were selected based on their common presence throughout all lipidomic libraries and samples. Identification and scoring were conducted identically to the LC-MS/MS Analysis section, except where the spectral libraries were modified to contain only the expected fatty acids to limit analysis time. The resulting identifications were then compared to those present in reference 26 to determine the proportion of matching identifications. Any additional identifications were ignored for the purpose of the comparison.

RESULTS AND DISCUSSION

Many of the complications associated with previous *in-silico* libraries for lipidomics stem from restrictions related to their size and accuracy. Primarily, these arise due to the influence these aspects have on the efficacy of a library during analysis.[170]

The size of a spectral library represents the total quantity of spectra it contains and, thus, the number of molecular species it accounts for. Any compound not present within a library cannot be identified when using it. Hence, more extensive and comprehensive libraries are often preferred. When considering lipidomic spectral libraries, size may be further segmented into terms of lipid class and lipid molecular species. Yet, when library size is considered, classes are often the primary concern of lipidomic spectral libraries. As a result, many libraries provide a shallow representation of lipid molecular diversity and, thus, a limited domain of utility. However, spectral libraries may likewise become excessive in size and potentially burden analysis workflows due to the computational requirements necessary for their generation and use.

Beyond size, spectral accuracy is also critical in determining the effectiveness of an *in-silico* spectral library. The accuracy of an *in-silico* spectrum depends on various factors, including the accuracy of mass labels and the completion and specificity of fragmentation patterns. However, spectrum accuracy can also depend on the circumstances of analysis, e.g. the MS used. The presence and intensity of product ions can often vary along with analysis factors

such as collision energy, causing otherwise accurate spectra may become invalid based on these circumstances.[170] Ideally, a spectral library must dynamically account for these variations, as they can influence the relative number of true-positive identifications made.

Correct spectra annotations are also of particular importance when considering spectra accuracy, as isomeric lipid species can often produce identical fragmentation patterns. As a result, spectral libraries can risk incorrectly assigning identifications to numerous candidates. Conventionally, conflicts of this sort can be resolved by assigning fragmentation patterns with structurally ambiguous annotations, such as those shown in Figure 19, which prevent the over-interpretation of features that are not evident in the spectra.[171, 172]

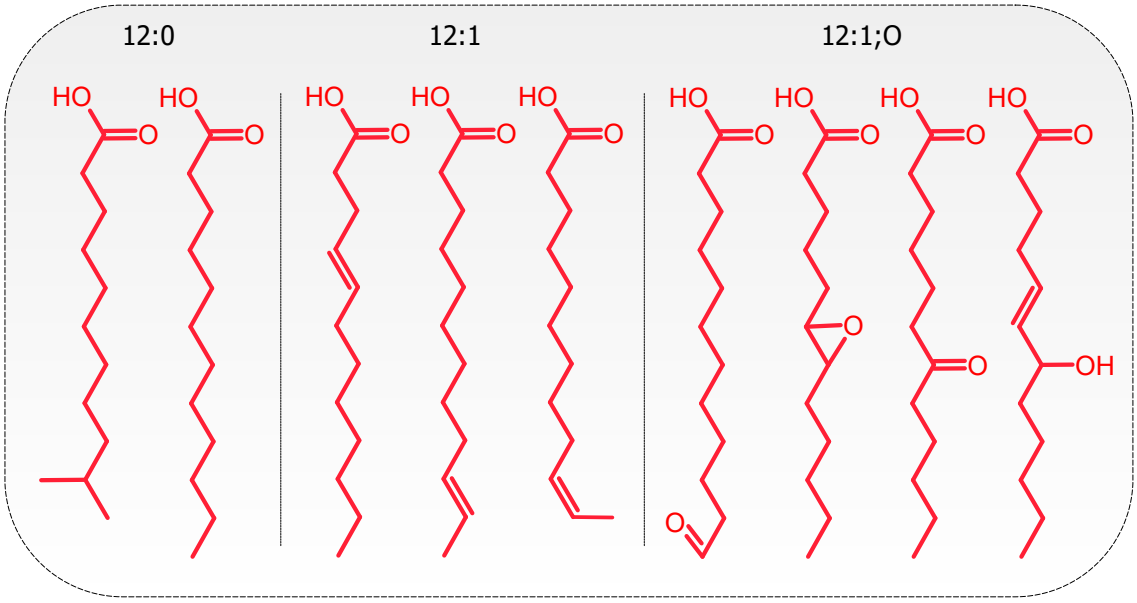

Figure 19 - Example annotations assigned to isomeric fatty acids. Species on the left may be represented with 12:0, indicating a chain of twelve carbons with zero points of desaturation. Species in the center may be represented with the name 12:1, signifying a chain of twelve carbons, one point of desaturation. Species on the right may be represented with the name 12:1;O, signifying a chain of twelve carbons, one point of desaturation and one point of oxidation. Assuming no further fragmentation or sample derivatisation is conducted, the true structure of the fatty acids cannot be determined from the parent lipids' fragmentation pattern. Instead, the systematic names (e.g. 12:1;O and 12:0) are used to ambiguously designate the fatty acids' structure.

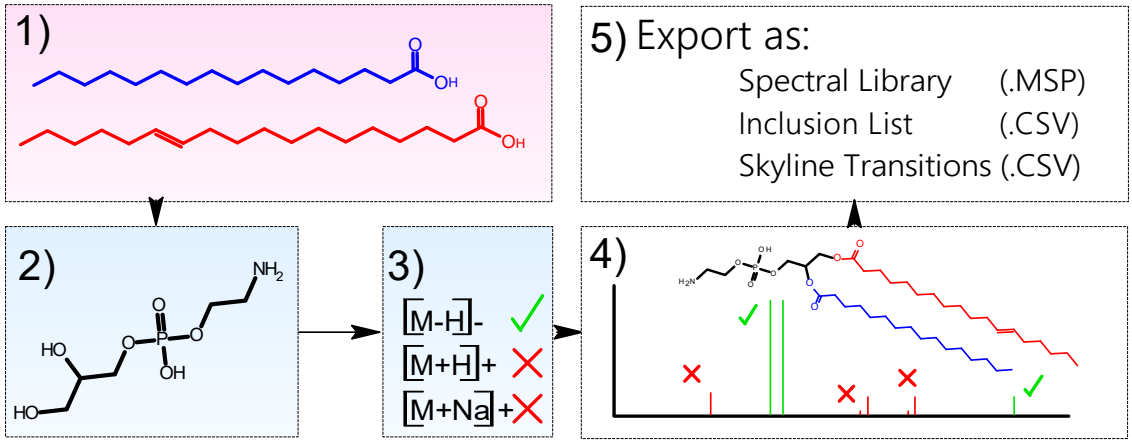

Figure 20 - Flowchart outlining the process of spectral library generation using LSG. 1) Fatty acid species are defined. Individual fatty acid species may be chosen or generated as a range. 2) Lipid classes of interest are selected. 3) Desired fragmentation spectra, based on adducts, are selected. 4) Desired fragmentation spectra may be further modified to better represent the experimental fragmentation patterns observed. 5) The export format is selected. Exporting as a .MSP file will produce a spectral library containing those classes selected. A .CSV inclusion list, formatted for a QE+ Orbitrap mass spectrometer, may be exported. A .CSV transition list, formatted for Skyline, may also be exported

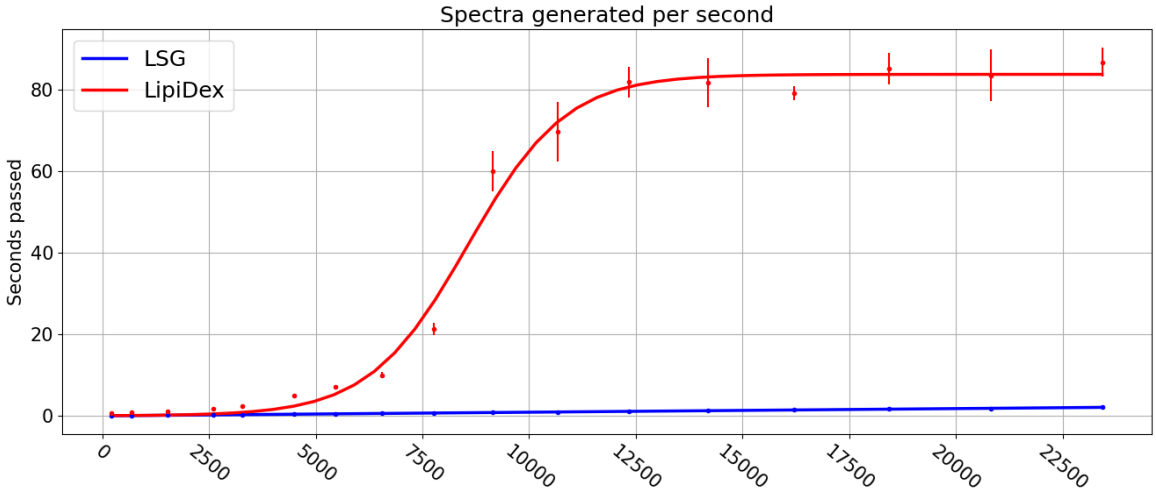

Figure 21 - Libraries of varying size were generated to reveal the trend between processing time and the number of generated spectra. For these tests, TGs were selected due to the large number of potential species that could be generated. Although TG species may more frequently be observed in the form $[M+NH_4]+$, the [M+Na]+ adduct was selected here due to the far greater spectra complexity represented by LSG than in LipiDex. I.e. in a LipiDex library, a TG species with 3 unique fatty acids would produce a spectra of 4 fragments in a $[M+NH_4]+$ spectra and 3 in a [M+Na]+ spectra; whereas with LSG, the same species would produce 8 fragments in a $[M+NH_4]+$ spectra and 13 in a [M+Na]+ spectra. This indicates that LSG may produce far more complex spectra than competing software in a minute fraction of the time. Each test was conducted a total of 5 times, and the average duration plotted. Other spectra may take more or less time to generate, based on their complexity.

LSG was designed to directly address these concerns and permit the generation of spectral libraries suitable for all analyses. As a result, many of the software's features are specifically designed to enable precise control over library size and spectral accuracy. Libraries are scaled in terms of classes and molecular species through the selection of fragmentation templates

and the specification of fatty acids, whereas accuracy is controlled by adding, subtracting or manipulating the fragments in the templates. This enables dynamic modification of generated spectra through user input to account for variations that may arise due to instrumentation or method parameters. A simplified representation of the process of library generation is shown in Figure 20. The libraries produced are currently the most comprehensive of their kind, with a total of 136 fragmentation templates and 67 literature-sourced fatty acid species pre-defined in the software,[173, 174] though over 1000 fatty acid species may be created to expand coverage over both well-documented and hypothetical lipid species. The generated spectra are likewise dynamic and can be modified through user input to account for variations that may arise due to instrumentation or method parameters.

Libraries are rapidly produced, as highlighted in Figure 21, with approximately 12,000 unique fragmentation spectra generated per second, representing almost two orders of magnitude increase in speed compared to the LipiDex Library Generator, which can take up to 80 seconds to create a library of equal size. Furthermore, library sizes are efficiently reduced to prevent computational burden through the use of ambiguous fatty acid notation. The fatty acid species represented in software are intentionally ambiguous as to the arrangement of certain isomeric features, such as the isomerism of double bonds or the sites and types of oxidation. Maintaining this level of ambiguity permits a single fatty acid annotation to represent a plethora of isomeric molecular species, such as those in Figure 19. The resulting compression prevents molecular structure over-interpretation and considerably reduces library size without compromising the number of molecular species accounted for, as a single fragmentation spectra may apply to numerous molecular species. Structural ambiguity of this type is further utilised when considering the *sn*-arrangement for the fatty acids attached to a lipid's backbone. Typically, the fragmentation spectra produced from these types of isomers differ only in the ratios of certain fragments but are otherwise identical. Thus, by disregarding this variability and maintaining an equal ratio between these variable fragments, library size is further compressed by a proportion equal to the number of potential isomers at the expense of the specificity of the identification. However, for some specific lipids, such as cardiolipins, unique fragments are indeed produced based on this arrangement. For these classes, some *sn*-specificity is considered by default; however, this option may be disabled by the user if maintaining a smaller library is of more utility than the added specificity. In cases where the generated spectra are devoid of information regarding lipid fatty acid composition, whether by default or after user manipulation, lipid notation is changed to species-level notation [175], and the libraries are further compressed.

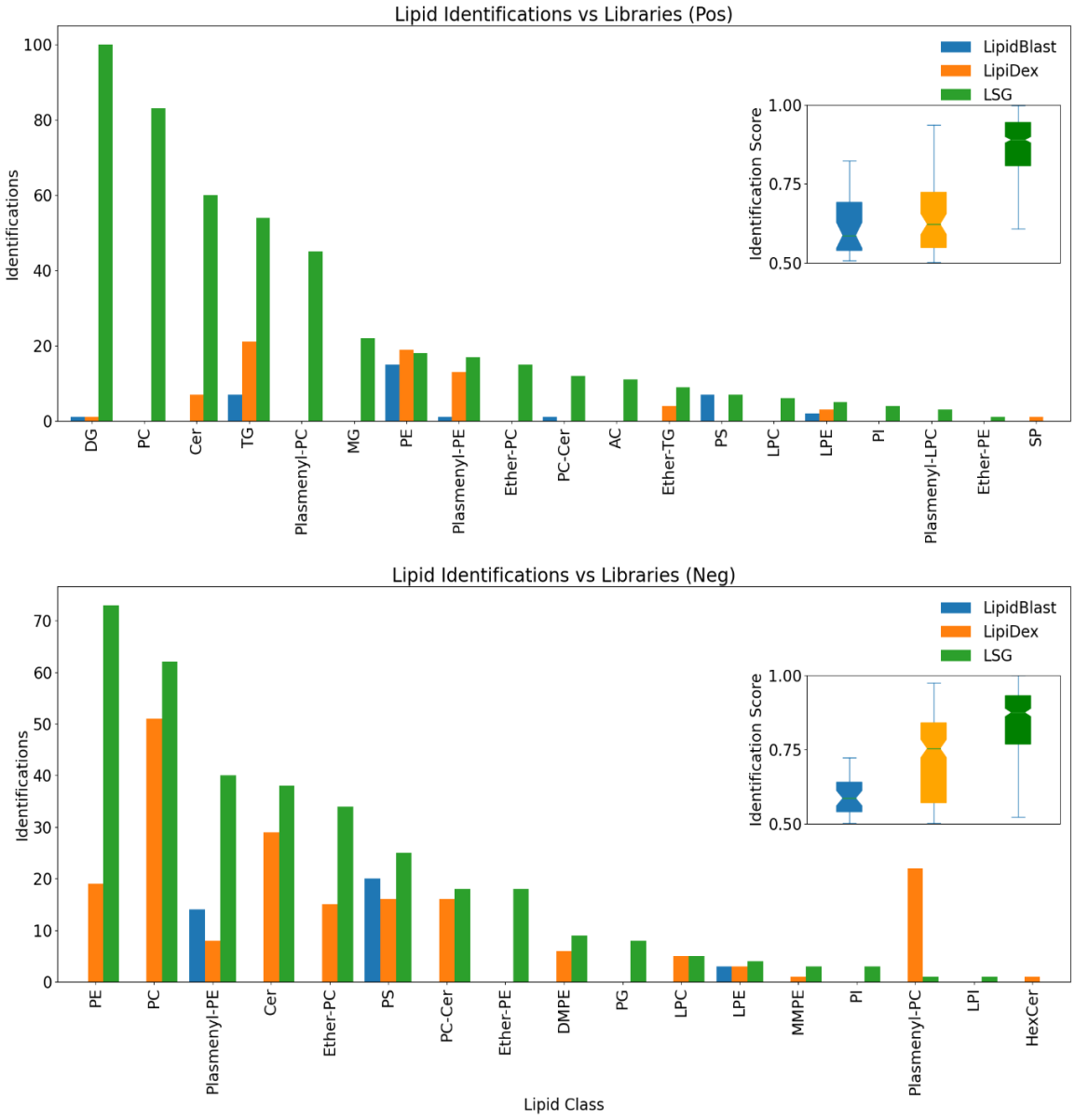

Figure 22 - Quantity of candidate lipid identifications achieved with differing in-silico libraries organized by class, along with identification scores. The LSG library results are represented in green, LipiDex in orange and LipidBlast is in blue. The cut-off value for the identification score was reduced to 0.5 to permit all the libraries used to obtain candidates. Due to this decreased cut-off, some identifications are likely to be false positives, and thus remain as candidates rather than true identifications. 474 lipid candidates were achieved with LSG in positive analysis mode, and 342 in negative. 70 lipid candidates were achieved with LipiDex in positive mode, and 192 in negative. 34 lipid candidates were achieved with LipidBlast in positive mode, and 37 in negative. Candidates include multiple adduct states for individual lipid species. AC – Acylcarnitine, CE – Cholesterol Ester, MG – Monoacylglycerol, DG – Diacylglycerol, TG – Triacylglycerol, PC – Phosphatidylcholine, LysoPC – Lyso Phosphatidylcholine, PE – Phosphatidylethanolamine, LysoPE – Lyso Phosphatidylethanolamine, MMPE – Monomethyl Phosphatidylethanolamine, DMPE – Dimethyl Phosphatidylethanolamine, PG – Phosphatidylglycerol, PI – Phosphatidylinositol, PS – Phosphatidylserine, SM – Sphingomyelin, HexCer –ceramide-1-hexose.

Minor fragments produced by some oxidised fatty acid species are ignored to maintain the benefits of this compression. While such fragments may provide evidence for the precise site

of fatty acid oxidation, they are not necessary for determining the degree of oxidation nor on which fatty acid the oxidation occurs. Rather, with their removal, the degree of structural information contained within the fragmentation spectra remains consistent with that regarding the types and sites of desaturation. A caveat of this simplification is that the generated fragmentation spectra are not representative of any particular species in which the type and site of oxidation interfere with the generalised fragmentation pathways, which serve as the basis for the templates. Fortunately, such deviations are limited to specific molecular arrangements, such as with ceramides containing α-hydroxy fatty acids.[176] However, it is essential to understand the limitations of the templates regarding these molecular species. Other factors which may also influence the relative intensity of certain fatty acid fragments, such as poly-unsaturation, are likewise not considered.

To verify whether the modifications provided by LSG translated to the more thorough annotation of a sample's lipidome, an LSG-generated spectral library was applied in analysis, and the number of features observed compared to those achieved with a LipiDex generated library, and the LipidBlast spectral library. For this comparison, triplicate injections of an SH-SY5Y extract were chromatographically separated in both positive and negative esi modes and processed according to the aforementioned conditions. The results of this comparison are provided in Figure 22. LSG achieves a 3.5 to 4-fold increase in lipid identifications over the LipiDex and LipidBlast libraries, while identification scores also increase markedly. It is suggested here that the greater quantity of annotations observed results from the greater coverage of molecular species provided by the LSG library. In contrast, the increase in

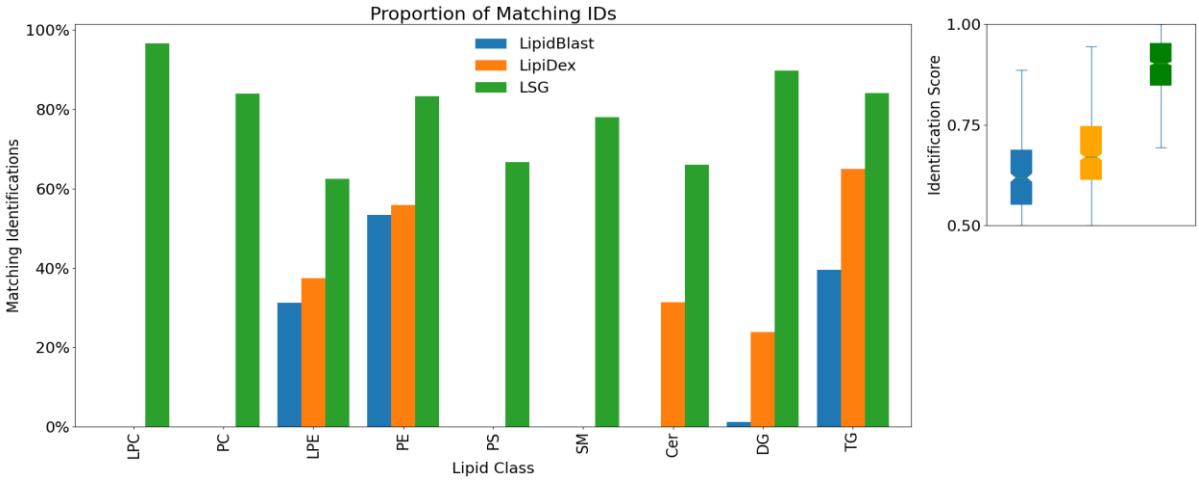

Figure 23 - Quantity of candidate identifications matching the literature contents of the AdipoAtlas reference samples. Sample processing and scoring was conducted in an identical manner to Figure 4. Analysis was conducted in positive mode only, as several of the evaluated classes do not ionize under negative ESI conditions.

identification score is suggested to be the result of its improved spectral accuracy. However, verification of identification quality is also necessary to conclude the differing performances of the libraries. The publicly available AdipoAtlas [169] samples were used as a reference to benchmark the extent of true-positive identifications that could be achieved with the libraries used. The results of this evaluation are presented in Figure 23. For the 9 lipid classes examined, the LSG-generated spectral library consistently achieved a near-complete level of true-positive lipid identifications, while each identification similarly matched with greater score. The expanded coverage of molecular species is evident where LSG contains a total of 136 pre-defined fragmentation templates encompassing a total of 87 classes, approximately 2-fold greater than the 54 fragmentation templates and 29 classes provided by LipidBlast. However, this disparity is also exacerbated due to LSG's more thorough representation of lipid species. Whilst the LipidBlast library is purported to contain over 200,000 fragmentation spectra, the library does not utilise many of the compression techniques featured in LSG. Thus, the LipidBlast library contains multiple duplicate spectra, which specify structural features that cannot be determined from the fragmentation pattern. This may be observed where LipidBlast provides 5476 spectra per phospholipid class to represent a total of 1176 unique fragmentation patterns. Such duplicates are elsewhere known to be burdensome, requiring additional data processing to eliminate duplicate identifications.[177]

In contrast, the LipidDex Library Generator provides 72 fragmentation templates covering 46 classes in their HCD_Acetate Library, whilst utilising many of the same compression techniques used in LSG. However, the templates used to maintain the same simplified patterns used throughout LipidBlast, which often ignore the presence of several major characteristic ions. As a result, the LipiDex-generated library cannot achieve the same quantity of identifications or scores as LSG.

CONCLUSION

LSG is an open-source *in-silico* spectral library generator specifically designed to aid in the identification of lipids in LC-MS/MS analysis. LSG-generated libraries are exportable in the universal .MSP format, permitting their use in any third-party or vendor-specific mass spectral library application. Furthermore, the features included in this software allow for the production of user-catered spectral libraries by selecting individual lipid classes and modifying their fragmentation spectra. With a pre-set 136 lipid templates covering 87 lipid classes, and over 1000 fatty acids, LSG is the most comprehensive software of its type. In direct comparison to previously published in-silico libraries used for lipidomic analysis, the greater comprehensivity of LSG had enabled a 3.5 to 4-fold increase in lipid identifications, greater coverage of true-positive identifications, along with a marked increase in identification score.

SUPPLEMENTARY DATA

Supplementary Table 1 – Lipid Classes included in the LSG software. References are provided as the source of the fragmentation templates.

Lipid Class	Reference
Monoacylglycerol	147, 178, 179
Diacylglycerol	147, 178-181
Triacylglycerol	147, 178, 179, 181, 182
Ether Triacylglycerol	179
Diacylglycerol glucuronide	179, 183
Acyl-diacylglycerol glucuronide	179
Monoglycosyl diacylglycerol	147, 184, 185
Ether Monoglycosyl diacylglycerol	179
Sulfoquinovosyl diacylglycerol	179
Diglycosyl diacylglycerol	184, 185
Diacylglycerol-3-O-carboxyhydroxymethylcholine	179
N-trimethyl homoserine diacylglycerol	179, 186
Phosphatidylinositol-mannoside diacylglycerol	147
Phosphatidylinositol-dimannoside diacylglycerol	147
Acyl-phosphatidylinositol-mannoside diacylglycerol	147
Diacyl-phosphatidylinositol-mannoside diacylglycerol	147
Bismonoacylglycerophosphate	179
Hemibismonoacylglycerophosphate	179, 187, 188
Dilysocardiolipin	179
Lysocardiolipin	179
Cardiolipin	147, 179
Phosphatidic acid	147, 179, 189
Ether phosphatidic acid	190
Phosphatidylmethanol	179
Phosphatidylethanol	179, 191
Phosphatidylcholine	147, 158, 189, 192-198
Ether phosphatidylcholine	179, 199
Plasmenyl phosphatidylcholine	147, 199
Phosphatidylethanolamine	147, 158, 179, 188, 189, 197, 200
Ether phosphatidylethanolamine	179
Plasmenyl phosphatidylethanolamine	179, 198, 201-203
N-methyl-phosphatidylethanolamine	179, 204, 205
N,N-dimethyl-phosphatidylethanolamine	179, 197, 206, 207
N-Acyl phosphatidylethanolamine	208
Phosphatidylglycerol	147, 189, 198
Phosphatidylinositol	18, 147, 158, 189, 198
Ether phosphatidylinositol	190
Phosphatidylinositol phosphate	18
Phosphatidylinositol diphosphate	18
Methylated phosphatidylinositol phosphate	144

Methylated phosphatidylinositol diphosphate	144
Phosphatidylserine	147, 158, 179, 189, 197, 198
Ether phosphatidylserine	179, 201
Plasmenyl phosphatidylserine	179, 201
Phosphatidylthreonine	190
Pyrophosphatidic acid	209
Cytidine-5'-diphosphate diacylglycerol	210-212
Lyso-3-O-carboxyhydroxymethylcholine	179
Lyso N-trimethyl homoserine	186
Lyso phosphatidic acid	179, 213, 214
Lyso phosphatidylcholine	147, 179, 189, 193, 215
Lyso phosphatidylethanolamine	147, 179, 189, 216
Lyso N-Acyl-phosposphatidylethanolamine	179, 188
Lyso phosphatidylglycerol	179, 217
Lyso phosphatidylinositol	18, 179, 218
Lyso phosphatidylserine	179, 218
Lyso N-Acyl-phosphatidylserine	179, 219
N-Acyl-sphinganine	220
N-Acyl-sphingosine	220-226
N-Acyl-phytosphingosine	223
N-Acyl-ceramide-1-phosphate	147, 220, 227, 228
N-Acyl-ceramide-1-phosphocholine	147, 229, 230
N-Acyl-ceramide-1-phosphoethanolamine	179
N-Acyl-ceramide-1-phosphoinositol	179
N-Acyl-ceramide-1-hexose	179, 231, 232
N-Acyl-ceramide-1-Acylhexose	179
N-Acyl-ceramide-1-dihexose	179, 233
N-Acyl-ceramide-1-trihexose	179
Sulfatide	156, 179
Free fatty acid	179
Zymosteryl Ester	147, 156, 179
Cholesteryl Ester	147, 156, 179
Brassicasterol Ester	179
Camposterol Ester	179
Sitosterol Ester	179
Sigmasterol Ester	179
Lanosteryl Ester	179
Acyl-carnitine	156
Acyl-CoA	234

Supplementary Data: LSG Candidate identifications in Pos and Neg modes for the SH-SY5Y analysis. Pos and Neg mode .raw datafiles used in SH-SY5Y analysis : https://pubs.acs.org/doi/10.1021/acs.analchem.2c04518?goto=supporting-info

Acknowledgments

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Chapter Three:

Enhancing Coverage of Phosphatidylinositol Species in Canola Through Specialised Liquid Chromatography-Mass Spectrometry Buffer Conditions

PREAMBLE

Following the completion of Lipid Spectrum Generator, libraries containing vast quantities of accurate *in-silico* LPI, PI, and PIP fragmentation spectra could be generated nearly instantaneously, as demonstrated in Figure 24. The generator was not limited to only these classes, and closely related lipids, such as oxidised PIs, were also included, along with the ether-PIs, acylated and mannosylated PIs, PI-ceramides and methylated PIPs, in addition to numerous other classes. Thus, with these libraries, a near-comprehensive evaluation of the PI-lipidome could be performed, so long as the compounds of interest were intense enough to produce a suitable fragmentation pattern. However, additional obstacles were still present that would limit their detection and prevent the comprehensive characterisation of these molecular species.

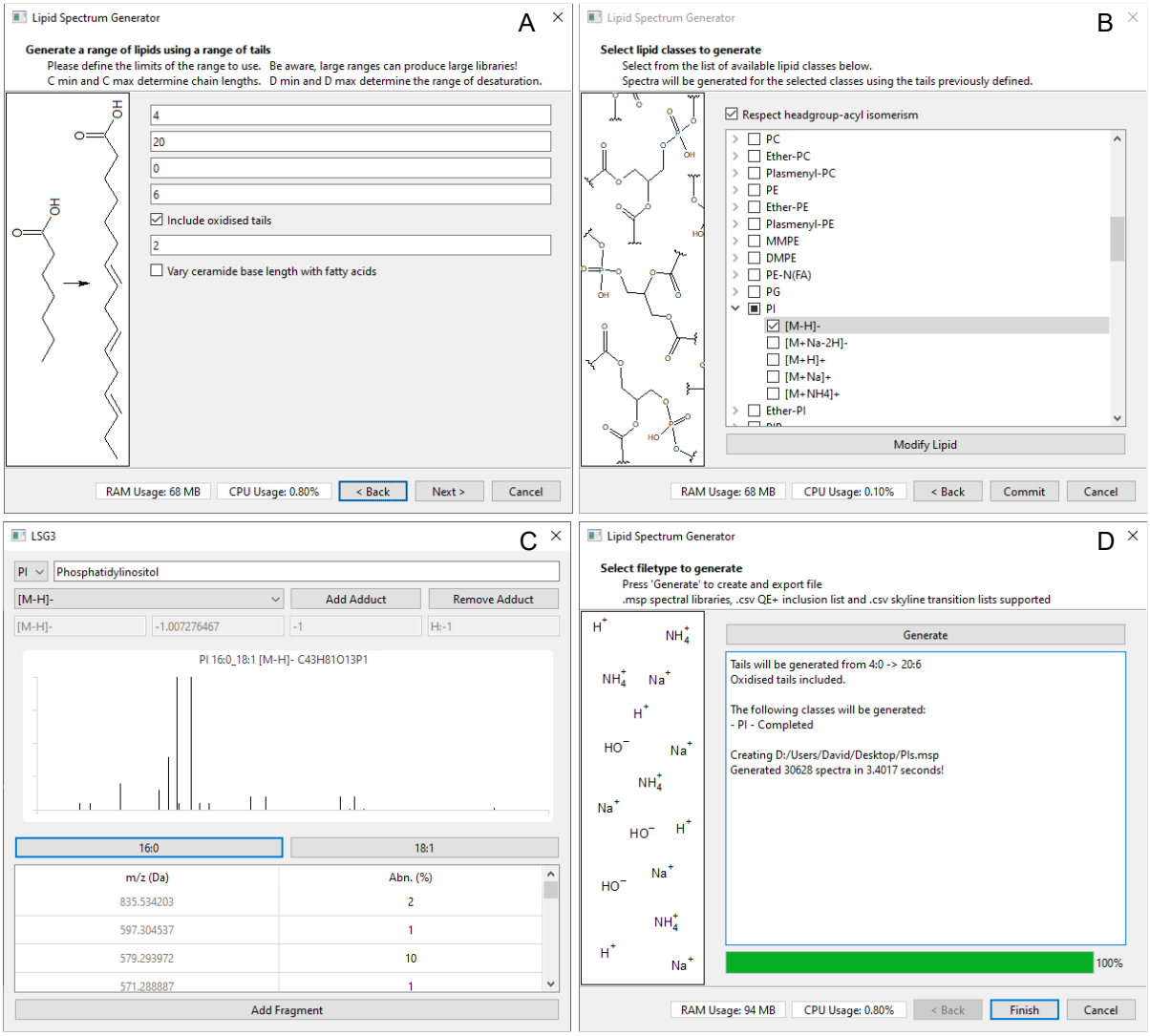

Figure 24 – An overview of spectral library generation using LSG. (A) A spectral library using a range of fatty acids is specified, and the fatty acid parameters are entered. (B) The desired fragmentation templates are selected. (C) An example fragmentation spectrum for the chosen template is reviewed. The fragmentation spectra may be modified at this point to better reflect the mass and intensity values observed experimentally. (D) The spectral library is generated. A library of 30,628 unique fragmentation spectra for the phosphatidylinositols is generated in approximately 3.4 seconds. This library represents a

26-fold increase in unique fragmentation spectra compared to LipidBlast in less time than it takes to download the LipidBlast library.

The analysis of PIs, and by extension, their LPI and PIP derivatives, is made difficult due to their low abundance, where PIs represent approximately 6-10% of total cellular phospholipids and PIPs as few as 0.2 to 1% of the PI fraction.[64] Yet, further complications arise as the chemical characteristics intrinsic to these compounds can detrimentally influence their ionisation efficiency, whilst matrix effects may also suppress ionisation and thus inhibit their detection.[145, 235] As a result, few, if any, are observed in so-called 'comprehensive' lipidomic characterisations. Considering the intended characterisation of these species in canola, these inhibiting factors are anticipated to significantly limit the distribution of molecular species observed, and they, therefore, must be addressed moving forward.

An approach to observe greater distributions of these compounds, often employed by characterisations, necessitates the analysis of undiluted and highly concentrated lipid extracts, potentially containing from 400-800,000 ppm total lipid.[138] However, analysis methods such as these can exacerbate the effects of matrix suppression, detrimentally influence chromatography due to overloading, or even contaminate instrumentation, and they are thus unfavourable. Instead, an approach that improves the efficiency and sensitivity of analysis would be preferred. The manipulation of solvent composition or the addition of mobile phase additives has been demonstrated to alter and bias conditions to favour the analysis of certain compounds,[145, 235, 236] and alterations such as these could potentially be used to enhance the characterisation of the PIs. There is some contradiction in the literature regarding the suitability of conditions for lipid analysis under negative ionisation mode, where both acidic and alkaline additives [72, 73] and neutral and alkaline buffers [71, 74] have been recommended. Hence, in this case, it is unclear what conditions may be suitable for analysing the PIs and their related compounds.

These contradictions may have arisen as these conditions are generally considered from the perspective of broad and multiclass characterisations, where methods do not intentionally favour the analysis of any particular class. Correspondingly, as certain compounds may require highly specific analysis conditions, which themselves may potentially result in the suppression of other classes, contradictions naturally arise based on the major classes considered. However, as the scope of this analysis is limited to only a few acidic lipid classes, these conditions may be re-evaluated from the perspective of those that specifically favour the analysis of the PIs.

The pursuit of this goal resulted in the following chapter, published in the *Journal of Chromatography A*, entitled 'Enhancing Coverage of Phosphatidylinositol Species in Canola

Through Specialised Liquid Chromatography-Mass Spectrometry Buffer Conditions', which demonstrated the utility of an ammonium fluoride-based buffer in the analysis of these compounds.

Compared to the previously optimised analysis conditions [71], the following publication provided an over 30-fold increase in ionisation efficiency for these compounds and a 38-fold increase in signal-to-noise ratio, enabling the identification of 14 PI species and 12 PI candidates from within a highly diluted canola extract.

CERTIFICATE OF AUTHORSHIP

The following chapter is an accepted manuscript published in the *Journal of Chromatography A (J. Chromatogr. A)*. I, David Gertner, certify that the work in the following chapter has not been submitted as part of any other documents required for a degree.

AUTHORSHIP CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
DAVID S. GERTNER	CONCEPTUALISATION, EXPERIMENTAL DESIGN, PERFORMED EXPERIMENTS, ANALYSED DATA, WROTE ORIGINAL DRAFT AND EDITED MANUSCRIPT	Production Note: Signature removed prior to publication.
ALEXANDRE OGLOBLINE	CONCEPTUALISATION, REVIEWED AND EDITED MANUSCRIPT	
DAVID P. BISHOP	CONCEPTUALISATION, REVIEWED AND EDITED MANUSCRIPT	Production Note: Signature removed prior to publication.
MATTHEW P. PADULA	CONCEPTUALISATION, REVIEWED AND EDITED MANUSCRIPT	Production Note: Signature removed prior to publication.

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Enhancing Coverage of Phosphatidylinositol Species in Canola Through Specialised Liquid Chromatography-Mass Spectrometry Buffer Conditions

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ABSTRACT: Phosphatidylinositols (PIs) constitute a minor class of phospholipid with widespread influence throughout various cellular functions. Monitoring the distribution of these lipids can therefore provide insight as to the state of cellular processes or reveal the development of various pathologies. The speciation of these compounds is often performed either as part of a comprehensive characterisation of lipids, or specifically targeted using the same methods, however, such methods were intended to maximise coverage of lipid classes rather than provide an in-depth analysis of any single class. In the particular case of PIs, the majority of reported molecular diversity is limited to a small proportion of the already minor class, as such the cursory glance enabled by such methods is insufficient. Therefore, this work compared the suitability of both established and novel LC-MS buffers with the aim of maximising the ionisation efficiency of PIs, in an attempt to enhance coverage of the class. Through experimentation, it was determined that a 0.25 mM ammonium fluoride buffer provided up to a 6-fold increase in signal intensity, and on average a 38-fold increase in the signal-to-noise ratio. Using these new conditions, 14 PI species, and 12 PI candidates were identified within a dilute lipid extract sourced from canola seed, compared to 0 species identified using the generalised method. As a result, it is suggested that this procedure has yielded the highest number of PI species identifications for a sample of this concentration. Methods which therefore intend to characterise PI species in dilute quantities, such as those extracted from mammalian cells, are henceforth provided with the means to conduct more comprehensive characterisations.

BACKGROUND

Phosphatidylinositols (PIs) constitute a class of phospholipid which are characterised by the presence of a myo-inositol ring substituted as the headgroup. Through cellular processes, the myo-inositol ring may be additionally phosphorylated at several of its hydroxy sites, giving rise to an additional seven known PI sub-classes.[32, 64] These additionally phosphorylated PI species (PIPs) play several key roles in various facets of maintaining cellular functions essential for growth and proper development, and thus are highly regulated within cells.[32, 64, 66, 79-81] Aberrations which may interfere with this process of regulation have been identified as a distinguishing feature associated with a number of serious pathologies such as Joubert syndrome [64, 66], Charcot–Marie–Tooth disease,[62-64, 66] Lowe syndrome,[62-64, 66, 237] Bipolar disorder,[62, 67] Alzheimer's,[62, 64, 67] and some cancers.[61-66] Due to their pervasive nature, these lipids are interesting biomarkers needing identification, characterisation, and quantification; however, various properties, such as the low abundance of these lipids, makes analysis difficult. In the case of the PIP sub-classes, several specific approaches have been developed which enable targeted analysis, such as the use of ion-pairing or derivatising agents to improve chromatography and electrospray ionisation, each with their own compromise.[143, 238-242] However, despite their greater abundance and thus theoretically easier analysis, methods which observe the precursor unphosphorylated PIs are unspecific and are often derived from a set of parameters which have been generalised for comprehensive lipidomic analysis, and thus are not necessarily well suited for this class of compound.[67]

Typically, due to their hydrophobicity, reversed phase liquid chromatography (RP-LC) in conjunction with electro-spray ionisation mass spectrometry (ESI-MS) (Collectively referred to as RP-LC-MS) is utilised for the separation and characterisation of lipid species in complex samples.[137, 243] Those generalised conditions which are intended for comprehensive characterisations of lipids in a single chromatographic separation compromise conditions rather than use those which are ideal for any class as to achieve maximum coverage of the lipidome. As a result, these experiments often require concentrated samples, do not resolve certain isobaric species, and enable purview of only those most intense species. It is here argued that due to their unique characteristics, such generalised parameters are particularly insufficient for the analysis of PIs.

PIs are relatively sparsely occurring in comparison to the other phospholipid classes, as a mix of multiple individual PI species they represent 11-18% of canola lecithin and only about 10% of total cellular phospholipids.[64, 66, 79] Additionally, alike other lipid classes, these molecules are observed with a range of slight structural variations which alter their specific physical and chemical properties, and therefore their biological activities and functions.[79] However, a

distinctive feature of PIs is that in mammalian cells a significant proportion is biased towards a specific species, namely the 1-stearoyl-2-arachidonoyl form.[64, 79] It has been suggested that this form may allow for adequate membrane packing, exposing the headgroup for efficient interaction.[79] As a result of this bias, the majority of the reported molecular diversity for this class is limited to a small proportion of an already minor lipid class. However, as these lipids are highly regulated, it is suggested that these minor species are not biologically inconsequential. It is when considering the analysis of these minor species, whether it be for characterisation or observing potential aberrations in their regulation, that the limitations of the generalised parameters inherited from comprehensive lipidomic characterisations become most apparent.

Fortunately, previous work has shown that that these generalised conditions may be biased to improve the analysis of particular classes. For example, Cajka and Fiehn [71] have previously demonstrated through testing a variety of common buffers and acids, that the ionisation efficiency for particular lipid classes could be selectively bolstered. Their results had suggested that a 10 mM ammonium acetate (AmAc) buffer enabled the greatest ionisation efficiency for several lipid classes in negative ESI, including PIs. However, more recently the utility of AmAc has come into question, as conflicting data has suggested otherwise. In a similar assessment of common buffers and acids, Monnin C. *et al*. [72] have shown that the addition of AmAc severely hinders ionisation efficiency for a range of lipid classes, including PIs, whilst only promoting the ionisation efficiency of phosphatidic acid, ceramides and phosphatidylcholines. It had been their conclusion that for the analysis of lipids in negative ESI, 0.02% acetic acid enabled the greatest ionisation efficiency.

Undoubtedly, for a comprehensive characterisation of PI species, further research is required to assess the validity of AmAc, and to examine potential alternative conditions which may supersede it in ionisation efficiency. An improvement in the ionisation efficiency for these compounds would hence enable the characterisation of the diverse minor species endemic to this class.

In terms of potential substitutes for AmAc, there is some precedent for the use of other buffers such as ammonium hydroxide (AmOH) and more recently ammonium fluoride (AmF). Although their use has been limited, both have been suggested to improve ionisation efficiency for various small molecules.[74, 244-246] As there has only been limited assessment for these buffers in terms of their ability to ionise phospholipids, their utility in comparison to AmAc was measured in this work. Furthermore, a variety of basic modifiers have been used in negative ESI as post column additives to enhance the ionisation efficiency for compounds of interest.[247] Therefore, in similar fashion, a select few would need to be considered for the purpose of

further enhancing ionisation efficiency. However, as affirmed elsewhere and iterated in the aforementioned works of Cajka and Feihn [71] and Monnin C. et. Al,[72] the ionisation efficiency of phospholipids appears to be sensitive to various factors, including the interactions between the lipid headgroup and solvents used.[145] As such, it would be necessary to further explore the influence of the solvents used during chromatography on the efficiency of ionisation of PIs and PIPs.

To assess the degree any improvement in ionisation efficiency would translate into an improvement in analysis, an attempted characterisation of PI species present within a canola extract was conducted using the finalised conditions, after which it was compared to a characterisation performed using the initially suggested 10 mM AmAc additive. For the purpose of these characterisations, a canola extract had been elected as a model due to a proportionally high content of PIs (representing 11 – 18% of phospholipids) and unique fatty acid profile, as outlined in a previous characterisation of rapeseed lecithin.[148, 248] Hence it was suspected that a diverse group of relatively intense PI species may be observed without the need for preconcentration.

Thus, the aims of this investigation is to determine the solvent, additive and buffer conditions which would specifically enhance PI detection and quantification through enhancing electrospray ionisation efficiency, and to then determine whether the increase in ionisation efficiency would in fact enhance the characterisation of these lipids.

MATERIALS AND METHODS

CHEMICALS AND STANDARDS

Methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA) used were of LC/MS grade, B&J Brand, purchased from Honeywell.

Ultra-pure water (UP) (18.2 MΩ-cm) was sourced from an in-lab water purification system (Sartorius).

Phospholipid standards (17:0-14:1 PI, 18:1-18:1 PI, 18:0-20:4 PI, 18:1-18:1 PI(3)P) were purchased from Avanti Polar Lipids Inc.

Ammonium acetate (AmAc), ammonium hydroxide (AmOH) and ammonium fluoride (AmF) were purchased from Sigma-Aldrich and were of analytical reagent grade or higher.

Pyridine, piperidine and N,N-diisopropylethylamine were also purchased from Sigma-Aldrich and were of 98% purity or greater.

Canola seed was of an unknown cultivar, purchased from Reptile Direct Australia.

STANDARD SOLUTIONS

Canola extract was prepared through an MtBE extraction protocol, using 100 mg canola seed which had been homogenised in a ball mill. The resulting extract was then reconstituted into 2 mL MeOH.[249]

Briefly, 1.5 mL of MeOH and 5 mL of Methyl tert-Butyl Ether (MtBE) was added to the 100 mg sample of homogenised canola seed in a 15 mL falcon tube. The sample was mixed on a rotisserie for an hour and then left to rest for 10 minutes. Next, 1.25 mL of UP water was added, and the sample shaken. The sample was then centrifuged at 1000 g for 10 minutes. The upper layer was then removed and dried under N_2 before reconstitution in 2 mL of MeOH.

Two standard solutions were prepared for the purpose of conducting both flow injections and chromatographic separations. Standard solution 1 consisted of 1 ppm 17:0-14:1 PI, 18:1-18:1 PI, 18:0-20:4 PI, and 18:1-18:1 PI(3)P in 100% ACN. Standard solution 2 consisted of 1 ppm 17:0-14:1 PI added to a mixture of 50% canola extract and 50% ACN.

Despite the experimentation not targeting additionally phosphorylated PIs, the 18:1-18:1 PI(3)P standard was included within the standard mixture to observe whether the conditions that favoured the analysis PIs likewise produced favourable conditions for their derivatives.

LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS)

LC-MS/MS analysis was conducted using an Agilent 1290 Infinity LC system, coupled to an Agilent 6510 qTOF mass spectrometer. Chromatography was conducted using a Waters Acquity CSH column (1.7 µm, 135 Å, 150 mm x 2.1 mm). Data was acquired under electrospray ionisation (ESI) negative mode with the optimised source parameters as follows; gas temperature was 365º C; drying gas (nitrogen), 5 L/min; nebuliser gas (nitrogen), 30 psig; capillary voltage, -4 kV; fragmentor voltage, 80 V; skimmer voltage, 65 V. MS^1 spectra were collected by accumulating over *m/z* 500 – 1000 for 0.25 s; MS^2 spectra were then collected when a precursor ion exceeded 3000 counts, and fragments were observed over *m/z* 125 – 1000.

Through a series of flow injections, the ideal buffer conditions under which PIs would ionise were to be determined. Buffers and modifiers examined included glacial acetic acid, AmAc, AmOH and AmF (shown in Table S 1), as well as piperidine, pyridine and N,N-diisopropylethylamine (shown in Table S 2) at varying concentrations. During the flow injection experiments only MS1 data was gathered, whereas during the chromatographic experiments, both MS1 and MS2 data were gathered.

After optimisation, results for the new buffer condition were then compared to those results gathered using the AmAc buffer. Four replicate injections were completed using each

condition. For each separation, 5uL of standard solution 2 was injected, the column was maintained at 65º C, and flow was maintained at 0.5 mL / minute. The gradient follows: 0 minutes 15% B, 0 – 2 minutes 30% B, 2 - 2.5 minutes 48% B, 2.5 – 11.5 minutes 99% B, 11.5 – 12 minutes 99% B, 12 - 12.1 minutes 15% B, 12.1 – 15 minutes 15% B, where mobile phases A and B consisted of 60% ACN + Buffer, and 90% IPA with 10% ACN + Buffer respectively.

Chromatograms were then processed using Agilent MassHunter Workstation and characterisation was performed for each injection by manually assessing the fragmentation spectra. PI species could easily be identified through their precursor mass and the presence of certain characteristic fragments which corresponded to the inositol headgroup.[18]

RESULTS AND DISCUSSION

Prior to flow injections, an initial chromatographic separation was conducted to estimate the solvent conditions under which the compounds of interest would elute, so that these conditions could be replicated as the flow injection solvent. Conditions were based off those recommended by Cajka and Feihn.[71] Briefly, a 5 uL injection of Standard solution 1 was separated over 10 minutes at 0.2 mL / minute, following a gradient which ramped from 20 – 100% B using the aforementioned column which was maintained at 50° C. Mobile phases A consisted of 60% (v/v) ACN and B 90% IPA, 10% ACN, with the addition of 10 mM AmAc in each. Through this separation, it was determined that 50% B approximated the solvent conditions under which PIs would elute, thus these conditions would later be used as the flow injection mobile phase parameters. A peak corresponding to the additionally phosphorylated PI (i.e., 18:1-18:1 PI(3)P) could not be observed in this initial chromatographic separation, although at this point it was not determined as to whether this was due to an incompatibility of the chromatography or the ionisation conditions.

BUFFER SUBSTITUTION

Flow injection experiments were conducted by substituting the buffer used, in accordance with Table S 1. Through 10 replicate injections for each condition, the peak areas for the masses corresponding to 17:0-14:1 PI, 18:1-18:1 PI and 18:0-20:4 PI were recorded.

In varying the concentration of each buffer, an optimal condition was determined. Whereby all data sets could then be normalised against the mean intensity of a common condition (no added buffer) to allow for direct comparison. Though a series of flow injections including glacial acetic acid as a modifier were conducted here, the solvent conditions used here differ to those conducted by Monnin C. et al..[72] The results shown Figure 25 are the summed intensities of all the lipids analysed. The results in Figure 25A show that AmF and AmOH outperformed AmAc with up to a 6-fold increase in intensities observed in comparison to minimal changes with AmAc. In addition, the results in Figure 25B show that a commonly used concentration of

acetic acid appear to cause considerable suppression, though the specific condition of 0.02% suggested by Monnin C. et al. was not tested.

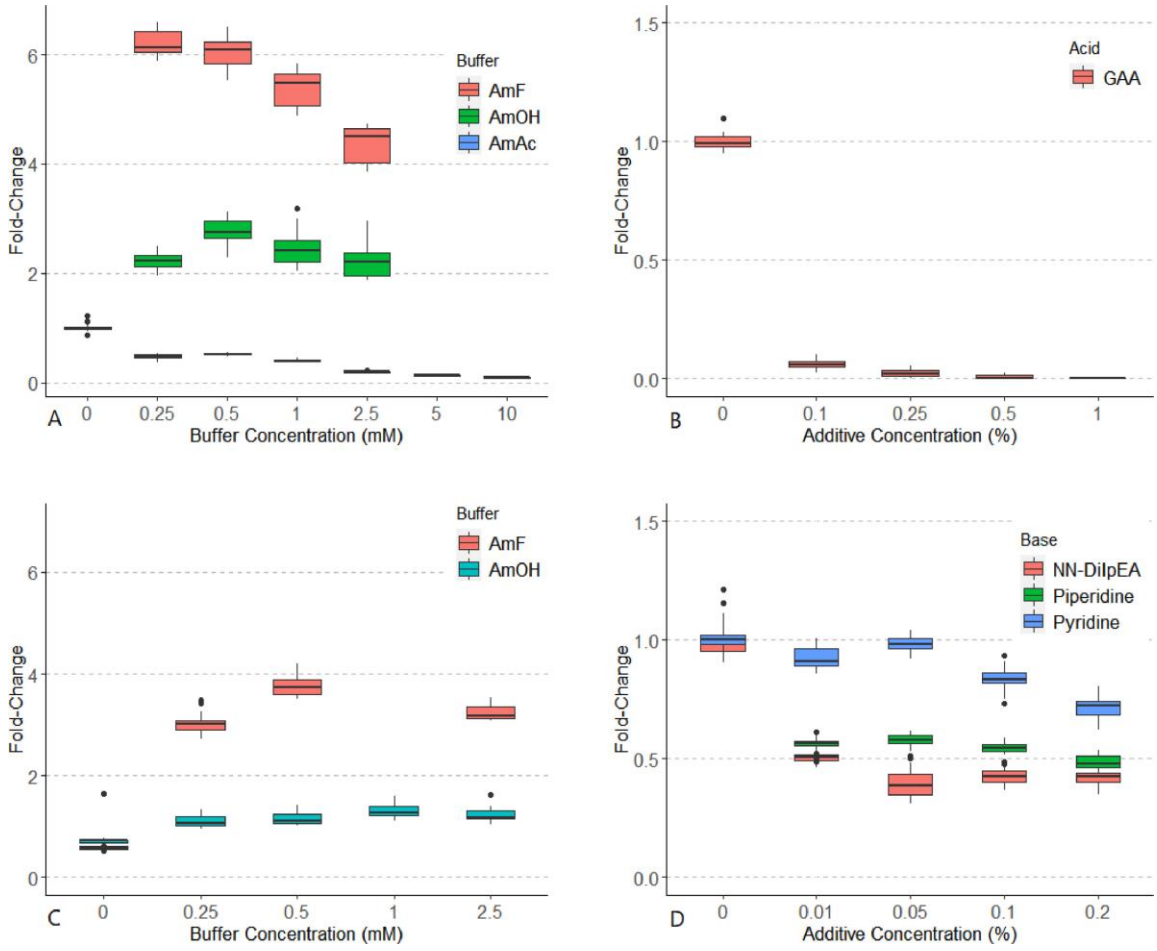


Figure 25 - A & B Summed intensities relative to the common condition (of no added buffer), for conditions mentioned in Supplementary Table 3, using an acetonitrile based mobile phase. AmF (A) and Glacial Acetic acid (B) are shown in red, AmOH green, and AmAc blue, with AmF providing the greatest increase in signal intensity. C Summed intensities relative to the common condition (of no added buffer), for AmF and AmOH for those conditions mentioned in Supplementary Table 3, using a methanol based mobile phase. Data for the 1mM AmF experiment using the methanol based mobile phase was excluded due to an error in experimentation. D Summed intensities relative to the common condition (of 0.25mM AmF with no added basic modifier), for those conditions mentioned in Supplementary Table 2. NN-DiIpEA is shown in red, piperidine in green, and pyridine in blue. All additives decreased the intensity at all concentrations.

It may appear that for the AmOH or AmF buffers a greater variation in signal was observed. However as shown in Supplementary Figure 1, the variations in intensity for a single species remained low, and it was the mean intensity for each individual species that drifted apart. It is suspected that with the greater ionisation efficiency observed, the slight variations in efficiency caused by saturation and chain length became more apparent, thus broadening the fold change distribution for the lipid class.[145] Whilst AmF has not previously been used as a buffer

for the separation of PIs, it is known to enhance the ionisation efficiency for a broad range of compounds under both positive and negative ESI conditions.[250, 251] In these cases, the increase in signal intensity was sufficient to enable a more comprehensive level of coverage for the compounds of interest. The enhanced ionisation efficiency observed in studies using AmF has been attributed to the strong basicity of the fluoride (F^-) anion in the gas phase, causing the formation of a $[M + F]^-$ adduct, which then decays into the $[M - H]^-$ form with the neutral loss of a halide. A similar mechanism has been reported for chloride (Cl^-), where the ratio of $[M - H]^-$ and $[M + Cl]^-$ adducts observed depended on the gas phase acidity of the compound relative to HCl.[250, 252, 253]

In addition to buffer conditions, the choice of solvent is known to have a substantial effect on the intensities of signals observed as a result of altering the ionisation efficiency for particular compounds.[236] To account for whether ACN provided favourable conditions for the ionisation of PIs, the flow injections performed using AmOH and AmF were repeated, wherein the ACN in the mobile phase was substituted with MeOH. MeOH is commonly used in place of ACN for lipidomic separations and hence is suspected to perform similarly, while potentially providing differing conditions for ionisation due to the increased polarity of the alcohol.[137] The results for these additional experiments may be seen in Figure 25C, with the intensities not increasing in MeOH to the same degree as in ACN. Due to the unique headgroups of the individual phospholipid classes, this particular preference for an ACN based mobile phase may be particular to PIs. A similar assessment has not been completed for all lipid classes, however, contrary to the results observed here, MeOH has been shown to enhance ionisation efficiency for particular phosphorylated metabolites by several fold under negative ESI conditions, in comparison to ACN.[236]

From the results shown in Figure 25 it was therefore determined that an ACN based mobile phase containing 0.25 mM AmF performed best in terms of maximising MS signal intensity for the selected PI standards.

POST-COLUMN ADDITIVES

In an attempt to further enhance the observed intensity, an additional set of flow injection experiments were conducted, in which a basic modifier taken from Supplementary Table 2 was added at a particular concentration. To represent the broad variety of bases used in literature, three in particular were chosen, representing an aromatic (pyridine), secondary (piperidine) and tertiary amine (N,N-Diisopropylethylamine). For each condition, the peak areas for the masses corresponding to 17:0-14:1 PI, 18:1-18:1 PI and 18:0-20:4 PI were recorded over 10 replicate injections and then normalised to the mean intensity for a common condition. The results of which may be seen in Figure 25D where these additives decreased

the signal intensities of the PIs at all concentrations examined. This decrease in intensity appears to be contrary to the notion that an increase in pH would further facilitate deprotonation (and thus increase ionisation efficiency) and as such, may be an example of "wrong-way-round ionisation", in which more acidic conditions favour deprotonation.[72] However, having shown that an AmOH buffer somewhat favoured the ionisation of PIs (Figure 25A), it is likely that the combination of AmF and the bases tested resulted in the supressed ionisation, rather than the bases themselves.

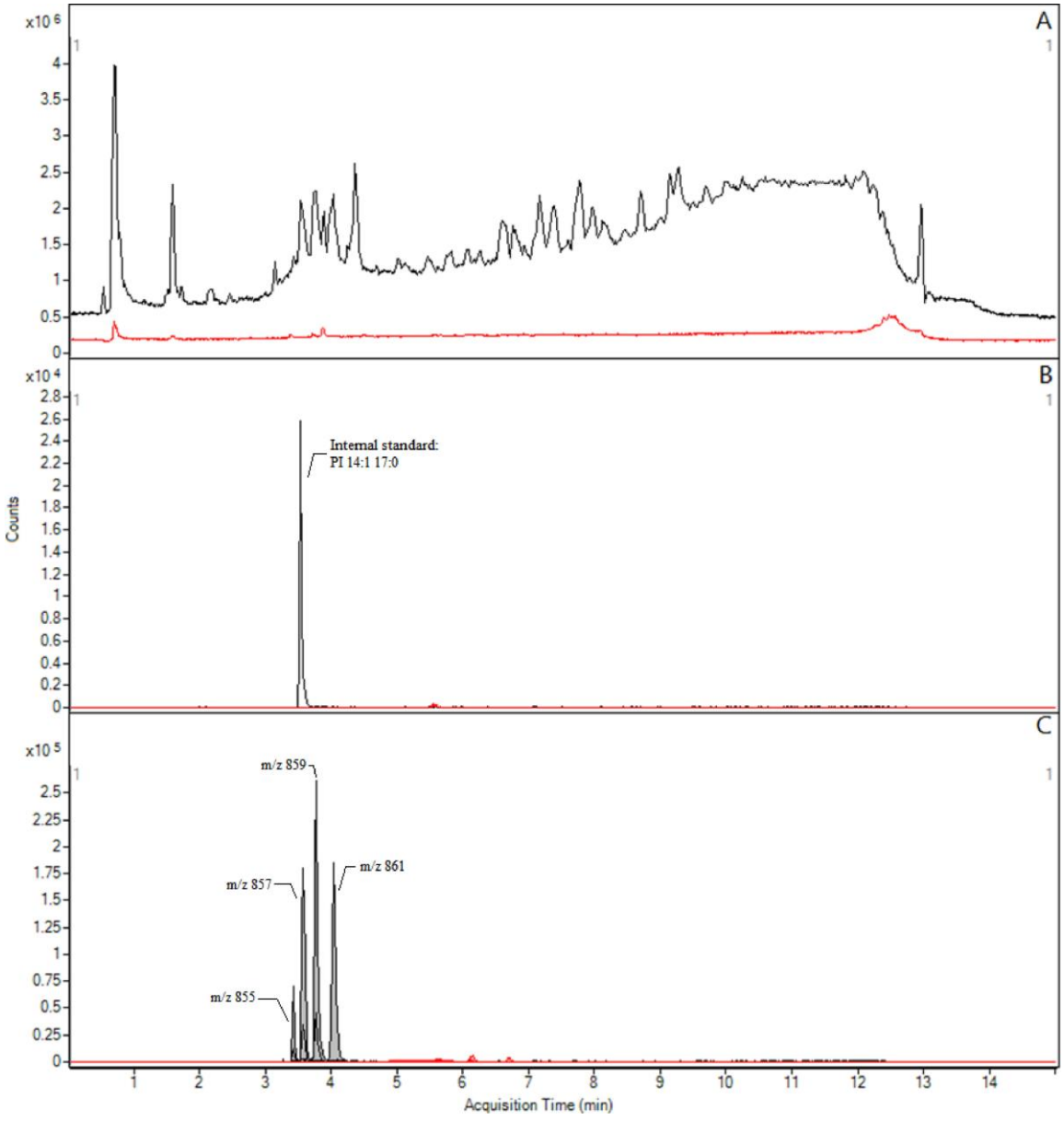


Figure 26 - Chromatograms for the AmF separations are shown in black, Chromatograms for the AmAC separation are shown in red.(A) Example TICs of standard solution 2. (B) Example EICs of internal standard 17:0-14:1 PI. (C) Overlayed example EICs of 4 PI candidates observed under both conditions.

As such, through the results of these experiments it is suggested that the selected additives only served to supress the signal intensity for the observed PI standards. Therefore, the optimal conditions were determined to be that of no added basic modifier.

CHROMATOGRAPHIC COMPARISON

Having determined the buffer and solvent conditions for which the chosen PI standards were optimally ionised, the developed conditions were then applied to a chromatographic separation. These results could then be compared directly to those acquired using the initial 10 mM AmAc modifier. Henceforth, attempted characterisations were performed on standard solution 2, which contained a dilute canola extract and internal standard. Using this standard, four replicate injections of 5uL were conducted using each condition, as mentioned previously.

For these injections, an example total ion chromatogram (TIC) comparing the intensities produced by using either buffer may be seen in Figure 26A, where it was determined that the signal intensity produced using the AmF buffer was significantly greater than that using the AmAc buffer. Furthermore, as shown in Figure 26B, a significant shift in retention time is observed for the internal standard (and thus the class as a whole, shown in Figure 26C), eluting at approximately 5.5 minutes under AmAc to 3.5 minutes under AmF. It is believed that this shift in retention had occurred as a result of a change in mobile phase pH and may result in less resolution between the species of the class. Due to the greater intensity, the AmF chromatograms were used to identify 14 PI candidates through the use of their MS2 spectra, an example of which is shown in Figure 27, where the species shown was identified as PI 18:2_16:0. Alongside these identified species, a list of 12 additional candidates were observed with precursor masses corresponding to potential PI species. Previous studies have used accurate mass to propose the identity of compounds without the use of MS2 information, although with no ability to distinguish between isomers the identity is limited to the 'bond-type level' nomenclature, listing only the carbon number and degree of desaturation.[171, 254] Ideally, without MS2 information, the identity of such compounds could be supported with the retention time information predicted through software or measured with an appropriate standard. Simple modifications of lipid species within a single class, such as an additional carbon or double bond, are known to produce systematic and highly predictable shifts in retention time.[255, 256] With a sufficient number of candidates, such trends may be identified and extrapolated to vet candidate masses. Therefore, in order to support the proposed identity of these species through a means independent of their precursor mass, retention time information was compared to the retention pattern produced by the 14 structurally similar candidates with available MS2 information in addition to the internal standard. All observed candidates correlated highly with the constructed retention pattern, and therefore supported their status

as potential PI species. The completed pattern for candidates observed in the AmF separations is shown in Supplementary Figure 2.

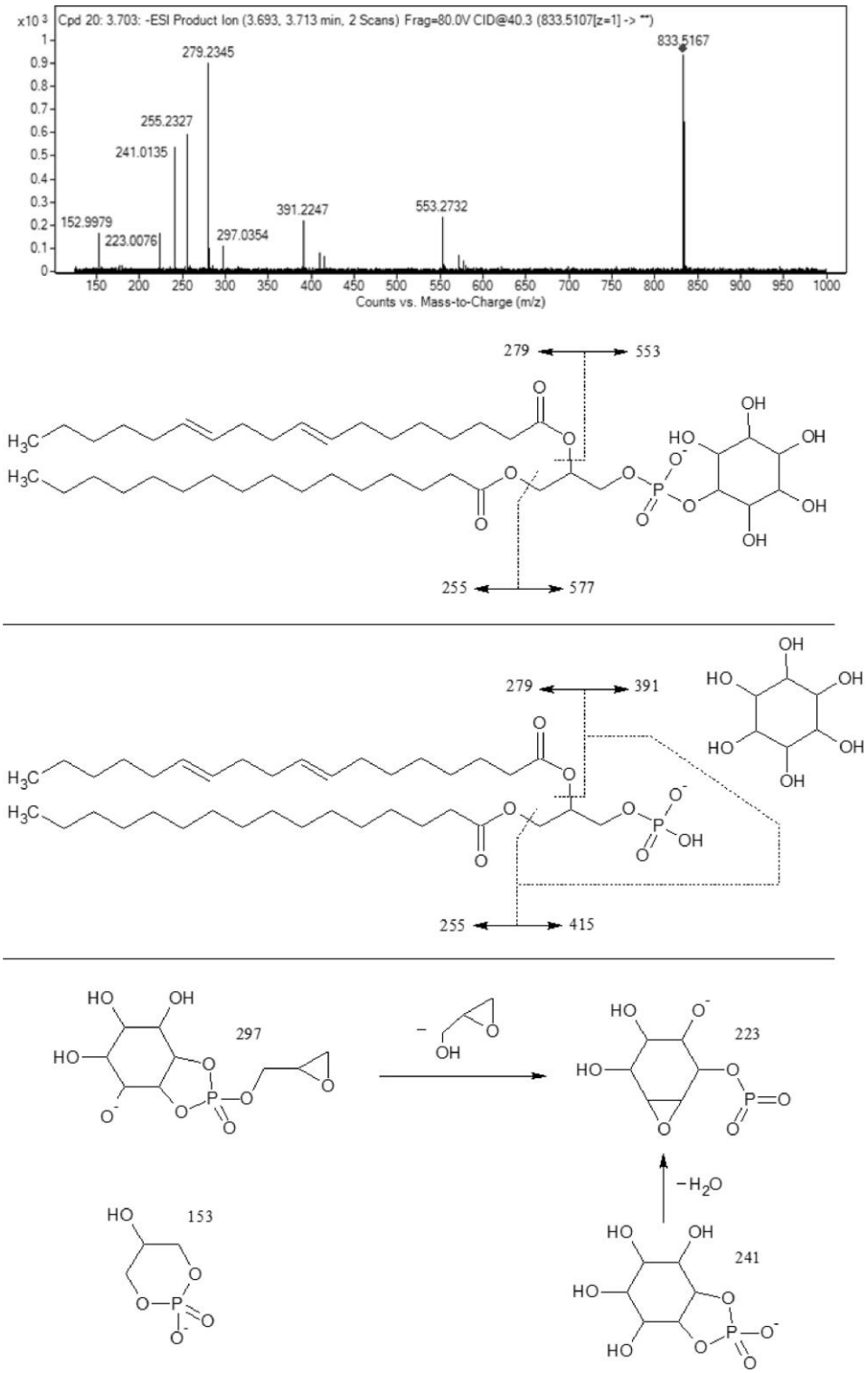


Figure 27 - Example product ion spectra of a PI candidate species, identified as PI 18:2_16:0. *m/z* 153 corresponds to a fragment characteristic of glycerophospholipids, whilst *m/z* 223, 241 and 297 correspond to fragments characteristic of a phosphatidylinositol. Fragments *m/z* 255, 279, 391, 553 and 833 correspond the free fatty acids, fatty acid neutral losses and parent ion *respectively.*

No MS2 data had been gathered for the AmAc separations, due to the low intensities of the parent ions, and thus their identity had to be inferred. Both the known species and the unknown PI candidates which were observed in the AmF separation (Figure 26C) were then used to assign identity to ions of the same mass and order of elution observed in the AmAc separation. Table 3 shows the species identified under either condition and compares the level of information which could be determined. Those species which could be observed under either condition where then used to determine the difference in signal-to-noise, shown in Figure 28. The increase in signal intensity observed with the AmF buffer did not correspond with a concurrent increase in the noise, resulting in on average a 38-fold increase in signal-to-noise over those obtained with the AmAc buffer.

Table 3 - PI candidates observed under either condition (excluding internal standard). For those species where fatty acid composition is not specified, MS2 data had not been gathered due to low intensity, and their identity is suggested through their precursor mass.

PI Species Observed with AmF Buffer	PI Species Observed with AmAc Buffer
PI 16:0_18:1	PI 34:1
PI 16:0_18:2	PI 34:2
PI 16:0_18:3, PI 16:1_18:2	PI 34:3
PI 16:2_18:2	-
PI 18:0_18:1	-
PI 18:0_18:2, PI 18:1_18:1	PI 36:2
PI 18:1_18:2	PI 36:3
PI 18:1_18:3, PI 18:2_18:2	PI 36:4
PI 18:2_18:3	-
PI 18:3_18:3	-
PI 32:1	-
PI 32:2	-
PI 32:3	-
PI 33:1	-
PI 33:2	-
PI 33:3	-
PI 34:5	-
PI 35:1	-
PI 17:0_18:2	-
PI 35:3	-
PI 35:4	-
PI 38:5	-
PI 38:6	-

In total, through the use of the AmF buffer, 14 PI species were identified, and 12 PI candidates are suggested; whereas with the AmAc buffer, 0 PI species could be identified and only 6 candidates are suggested. All species observed using the AmF buffer, with their intensities relative to the internal standard, are shown in Supplementary Figure 3.

From this data it is apparent that there is a broad distribution of minor PI species within the canola extract, whereas only a few contribute to the bulk of the class. As result of the disparity in terms of signal intensity between the newly developed and previously reported conditions, it is clear that this molecular diversity could not have been observed using previously applied methods from the literature. Previous comprehensive characterisations of plant phospholipids lack the same depth of analysis in terms of coverage of PI species, despite employing either highly concentrated samples or more sensitive instrumentation.

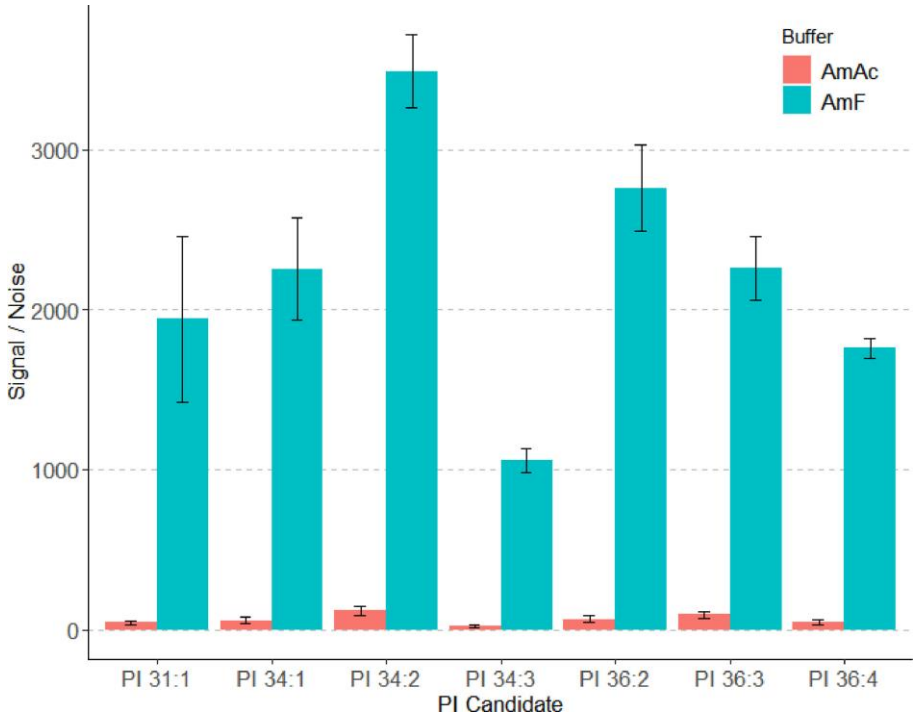


Figure 28 - Signal-to-Noise ratio of PI species observed under both conditions. Values were calculated based on peak height and RMS of noise intensity. On average the signal to noise ratio produced for each species using the AmF buffer was 38-fold greater than that produced using the AmAc buffer.

For example, one such analysis targeting PIs observed only 10 species in an extract from 20 mg of infant formula, concentrated into an unspecified volume.[67] In cases of general phospholipid characterisations, one analysis observed 7 PI species in an extract from 2.5 g flax seed concentrated into 1 mL,[257] a second observed only 3 PI species in an extract from 0.5 g of wheat roots concentrated into 1 mL [258], a third observed 25 PI species in a variety of nuts from 0.5 g concentrated into 0.5 mL,[138] and a fourth observed 12 PI species in a variety of sunflower seeds from up to 0.5 g concentrated into an unspecified volume after a series of extractions and digestions.[259] In comparison, this procedure observed 26 PI species in an extract from 0.1 g canola seed, concentrated into 2 mL, and then diluted to half its concentration.

Of those observed, many of the more intense PI candidates shown in Supplementary Figure 3 constitute more than one co-eluted isobaric species. Alike all phospholipids, PIs are known to contain a number of double bond and positional isomers which are difficult to separate under standard reverse phase conditions.[137] Therefore, it is suggested that further chromatographic development or the use of ion mobility spectrometry is required to interrogate these isomeric species.

Using those conditions which were previously suggested in literature (i.e. the 10 mM AmAc buffer suggested by Cajka and Feihn), chromatographically separating these isobars and isomers would have proved impractical as the already minor peaks would be further diminished in intensity as each previously co-eluting species is resolved. However, it is apparent that under these newly proposed conditions (i.e. 0.25 mM AmF), the opportunity for further chromatographic resolution, and thus a more comprehensive level of characterisation, is available for future consideration.

CONCLUSION

In summary, it is suggested that the conditions generalised for lipidomic analysis are in particular insufficient for the analysis of phosphatidylinositol (PI) species, and it is proposed that a more comprehensive characterisation of PI species may be conducted by biasing the generalised conditions as to favour their ionisation. Experimentation revealed that out of those conditions tested, a mobile phase based on ACN, containing 0.25 mM AmF, sans modifier, significantly outperforms previously suggested 10 mM AmAc, sans modifier, mobile phase for the purpose of maximising PI ionisation efficiency.

To demonstrate the utility of these newly proposed mobile phase conditions, a characterisation of PIs extracted from canola seed was performed using both sets of conditions, through which it is apparent that the newly developed conditions has enabled a more comprehensive analysis and revealed the diversity of minor PI species present. Furthermore, it is suggested that these newly proposed conditions provide the opportunity to further enhance chromatographic separation, where previously it may have been limited due to the low ionisation efficiency.

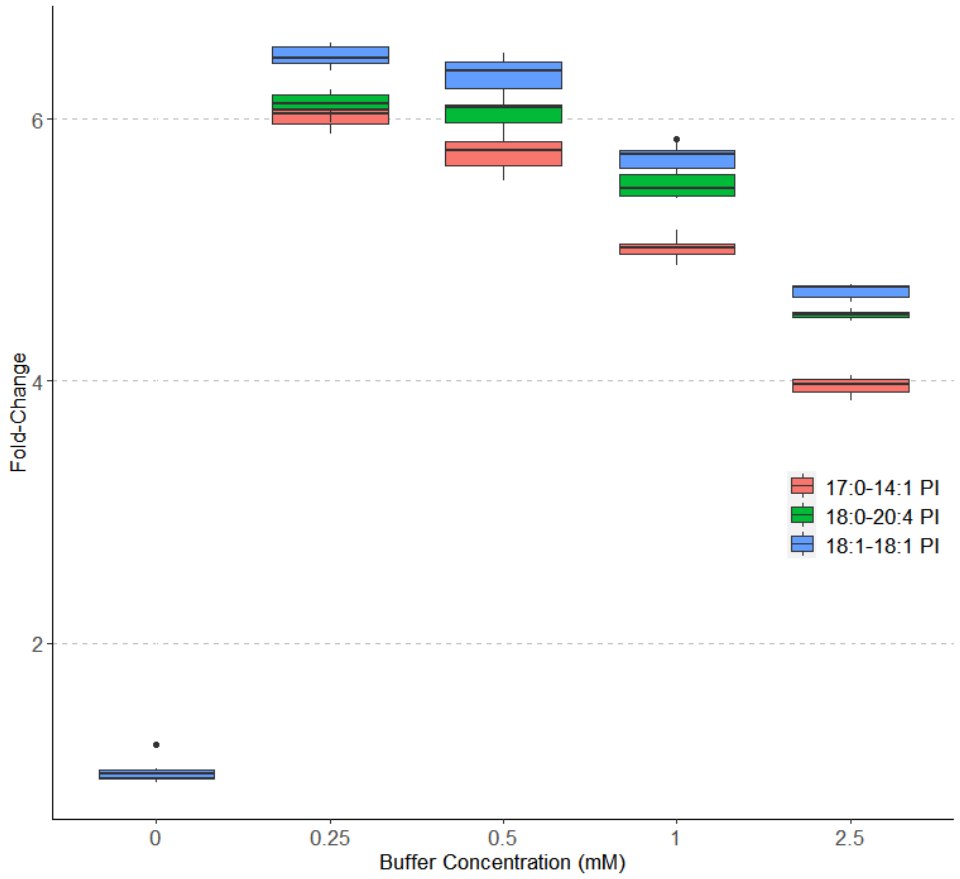
SUPPLEMENETARY DATA

Supplementary Table 2 - Bases and concentrations tested in flow injections. All bases were added to a mobile phase buffered with 0.25mM Ammonium Fluoride.

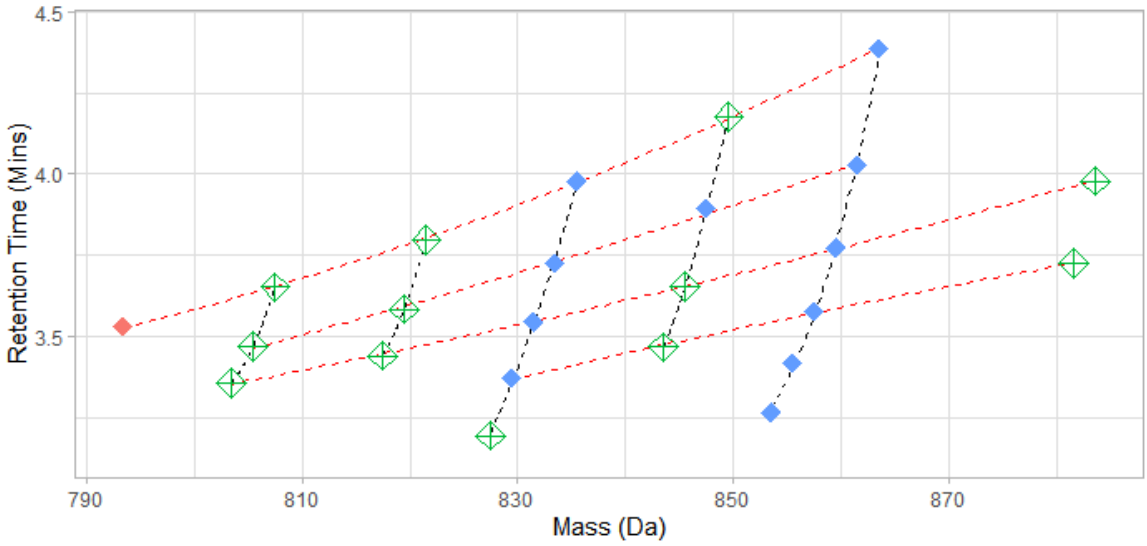
Piperidine	Pyridine	NN-Diisopropylethylamine
0.00%	0.00%	0.00%
0.01%	0.01%	0.01%
0.05%	0.05%	0.05%
0.10%	0.10%	0.10%
0.20%	0.20%	0.20%

Supplementary Table 3 - Buffers and concentrations tested in flow injections.

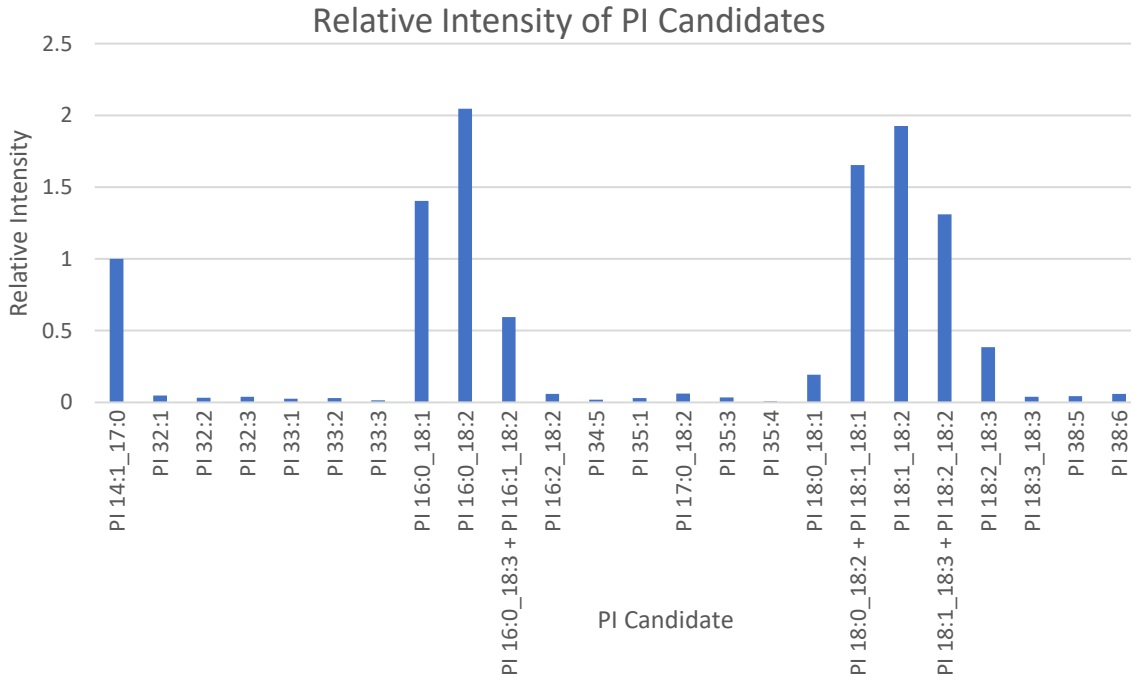
Glacial acetic acid	AmAc	AmOH	AmF
0.10%	0.00 mM	0.00 mM	0.00 mM
0.25%	0.25 mM	0.25 mM	0.25 mM
0.50%	0.50 mM	0.50 mM	0.50 mM
1.00%	1.00 mM	1.00 mM	1.00 mM
	2.50 mM	2.50 mM	2.50 mM
	5.00 mM		
	10.00 mM		



Supplementary Figure 1 - Intensities of the individual PI species ionised in the presence of AmF



Supplementary Figure 2 - PI Candidates organised by mass and retention time. The red diamond indicates the internal standard added, whereas the blue diamonds indicate PI species identified through the use of MS2 data. The hollow, green diamonds indicate candidates which have been proposed due to the pattern formed by identified species.



Supplementary Figure 3 - All PI species observed in the canola extract with their intensity relative to the internal standard PI 14:1 17:0. From the distribution, it is apparent that a large proportion of the species are relatively non-abundant. Furthermore, it may be seen that several of the more intensely observed PI species are composed of more than one isobar.

Acknowledgments

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Chapter Four:

Simultaneous Analysis of Phosphatidylinositol and Inositde Species Using Novel Liquid Chromatography-Mass Spectrometry Buffer Conditions

PREAMBLE

The enhanced ionisation efficiency imparted by the ammonium fluoride buffer proved valuable in increasing the depth of characterisation afforded to the PIs. These novel analysis conditions would now permit their characterisation where they had previously been obscured, especially when considering matrices with a lower abundance of these species. However, despite the improved sensitivity afforded to these molecules, these analysis conditions would remain unsuitable for a comprehensive characterisation due to a few major complications. Primarily, none of the PI derivatives, such as the LPIs or PIPs, were identified in any of the prior analyses, whilst the analysis conditions also proved unsuitable for the chromatography of the PIs themselves. This latter complication was evident due to the presence of particular ions in the fragmentation spectra for various identifications, some of which are provided in Figure 29, though unique fragments are not necessarily produced for all forms of isomer. Hence, due to the co-elution, mass spectrometry alone cannot indicate the distribution of species present, and separation would be necessary to distinguish these species. Additional examples of non-differentiable isomers, including structures, are provided in Figure 30.

The major portion of these complications are suspected to have arisen due to the brief retention of the PIs, compared to the retention of other phospholipid classes, such as the PEs, shown in Figure 31. Those additionally hydrophilic species, such as the LPIs, are thus suspected to have not been retained at all and were perhaps suppressed from detection by other unretained molecules. The precursor mass for a candidate LPI species had been observed in the unretained region, as shown in Figure 32 although its identity could not be confirmed due to the lack of MS^2 data. Similarly, this brief retention is expected to have been insufficient to achieve chromatographic resolution for those isomeric PI species observed. Thus, a re-evaluation of the chromatographic gradient and solvents would be necessary to ensure the retention and separation of these species. However, additional factors independent of the chromatography were also likely preventing the detection of the PIPs, as these species were also not observed in the preceding flow injections. Diffusion due to the chelation behaviour of the additional phosphate moieties was expected to be a significant contributing factor in this case, and this would need to be considered moving forward.

Removing isopropanol as a mobile phase solvent was expected to improve the chromatography significantly, as eliminating the strong elution solvent would increase retention for all hydrophobic molecular species. It was also suspected that the polar alcohol group contained in isopropanol, to some extent, resulted in the diffusion of peaks due to non-specific polar hydrogen bonding interactions. Hence, with its removal, peak widths were expected to remain narrow despite the increase in retention. Furthermore, as demonstrated

by the suppressive effects imparted by alcohols such as methanol, it was anticipated that the elimination of isopropanol might simultaneously enhance the sensitivity of analysis and improve the detectability of these species.[260] However, due to the increased retention observed for other lipid species, a reduction in elution strength would likely result in their carry-over, as a mobile phase lacking isopropanol might be insufficient to enable their elution at all. Adopting an SPE protocol to isolate the PIs was deemed necessary in this case.

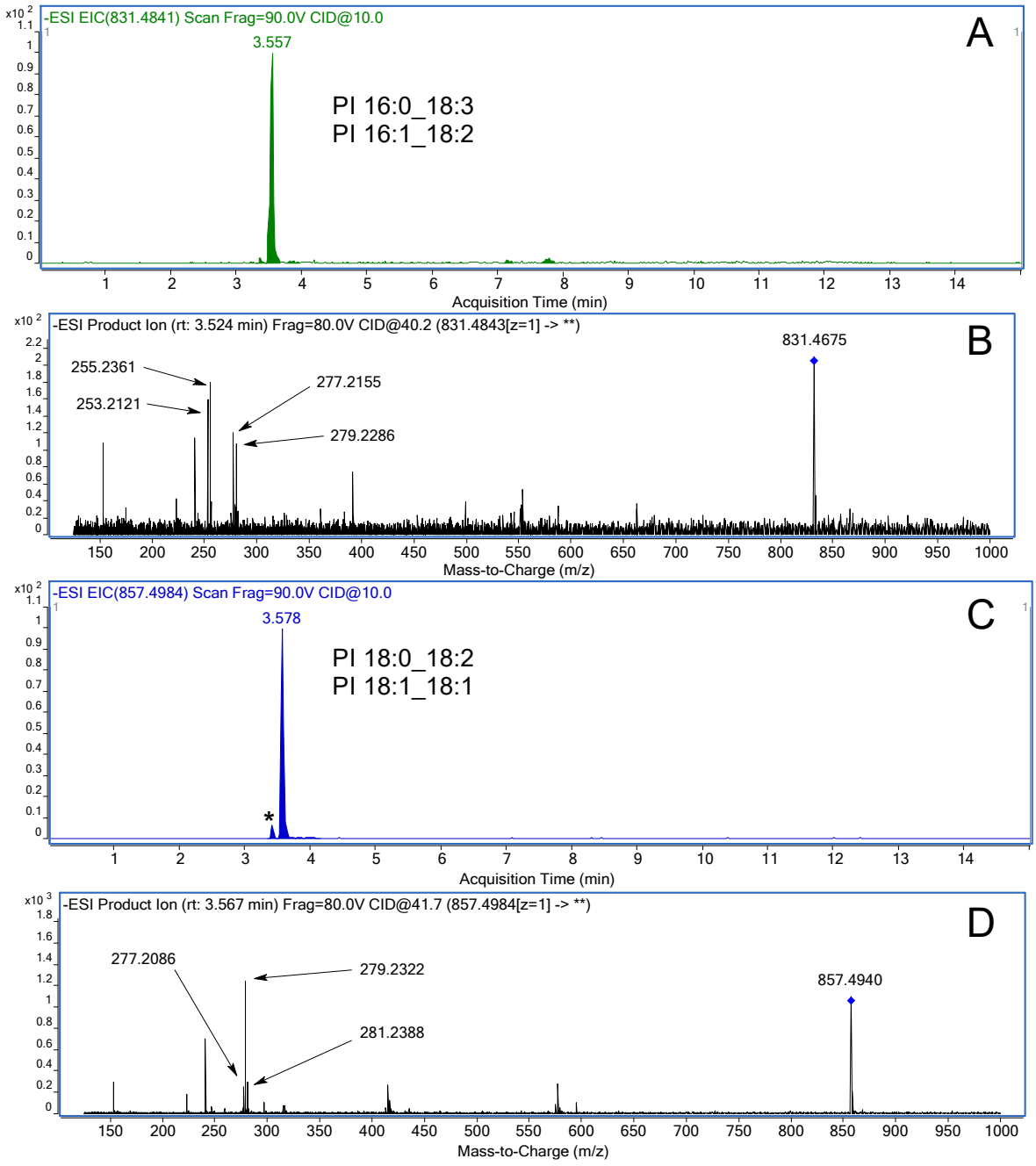

Figure 29 – The relatively poor retention of the PI species prevents sufficient resolution between isomers, which appeared to co-elute into a single peak. In A and C, two pairs of isomers are displayed as EICs to reveal the insufficient resolution achieved by these conditions. In B and D, fragmentation spectra for these peaks are provided, highlighting the unique fragments produced by certain isomers. Those peaks marked

by an asterisk (*) represent heavier isotopes of chromatographically resolved species and are not structural isomers.

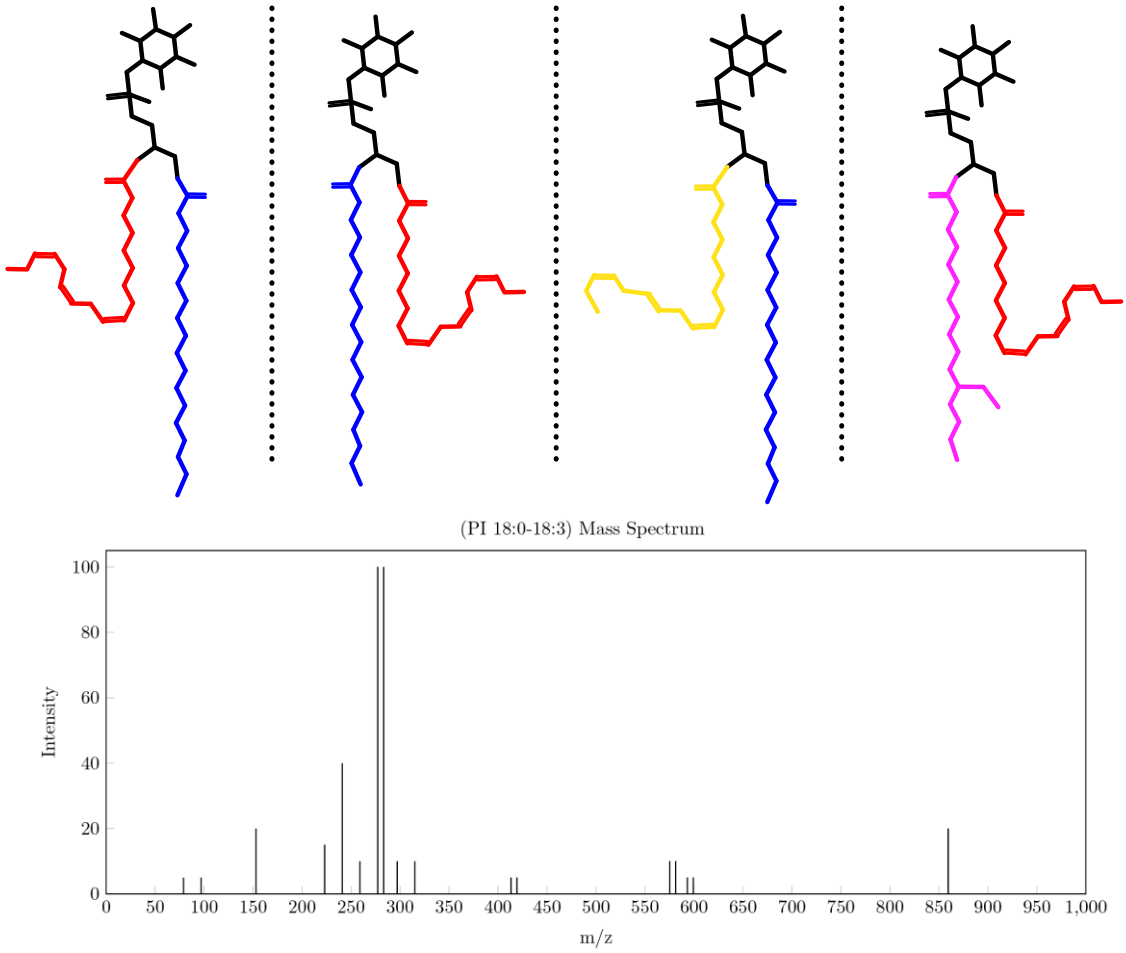

Figure 30 – A set of 4 isomeric PI species. The fragmentation spectra for all these species would produce ions of identical mass, as displayed, although their ratios may differ slightly.

The non-specific interactions exhibited by the PIPs would need to be addressed in an alternate manner. To this end, a series of instrumental modifications were incorporated, including the use of a passivated chromatograph and stationary phase specially designed for the chromatography of phosphorylated compounds.[261] However, as it was uncertain whether these changes alone would prove sufficient for the chromatography of the PIPs, buffer composition was re-evaluated to determine whether the competing chelating effect of carbonate [139] could protect against the diffusion of these compounds. This work culminated in the following chapter, published as a manuscript in the Journal of Separation Science.

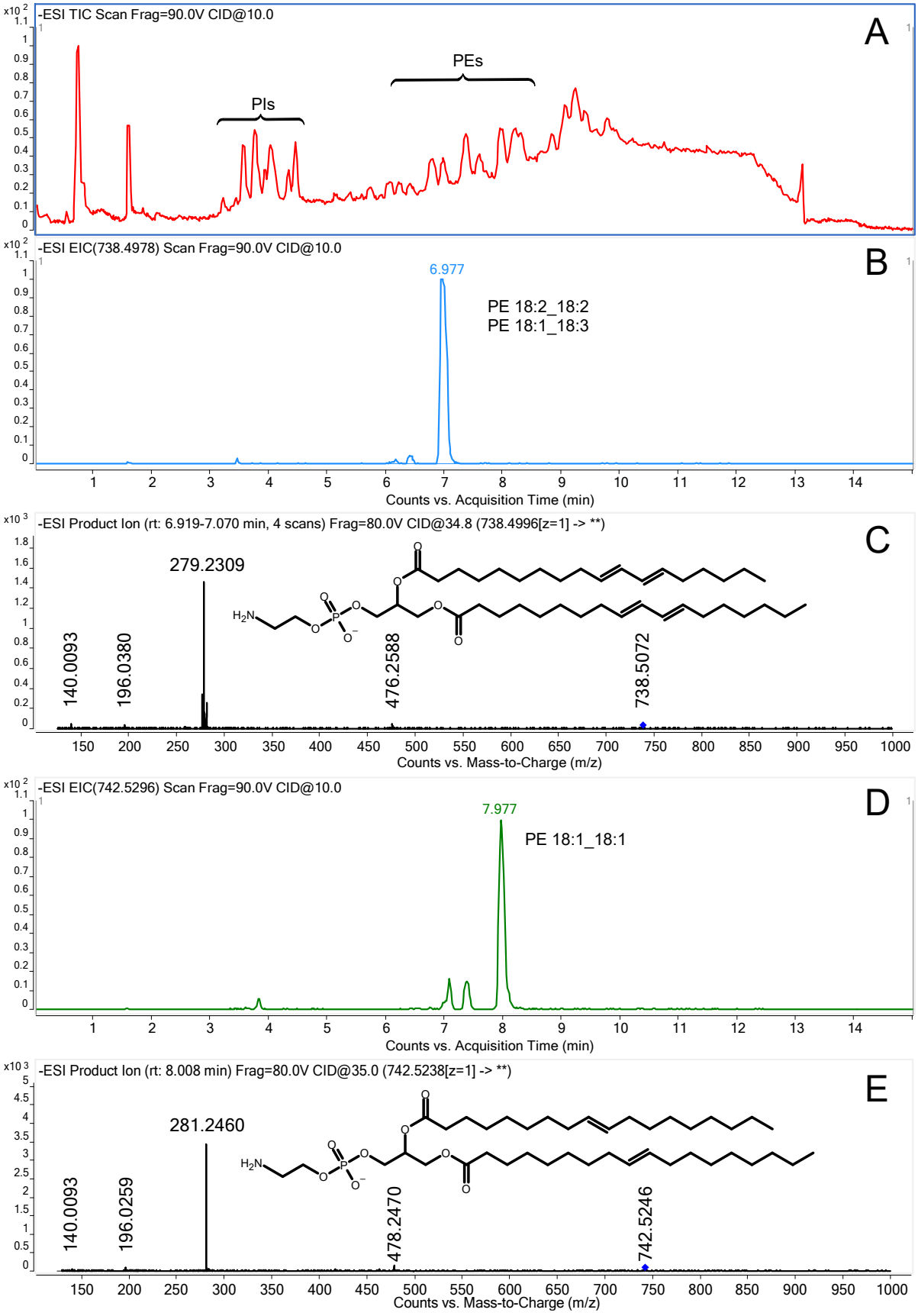

Figure 31 – (A) Under the ammonium fluoride buffer, the peaks associated with PIs elute before 4.5 minutes, whilst other phospholipid classes, such as the PEs, continue until approximately 8.5 minutes. A set of example peaks confirming the later elution of the PEs are shown in B and D. The MS2 spectra associated

with these species are also provided to confirm their identity (C, E); however, the precise structure of the fatty acids is ambiguous.

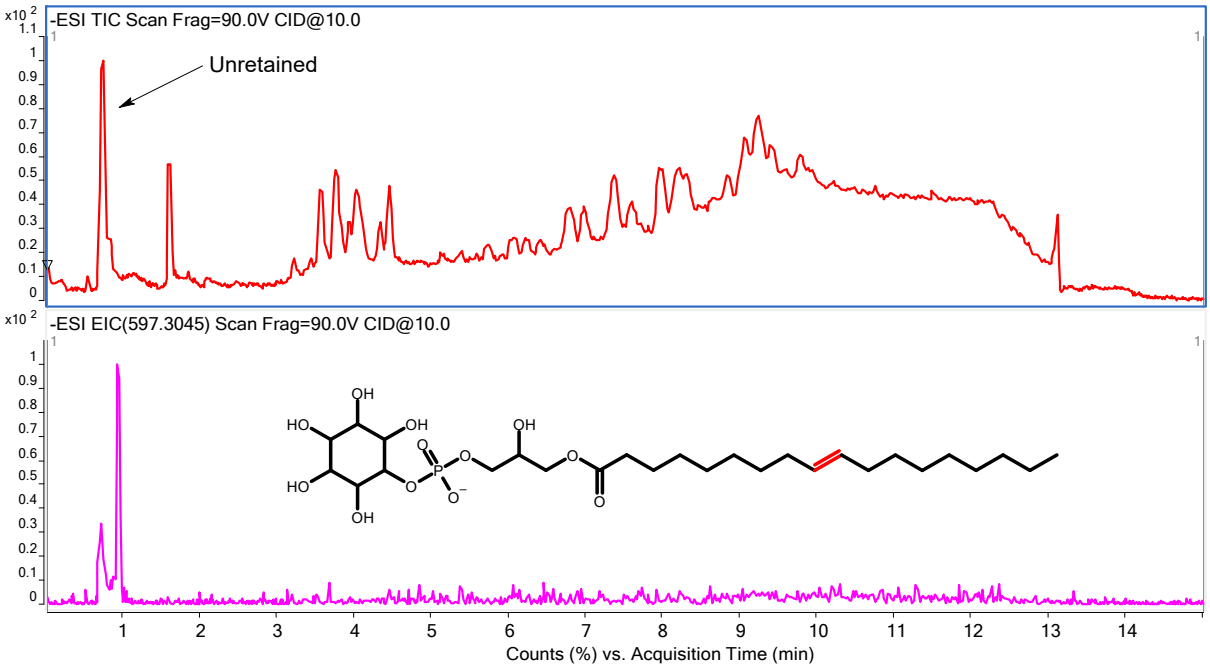

Figure 32 – An example EIC corresponding to the mass of a LPI species, LPI 18:1, although this identity could not be confirmed due to the absence of MS2 data. This candidate peak had eluted in the unretained region and suggested the unsuitability of the method in its current form for the analysis of these species.

CERTIFICATE OF AUTHORSHIP

The following chapter is an accepted manuscript published in the *Journal of Separation Science (J. Sep. Sci.)*. I, David Gertner, certify that the work in the following chapter has not been submitted as part of any other documents required for a degree.

AUTHORSHIP CONTRIBUTIONS

AUTHOR	CONTRIBUTION	SIGNATURE
DAVID S. GERTNER	**CONCEPTUALISATION, EXPERIMENTAL DESIGN, PERFORMED EXPERIMENTS, ANALYSED DATA, WROTE ORIGINAL DRAFT AND EDITED MANUSCRIPT**	Production Note: Signature removed prior to publication.
DAVID P. BISHOP	**REVIEWED AND EDITED MANUSCRIPT**	Production Note: Signature removed prior to publication.
MATTHEW P. PADULA	**REVIEWED AND EDITED MANUSCRIPT**	Production Note: Signature removed prior to publication.

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RESEARCH ARTICLE

JOURNAL OF SEPARATION SCIENCE

Optimisation of Chromatographic Buffer Conditions for the Simultaneous Analysis of Phosphatidylinositol and Phosphatidylinositol Phosphate Species in Canola

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ABSTRACT: The phosphatidylinositols and phosphatidylinositol phosphates are a set of closely related lipids known to influence various cellular functions. Irregular distributions of these molecules have been correlated with the development and progression of multiple diseases, including Alzheimer's, bipolar disorder, and various cancers. As a result, there is continued interest regarding the speciation of these compounds, with specific consideration on how their distribution may differ between healthy and diseased tissue. The comprehensive analysis of these compounds is challenging due to their varied and unique chemical characteristics, and current generalised lipidomics methods have proven unsuitable for phosphatidylinositol analysis and remain incapable of phosphatidylinositol phosphate analysis. Here we improved upon current methods by enabling the sensitive and simultaneous analysis of phosphatidylinositol and phosphatidylinositol phosphate species, whilst enhancing their characterisation through chromatographic resolution between isomeric species. A 1 mM ammonium bicarbonate and ammonia buffer was determined optimal for this goal, enabling the identification of 148 phosphatidylinositde species, including 23 lyso-phosphatidylinositols, 51 phosphatidylinositols, 59 oxidised-phosphatidylinositols, and 15 phosphatidylinositol phosphates. As a result of this analysis, four distinct canola cultivars were differentiated based exclusively on their unique phosphatidylinositde-lipidome, indicating analyses of this type may be of use when considering the development and progression of disease through lipidomic profiles.

BACKGROUND

The phosphatidylinositols (PIs) are a class of biologically important, non-abundant phospholipids, distinguished by a common *myo*-inositol headgroup. These lipids, alongside their phosphorylated derivatives (i.e., the PI phosphates, or PIPs), are ubiquitous in eukaryotic life and aid in coordinating various key cellular processes, including signal transduction, membrane trafficking, and cell proliferation.[32, 64, 76, 239, 262, 263] As a result of their widespread

activities, aberrations that influence a cell's ability to regulate these lipids can result in disease, and abnormal levels are often noted as a distinguishing feature in Alzheimer's disease,[62-64] Bipolar disorder [62, 63] and cancer.[62-66] Such lapses in regulation can likewise result in their irregular molecular distribution, permitting analyses to reveal the development of such pathologies. Hence, continual interest in the activity of these molecules motivates their in-depth characterisation.[32, 235, 239, 263, 264] Unfortunately, conventional lipidomic methods have proven inadequate when considering these species, as their physical and chemical properties are often prohibitive to their analysis.[90]

Current lipidomic characterisations often employ reversed-phase liquid chromatography electrospray-ionisation-tandem mass spectrometry (RPLC-ESI-MS/MS), as the combination of these techniques enables a highly selective and sensitive analysis.[137, 243] Lipidomic methods based on this format regularly pursue the rapid and simultaneous characterisation of numerous classes at once and have been optimised for purposes of high throughput and sample fingerprinting.[71, 137] So-called comprehensive methods have rapidly become the standard approach in lipidomic analysis and are likewise employed in characterisations where only a single lipid class is considered.[67, 71, 137, 265] However, these generalised protocols often compromise conditions favourable for individual classes to achieve maximum lipidome coverage and, as a result, may prove non-conducive to the analysis of certain lipid classes. PIs, for example, exhibit suppressed ionisation under these generalised conditions, which likewise achieve poor chromatographic resolution between isomers of this class, despite readily doing so for other lipid classes.[70, 260, 266-268] In extreme cases, whole lipid classes may be overlooked despite the potential for their presence in a sample. Such is the fate of the PIPs, which are regularly excluded from these characterisations due to their high polarity, poor chromatographic performance, poor ionisation efficiency, their propensity to chelate to various surfaces, and their requirement for specialised extraction conditions.[101, 142] Their analysis has instead been relegated to alternative and specialised protocols, resulting in a disconnect between PI and PIP characterisations, despite their biological connection. Hence, characterisations that consider these lipids are disjointed and often lack the depth achieved with other classes, and there are no alternative methods that address these complications.

We previously demonstrated that modifications made to the mobile phase buffers used in current generalised protocols are sufficient to bias conditions to favour the analysis of PIs, allowing for their characterisation with greater depth.[260] However, these conditions remained unsuitable for the analysis of PIPs. Therefore, this investigation aims to expand upon our previous work and enhance the characterisation of PI species and their phosphorylated derivatives by permitting their simultaneous analysis.

In addition to those advances previously made in the analysis of PIs, various sources throughout the literature have suggested a number of other variables which may be leveraged to pursue this goal. Experimental work regarding the influence of mobile phase pH and solvent composition has suggested that an alkaline and acetonitrile-based mobile phase may be sufficient to enhance the chromatographic retention, resolution, and ionisation efficiency for these lipid classes.[67, 143, 146, 260, 269] Furthermore, it is suggested that the chelation behaviour and poor chromatographic performance of PIPs may be addressed through the use of certain specialised stationary phase technologies [261] and mobile phase additives,[139] circumventing the previous necessity of ion-pairing agents and their potential for instrument contamination.[143, 270, 271]

Based on the abovementioned conditions, a new chromatographic method was optimised and applied to the characterisation of phosphatidylinositol species present across four cultivars of canola. The results were then compared against those of our previous study.[260] Canola was again selected as a matrix due to its proportionally high content of PIs (representing 11 – 18% of phospholipids) and unique fatty acid profile.[148, 248] Finally, the PI and PIP species identified in each cultivar were then used in principal component analysis to determine whether the distribution of molecular species present was specific enough to permit their differentiation. Such a result may indicate the utility of these conditions in distinguishing the molecular changes which occur in the development of pathology.

MATERIALS AND METHODS

MATERIALS AND STANDARDS

Acetonitrile (ACN) and Methanol (MeOH) were of LC/MS grade, Honeywell B&J brand, purchased from ChemSupply Australia. Chloroform ($CHCl_3$) was of LC grade, LiChrosolv brand, purchased from Sigma-Aldrich (Castle Hill, Australia). Glacial acetic acid (GAA) of 98% purity or greater and 12M Hydrochloric acid (12M HCl) were both purchased from Sigma-Aldrich (Castle Hill, Australia). Ammonium acetate, ammonium hydroxide, ammonium fluoride and ammonium bicarbonate (ammonium hydrogen carbonate) were also purchased from Sigma-Aldrich (Castle Hill, Australia) and were of analytical reagent grade or higher. Ultra-pure water (18.2 MΩ-cm) was sourced from an in-lab water purification system (Sartorius), henceforth referred to as H_2O. Phospholipid standards (17:0-14:1 PI, 18:1-18:1 PI, 18:0-20:4 PI, 18:1-18:1 PI(3)P, 18:1-18:1 PI(5)P) were purchased from Avanti Polar Lipids Inc. Oasis WAX 1 cc cartridges (30 mg stationary phase), and the ACQUITY Premier CSH C18 column (150 mm x 2.1 mm, 1.7 µm) were purchased from Waters Australia Pty Ltd (Rydalmere, Australia).

Four cultivars of canola seed (44Y90CL, 45Y93CL, 45T03TT and NuseedDiamond) were provided for analysis by the Department of Primary Industries NSW, Wagga Wagga.

SAMPLE PREPARATION

Canola extracts were prepared based on an acidified chloroform extraction protocol which has elsewhere been used for the analysis of phosphatidylinositide species.[143] Briefly, 500 µL of 1M HCl and 500 µL of MeOH containing 0.2 ppm 17:0-14:1 PI as internal standard was added to a mass of 20 mg homogenised canola seed in a 2 mL Eppendorf tube. After 10 seconds of vortexing and 30 seconds of sonication, the phase split was initiated by adding 500 µL of $CHCl_3$ to the sample. The sample was vortexed for an additional 10 seconds and then left on ice for 5 minutes. After one minute of centrifugation at 1000 g, the resulting chloroform layer was removed (approximately 500 µL), diluted with 500 µL of MeOH, and then applied directly to a 30 mg Oasis WAX cartridge that had been primed prior by passing through 2 mL of MeOH, followed by 2 mL of glacial acetic acid (GAA): MeOH (1:4) and finally, 2 mL of $CHCl_3$: MeOH (1:1). After the application of the sample, fractions containing both neutral and cationic lipids were eluted with an additional application of 2 mL of $CHCl_3$: MeOH (1:1). Finally, anionic lipids were eluted and collected from the cartridge using 2 mL of 12M HCl: $CHCl_3$: MeOH (1:6:6). After collection, the acidified solution was immediately washed with the addition of 1 mL of UP water, whereafter the chloroform layer was once again collected and dried under a flow of N_2, before reconstitution in a solution of 50% ACN containing 2 mM ammonium bicarbonate and 2 mM ammonia. After reconstitution, samples were applied directly for analysis. A blank extract, which contained only the internal standard, was prepared alongside five replicate extractions per cultivar. A quality control sample was prepared for each cultivar by pooling together the five replicate extracts. Analysis occurred on the same day of extraction to limit the possibility of sample degradation.

INSTRUMENTATION

Mass spectrometric analysis was conducted using a Thermo Fisher Q Exactive Plus mass spectrometry system in Top-N DDA analysis mode. An MS^1 scan accumulated from 500 to 1000 *m/z* for a maximum IT of 80 ms, and a mass resolution of 35,000. MS^2 scans targeted the eight most intense ions for a maximum IT of 60 ms each, with an isolation window of 1.5 *m/z*, fixed first mass of 120.0 *m/z* and an NCE of 30. The mass resolution for these scans was 17,500. The ESI ion source was held at position 'B'; the sheath gas, aux gas, and sweep gas were 50, 10 and 0 arbitrary units, respectively. Electrospray voltage was -2.5kV, and capillary and aux gas temperatures were at 350° C and 300° C, respectively. The S-lens RF level was at 70 arbitrary units.

Chromatography was conducted using an ACQUITY Premier CSH C18 column (15 cm x 2.1 mm), and Thermo Fisher Vanquish quaternary pump system. The optimised chromatographic conditions are as follows:

Flow was maintained at 0.5 mL/min, with a column temperature of 55° C. Mobile phase A consisted of 1 mM ammonium bicarbonate and 1 mM ammonia in H_2O, whereas B consisted of 95:5 ACN: H_2O. Gradient conditions began at 40% B and elevated to 50% B at 0.25 minutes, followed by 70% B at 6 minutes, 82% B at 12 minutes and 100% B at 14 minutes. For wash and equilibration, the gradient was then held at 100% B until 18 minutes, where it then immediately reverted back to 40% B until 25 minutes.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

Initial experimentation was designed to compare the chromatographic performance imparted by certain buffers in terms of their utility for both PI and PIP analysis. The buffer additives (ammonium acetate, ammonium fluoride, ammonium bicarbonate, and ammonium bicarbonate with added ammonia) were selected based on previous experimentation, which suggested their suitability both for PIs and the analysis of phosphorylated compounds.[71, 139, 260] These buffers were added to mobile phases A (H_2O + 1 mM buffer) and B (95:5 Acetonitrile: H_2O + 1 mM buffer) to utilise the favourable conditions imparted by acetonitrile whilst eliminating the potential for buffer precipitation through the removal of non-aqueous conditions. Additionally, an ACQUITY Premier CSH C18 column was chosen for these tests as the stationary phase due to its advertised improved chromatographic performance in separating phosphorylated compounds.[261] The column temperature was maintained at 45° C. The flow rate was held at 0.4 mL/min over a linear gradient that incremented from 30 to 100% B in 28 minutes, which was then held at 100% B for an additional 5 minutes before re-equilibration. A 1 ppm solution of the standards PI(3)P 18:1_18:1, PI 18:1_18:1 and PI 18:0_20:4 in MeOH was prepared to examine the chromatographic performance of PIPs and PIs simultaneously. In addition, the PI species selected were highly hydrophobic and would help determine whether the chosen solvent conditions were sufficient for their elution within a reasonable time frame. Triplicate injections of 1 µL were used to ensure retention time stability throughout the various conditions tested.

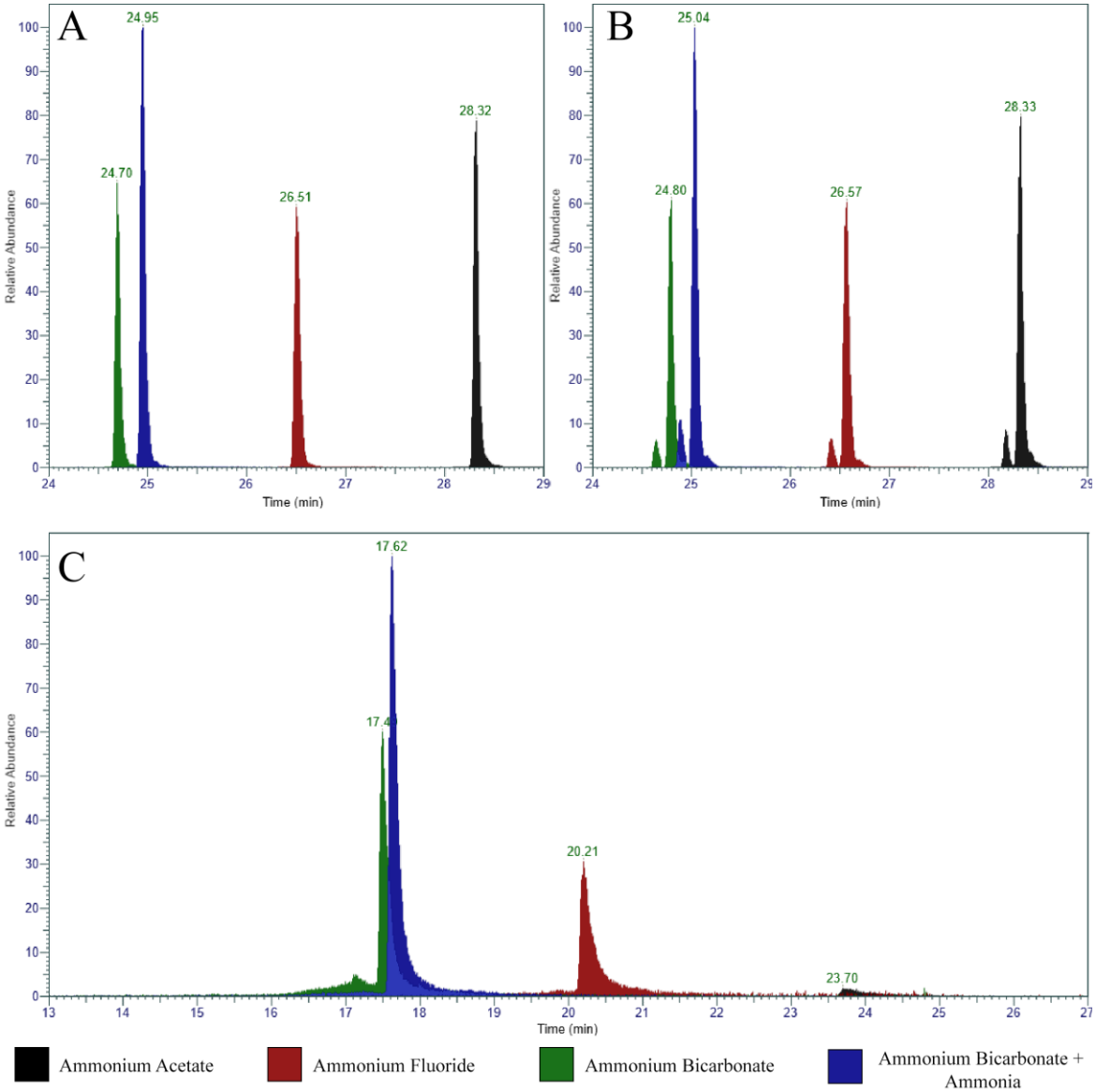

Figure 33 - Extracted ion chromatograms (EICs) taken from the analysis of a mixture of standards containing: (A) Phosphatidylinositols (PI) 18:1_18:1, (B) PI 18:0_20:4 and (C) PI(3)P 18:1_18:1. The *m/z* values for the [M-H]- ions which correspond to each of these species are as follows: (A) 861.5499, (B) 885.5499 and (C) 941.5162. Four overlayed EICs are displayed per standard, displaying the differing chromatographic behaviours observed with respect to the buffers examined. The four buffers assessed here were ammonium acetate, ammonium fluoride, ammonium bicarbonate, and ammonium bicarbonate with equal parts added ammonia. The ammonium acetate buffer provided sufficient chromatography for the PI standards (A and B) but was unacceptable for the phosphatidylinositol phosphate (PIP) standard (C). A shift in retention was observed for both the PI and PIP standards in the sequential ammonium fluoride and ammonium bicarbonate tests, while the peak shape of the PIP standard improved considerably. The addition of ammonia to the ammonium bicarbonate buffer further improved peak shapes and intensities for all standards considered.

Table 4 – A comparison of the measured buffer pHs (1mM, aqueous), pKbs, observed peak retention times (RT, minutes) and baseline widths (W, minutes) for standards A (PI 18:1_18:1), B (PI 18:0_20:4) and C (PI(3)P 18:1_18:1). Contrary to the expectations set out by the literature, the values for peak width for compound C, and values for retention time for all compounds appeared to vary independently of either pH or pKb.

BUFFER	PH	PKB	RT (A)	RT (B)	RT (C)	W (A)	W (B)	W (C)
AM. ACETATE	7.11	9.25	28.32	28.33	23.70	0.25	0.19	1.19
AM. FLUORIDE	6.03	10.80	26.51	26.57	20.21	0.25	0.20	0.56
AM. BICARBONATE	8.13	7.65	24.70	24.80	17.49	0.28	0.20	0.32
AM. CARBONATE	9.11	3.70	24.95	25.04	17.62	0.27	0.18	0.32

Figure 33 demonstrates that the peak shapes and retention times observed for the selected standards varied considerably with the buffers evaluated during the method development phase. Interestingly, all conditions produced suitable peak shapes for the PIs (Figure 33A and B), but the addition of ammonia to the ammonium bicarbonate buffer was necessary to maximise the chromatographic performance of the PIP standard (Figure 33C). This suggested a potential relationship between the mobile phase pH and the chromatographic performance of PIPs, which is consistent with the literature on the chromatography of phosphorylated compounds.[146] Additionally, changes in retention time such as those observed in Figure 33 have previously been related to the pKb of the buffer anion.[269] However, as shown by the data in Table 4, peak widths and retention time produced by the buffers did not correlate with either buffer pH, or the anions pKb. This, rather, suggests the presence of an alternate or competing mechanism relating the buffer to chromatographic performance, perhaps related to each anions ability to chelate, ion pair, or position in the Hofmeister series. However, these suggestions remain purely speculative and require further evaluation.

In terms of their suitability for analysis, we assessed the lower retention times produced by the ammonium bicarbonate and ammonium bicarbonate with added ammonia buffers to be conducive to the analysis of PIs, as under the ammonium acetate conditions, elution of the chosen standards occurred at approximately 99% mobile phase B. Under these conditions, the mobile phase may prove insufficient to elute more hydrophobic species within a reasonable timeframe, if at all. In comparison, these standards eluted at approximately 91% mobile phase B with both ammonium bicarbonate and carbonate buffers. In addition, despite

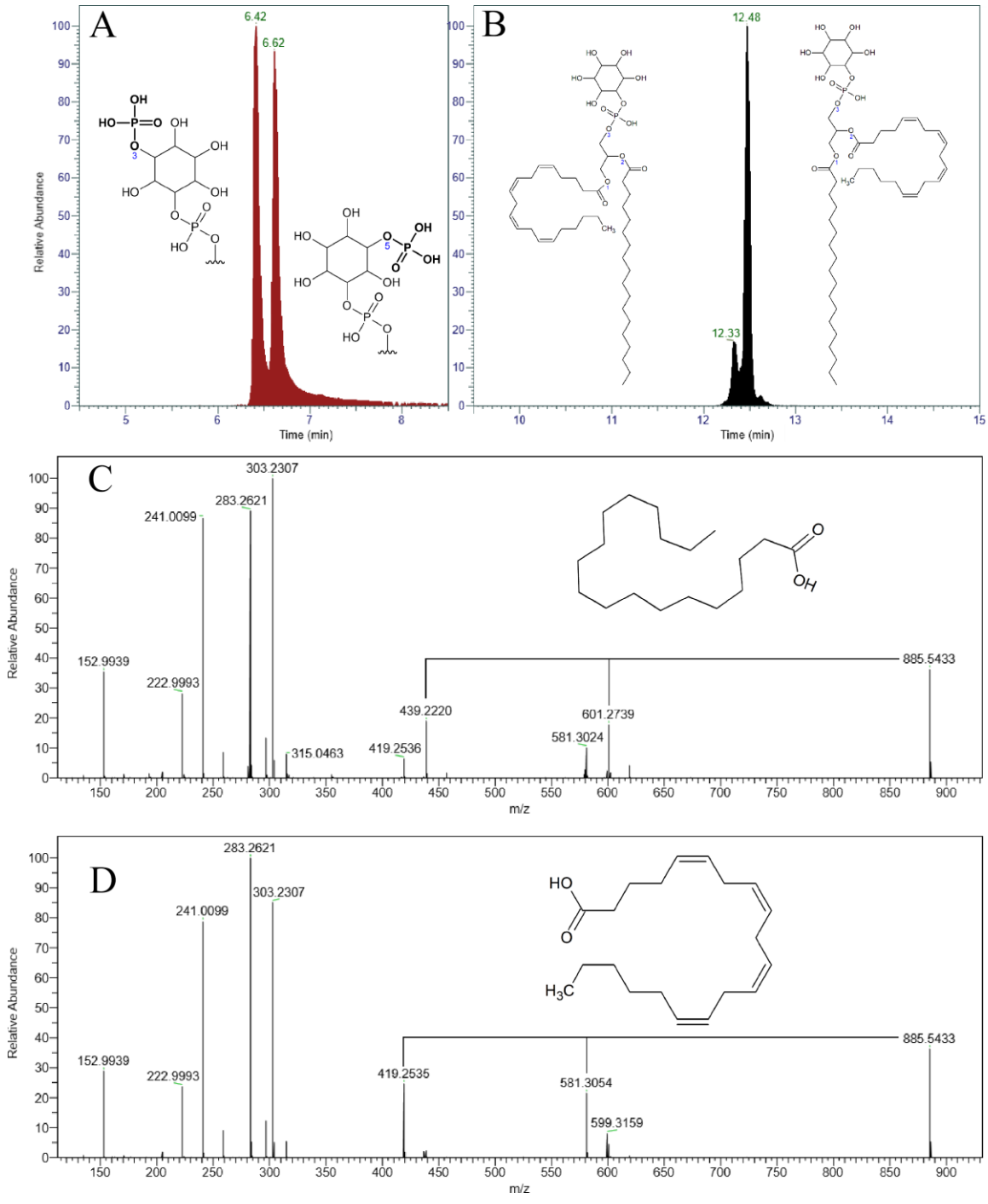

Figure 34 - The extent of the chromatographic resolution achievable between phosphatidylinositol phosphate (PIP) headgroup isomers (PI(3)P and PI(5)P) (A) and the tail regio-isomers phosphatidylinositols (PI) (PI 20:4/18:0 and PI 18:0/20:4) (B) is shown using the finalized chromatographic conditions. The MS^2 spectra for the two major PI tail regio-isomers, at retention times 12.33 and 12.48, are shown in Figures C and D, respectively. The identity of these isomers could be verified through their unique fragmentation, which corresponded to the identity of the *sn-2* fatty acid.

the reduction in retention time, clear resolution was maintained between the tail regio-isomers of the PI 18:0_20:4 standard. A slight decline in peak intensity is also observed in this order and may result from the retention time shift, as the differences in the mobile phase composition at the point of elution can influence desolvation and nebulisation.[236] However, our previous work suggested that the peak intensities observed under the ammonium fluoride buffer would far outperform those measured with ammonium acetate, yet this theme is not followed here.[260] The differences in ionisation efficiency previously observed may, therefore, be reliant on the mobile phase composition used in our previous study, namely either due to the presence of isopropanol in the mobile phase or the more aqueous conditions at the point of elution. In favour of the latter case, a marked increase in intensity is observed for the PIP standard in Figure 33C when compared to the ammonium acetate conditions; this, however, may exclusively be the product of the increased chromatographic performance. A final increment in peak intensity is then observed with the ammonium bicarbonate with added ammonia buffer, potentially as the alkaline conditions may enhance ionisation efficiency.

As a result of this process, the conditions of 1 mM ammonium bicarbonate with 1 mM ammonia were deemed satisfactory for the simultaneous analysis of PIP and PI species, and the method was further optimised for purposes of rapid sample throughput. For this optimisation process, a second standard solution containing 1 ppm PI(3)P 18:1_18:1, PI(5)P 18:1_18:1, PI 18:1_18:1 and PI 18:0_20:4 in MeOH was prepared to observe whether resolution could be achieved between both PIP headgroup isomers and PI tail regio-isomers. This development process saw the elimination of buffer from mobile phase B, which was highly organic and poorly solubilised the polar ammonium bicarbonate crystals. Not only did this further reduce the risk of buffer precipitation, but it comparatively increased the elution strength of mobile phase B, reducing the likelihood of exceptionally hydrophobic species carrying over between injections, and prevented the mobile phase from increasing in alkalinity with organic composition, which could potentially exceed the columns limit.[272] The final conditions are provided in section 2.3. As shown in Figure 34A and B, chromatographic resolution could be achieved between both PIP headgroup isomers and PI tail regio-isomers, the latter of which is verified through their unique fragmentation spectra (Figure 34C and D).

METHOD VALIDATION

Following the completion of method development, these conditions were then validated for the purpose of untargeted characterisation and applied in the analysis of canola PI and PIP species. Injection carry-over was assessed, with particular attention to the PIPs due to their activity as chelating agents, prior to all other validation steps to ensure accurate measurement of detection limits and linear correlation. Various efforts have been employed elsewhere to

mitigate PIPs chelating, including the use of a competing chelating agent such as EDTA.[142, 143] Therefore, a wash solution of 50% acetonitrile containing 25 µM EDTA was here used to aid in mitigating carry-over. In these tests, 2 µL injections of the same 1 ppm PI(3)P 18:1_18:1, PI(5)P 18:1_18:1, PI 18:1_18:1 and PI 18:0_20:4 solution was used, followed by a blank injection. The blank injection was then inspected for traces of the standard mix. No carry-over was observed for the PIP standards; likewise, there was no carry-over observed for the PI standards used.

Table 5 - The precision of peak area measurements throughout and beyond the calibration range. The values provided here are in terms of %RSD, calculated using three repeat measurements per concentration. The precision for peak area measurements remained high from between 5 to 500 ppb, though rapidly declined over measurements less than 1 ppb in concentration. The LOD values provided in Table 6 were estimated by determining the concentration for which % RSD values approximately equalled to 25%.

STANDARD	100 PPT	250 PPT	500 PPT	1 PPB	5 PPB	25 PPB	100 PPB	200 PPB	500 PPB
PI (3) P 18:1 18:1	19.09%	25.96%	8.07%	8.63%	5.71%	2.96%	1.12%	1.15%	0.37%
PI 18:1 18:1	13.10%	15.42%	5.64%	6.16%	0.83%	1.58%	1.28%	1.98%	1.28%
PI 18:0 20:4	51.59%	3.98%	8.36%	4.24%	2.41%	1.13%	1.93%	1.92%	2.57%

Table 6 - Summarised results from method validation. Limits of detection were determined by sequentially expanding the calibration curve on the lower end until the signals reproducability varied significantly. This was estimated where the % RSD values for peak area approximately equalled to 25%. Numerical values showing the % RSD for all measured concentrations are shown in Table 5. Linearity was determined over the range of 5 to 500ppb. Retention time stability was determined over the course of 12 repeat injections, equivalent to 5 hours of analysis time.

STANDARD	*M/Z* (PREDICTED)	LOD	r^2	RT (MINS)	RT % RSD
PI (3) P 18:1 18:1	941.516182	~ 250 ppt	0.9981	6.40	0.30
PI 18:1 18:1	861.549852	~ 100 ppt	0.9993	12.33	0.07
PI 18:0 20:4	885.549852	~ 250 ppt	0.9992	12.47	0.07

Validation was then continued to determine the peak area precision over the expected sample concentration range; furthermore, the limits of detection, calibration curve linearity, and retention time precision were also evaluated. These values are summarised in Table 5 and Table 6. Values for peak area precision were calculated as a %RSD value over 3 repeat injections and would be used to estimate the limits of detection (LOD), where the %RSD approximated 25%. As shown in Table 6, the LODs were calculated to be in the range of a few hundred ppt, and the linearity from 5 to 500 ppb exceeded an R^2 value of 0.99 for all three representative lipids. Finally, retention time variation was measured over 12 injections, equivalent to a period of 5 hours, and was calculated to be below 0.3%.

The pH of mobile phase A was measured to ensure that the alkaline buffer used did not exceed the pH limits of the ACQUITY Premier CSH C18 column, which reproducibly measured approximately pH 9.11. This pH was well below the column's limit of pH 11.

CANOLA ANALYSIS

Five replicate canola extracts were prepared for each of the four cultivars according to the protocol outlined in section 2.2. Each sample was then analysed with triplicate injections. Quality control samples were prepared by pooling together the five replicate extracts and analysed on every 10th injection. Data processing was conducted using MS-DIAL [157] in metabolite mode to permit an in-house spectral library catered specifically for the identification of phosphatidylinositols.[273] All identifications were then manually assessed, normalised against the internal standard, and used in principal component analysis (PCA). PCA plots were constructed in Python using sklearn and displayed with pyplot.

As a result of the analysis, a total of 148 phosphatidylinositol species were identified across the four cultivars, including 23 Lyso-PIs, 51 PIs, 59 Oxidised-PIs, and 15 PIPs. The identifications are available in Supplementary Table 1, and an example TIC is provided in Figure 36A, along with a set of representative EICs in Figure 36B which depict the approximate retention times for the observed lipid classes. These identifications represent an over 5-fold increase in number compared to our previous study, which used a generalised lipidomics gradient and observed 14 PI species and 12 PI candidates.[260] This considerable increase in identifications is suggested to have arised due to a number of compounding influences, such as the inclusion of SPE in the extraction process reducing potential matrix effects, and the improved suitability of the analysis method favouring the chromatography and ionisation of these species. However, despite the chromatographic conditions being optimised to permit their analysis, the largest increase in identifications did not come from the PIPs but rather from a subset of the PIs with degrees of oxidation. These identified species differed in fatty acid chain length compared to the non-oxidised PI species observed, suggesting that they were

unique and did not originate from sample oxidation during the extraction process. The exact form of oxidation was not determined; however, it is likely that they comprise hydroxy-functionalised fatty acids, as they are a well-known constituent of seed oils and have been observed previously as a minor constituent.[274, 275] The majority of these species shared retention times approximate to those of the PIPs and likewise exhibited diffuse peaks, suggesting that they were also highly polar and may exhibit similar chelation behaviour. This is consistent with the chromatographic behaviour of oxidised PIs observed elsewhere.[276] Two example EICs of oxidised PI species are provided in Figure 36.

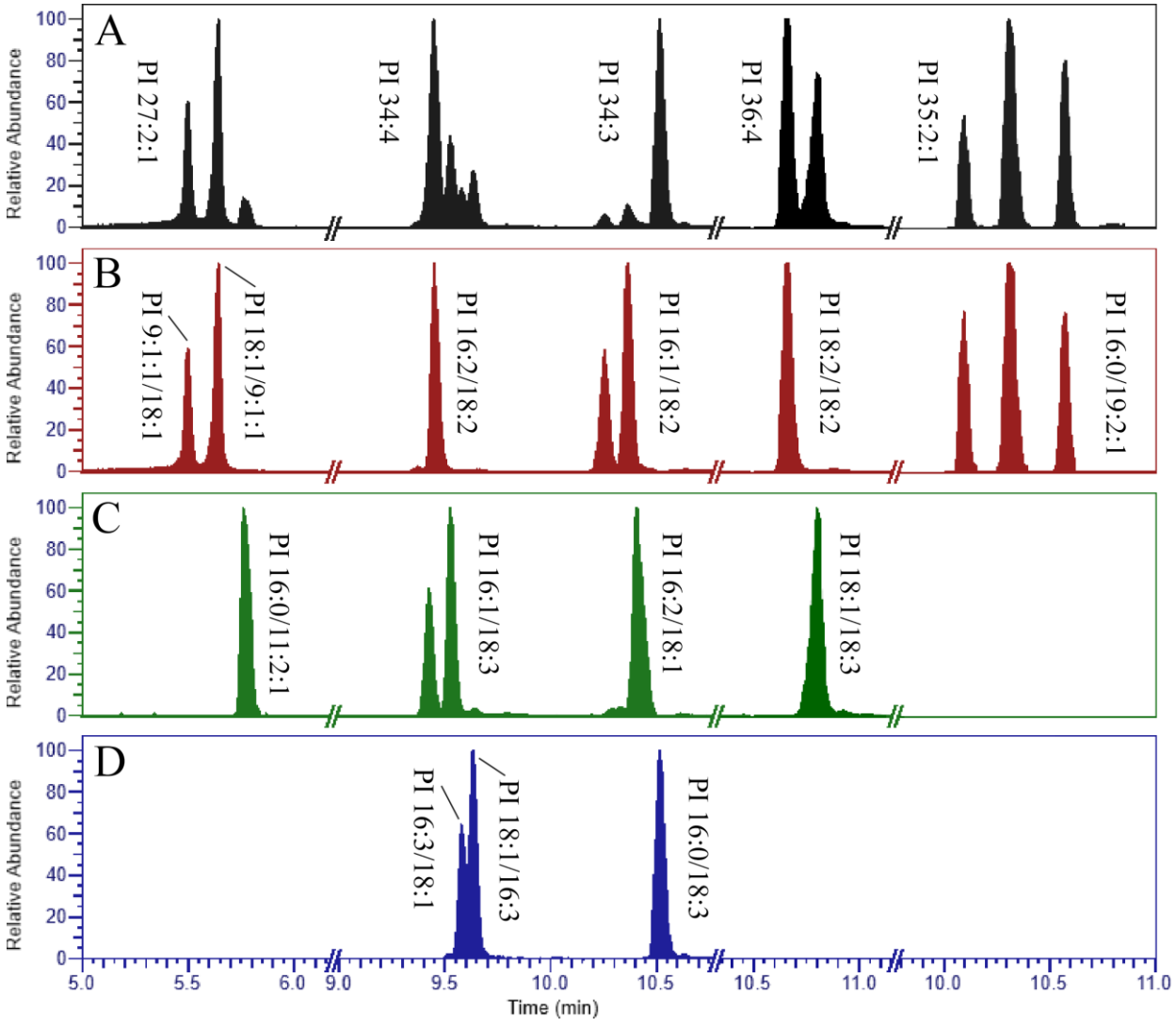

Figure 35 - EICs for various phosphatidylinositols (PI) species depicting the chromatographic resolution achieved between both tail isomers, tail regio-isomers, and double-bond isomers. In black (A), the extracted ion chromatograms (EICs) for five masses are provided (*m/z* 751.4027, 829.4899, 831.5006, 857.5229, and 861.5309), where each corresponds to a PI species (PI 27:2:1, PI 34:4, PI 34:3, PI 36:4 and PI 35:2:1, respectively). The subsequent red (B), green (C), and blue (D) chromatograms reveal the extent to which isomers may be resolved. For each mass, separation on the horizontal axis represents the resolution between both regio-isomers and double-bond isomers, whereas separation on the vertical axis represents a set of tail isomers.

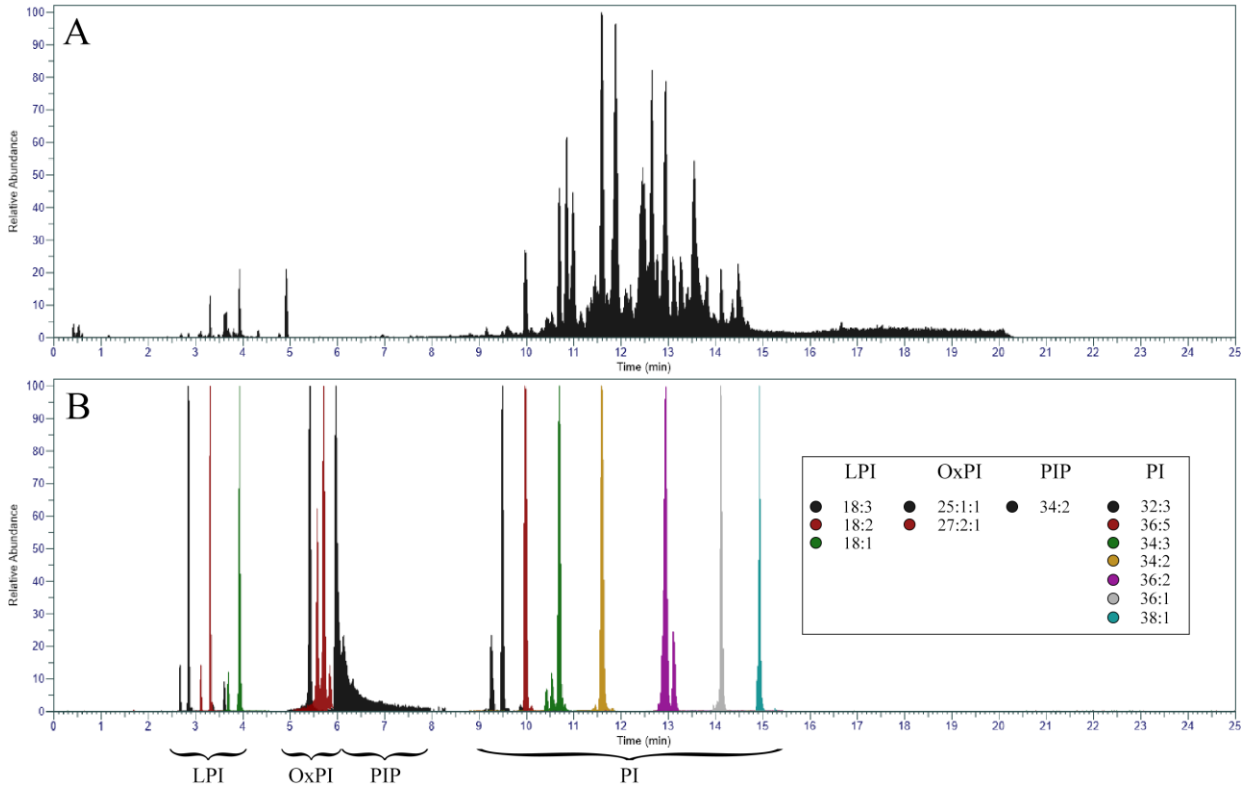

Figure 36 - (A) provides an example of TIC observed for one of the canola extracts. (B) provides an overlayed set of extracted ion chromatograms (EICs) depicting lyso phosphatidylinositols (PI) species, which generally appeared to elute between 2 and 5 min, phosphatidylinositol phosphate (PIP) species, which elute between 5 and 9 min, oxidized PI species from 5 min onward, and PI species from 9 min onward. All of the overlayed EICs are shown on independent scales.

Additionally, 68 of the identified species shared their precursor mass with at least one other identification and thus represented some form of lipid isomer. In comparison to our previous analysis, where only 6 co-eluting isomeric species were observed due to the presence of unique fatty acid fragments, the present method additionally resolved isomeric species that differed in *sn*-position, or double bond arrangement, as revealed by MS/MS analysis. The observation of these additional species may have arisen due to an improvement in ionisation conditions; however, an improvement in chromatographic conditions is also suspected to play a role. The solvents used in our previous study were based on those used in generalised lipidomic characterisations and included isopropanol to elute highly hydrophobic lipid species such as the triacylglycerols. The removal of isopropanol from the present mobile phase therefore sacrificed maximum elution strength in favour of improved selectivity. Additionally, it is suspected that protic solvents such as methanol or isopropanol may enable accelerated diffusion, allowing their removal to reduce peak broadening. Example EICs for some of these isomers are provided in Figure 36, which depicts the extent of the resolution which may be achieved between them. However, whilst these conditions aided in the resolution between these species, identification was only possible regarding fatty acid combination and *sn*-

position, as the double bond arrangement was not represented in the MS^2 spectra. Furthermore, due to these species still being closely related in a structural sense, many of these species were incompletely resolved; as shown in Figure 36, some EICs display shouldering or broad peaks where isomers partially co-eluted with one another. Such compounds thus represent an additional challenge in the pursuit of comprehensive characterisation.

The double bond isomer co-elution issue is not unique to this analysis but represents a challenge observed throughout lipidomics in general. Current alternate approaches enable some resolution between these forms of isomers and may therefore allow an analysis of further depth. Ion-mobility mass-spectrometry (IM-MS) based techniques are one such alternate approach where it is possible to achieve resolution between lipid isomers under certain conditions.[277] However, additional resolution in this regard permits only differentiation and not identification, as the double bond geometry is not represented in the fragmentation spectra. Recent improvements to general IM-MS systems are enabling the generation of unique fragments corresponding to a lipid's double bond isomerism. Online ozonolysis [278] and Paternò–Büchi reactions [279-281] are two alternative approaches that chemically modify lipids at double bond sites to generate these fragments. These techniques require additional hardware or modifications applied directly to the instrument and are thus not readily accessible. Furthermore, due to the chemical modifications, both the precursor mass and fragmentation spectra of target compounds are modified accordingly, requiring the modification of current spectral libraries.

Fortunately, there is a practical distinction between an absolutely comprehensive characterisation and one sufficient for the speciation of a matrix. To determine whether the identifications made were sufficient for the differentiation of canola based on cultivar, a PCA plot was prepared using the normalised peak areas from the data accumulated. The resulting PCA separation is provided in Figure 37, where four distinct groups may be observed, one belonging to each cultivar. As a product of the PCA, the specific lipid species which significantly correlated for or against the formation of the clusters could be determined, and those species are provided in Supplementary Table 2. Interestingly, amongst those lipid species which correlated, nearly all were PI species containing a single 18-carbon chain, with one or two degrees of desaturation, and a second carbon chain that varied significantly in length and desaturation and often contained at least one degree of oxidation. The similarities between these species may indicate that this second variable fatty acid is one of the most significant sources of variation between the examined cultivars, so far as the PI-lipidome is concerned. An ability to differentiate closely related matrices based entirely on their PI-

lipidome may serve as a valuable tool to understand the development and progression of certain diseases, specifically due to the variations these pathologies are known to impart on the PI-lipidome.[32, 235, 239, 263, 264]

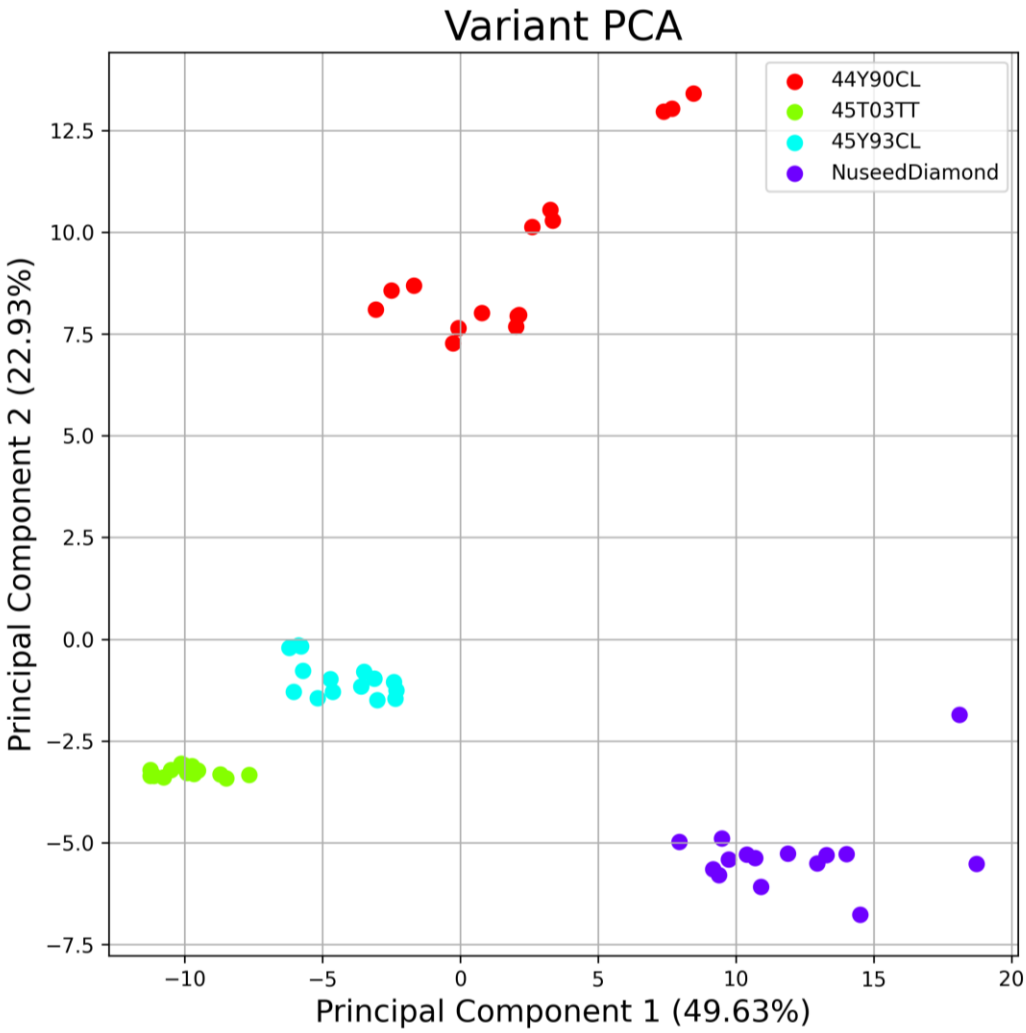

Figure 37 - A principal component analysis plot of the four canola cultivars examined in the experimental (44Y90CL, 45T03TT, 45Y93CL, and Nuseed Diamond). The principal component analysis (PCA) was conducted in Python using sklearn and displayed via pyplot. Only the identified Lyso-PI, PI, Oxidized-PI, and PIP species were used as input. As a result of the PCA, four distinct clusters were produced, each corresponding to a single cultivar.

CONCLUSION

Current generalised conditions utilised for comprehensive lipidomic analysis have proven insufficient for the characterisation of phosphatidylinositol (PI) species and do not consider phosphatidylinositol phosphates (PIPs). In this work we have demonstrated that a series of simple modifications to pre-existing methods is sufficient to bias analysis conditions in favour of PIs whilst enabling the simultaneous analysis of PIPs. It is suggested that the alkaline conditions imparted by the ammonium bicarbonate with ammonia buffer (at pH 9.11) may be

partly responsible for the improvement in chromatographic performance due to the comparatively worse chromatography observed under the other tested chelating buffers such as ammonium bicarbonate (at pH 8.13) and ammonium acetate (at pH 7.11).

In total, 148 PI and PIP species were identified across four cultivars of canola, including 23 Lyso-PIs, 51 PIs, 59 Oxidised-PIs, and 15 PIPs. Although the presence of oxidised lipids has previously been reported in seed oils as a minor constituent, it was unexpected that the majority of PI species observed appeared to be oxidised. These species appeared to elute earlier than other PI species with somewhat diffuse peaks, suggesting that they were highly polar and may exhibit some chelation behaviour. Additionally, the unique fatty acid content observed for these species suggests that they were not the product of oxidation during the extraction process. The large quantity of these species observed here may suggest that similar species are being under-reported in other studies, perhaps due to inadequate chromatographic conditions.

From the 148 identifications, 68 shared their precursor mass with another potential identification and represented some form of lipid isomer. Whilst lipid regio-isomers may be differentiated due to the presence of unique fragments, the resolution and identification of lipid double-bond isomers remain difficult; however, a future analysis may be able to resolve this by including complementary techniques such as ion-mobility spectrometry.

Despite the issue of co-eluting isomers obscuring some species, a PCA of the canola PI-Lipidome produced a distinct clustering for each cultivar, permitting their differentiation and identifying which species most significantly contributed to the clustering. In the future, analyses of this type may be useful when considering a disease's development and progression.

SUPPLEMENTARY DATA

Supplementary Table 6 - Top Ions for variance characteristic for each of the four canola cultivars. These ions were selected by determining the lengths and directions of the vectors associated with all ions in the (PC1, PC2) plane, relative to the origin. Vectors which pointed in the direction of each cluster were determined to correlate positively with the cluster, whereas those which pointed in the opposite direction correlated negatively. These vectors were culled by ensuring they simultaneously remained within the range of the cluster and within an angle of ±18° from the cluster mean (±π/10). This value was arbitrarily selected to bias towards those vectors which approximately pointed towards the mean. The ions are listed in descending order of vector length; thus, those ions which correlate more strongly with cluster appear first.

Correlation	44Y90CL	45T03TT	45Y93CL	NuseedDiamond
Positive	PI 10:1;2O_18:1 PI 12:3;2O_18:1 PI 11:2;O_18:1 PI 22:5_16:0 PI 8:1;O_18:1		PI 22:0_16:0	PI 18:2;O_16:0 PI 18:3;O_16:0 PI 18:2;O_18:1 PI 18:2;O_18:2 PI 18:3;O_16:0
Negative	PI 20:0_18:2	PI 9:1;O_18:0 PI 11:1;O_18:1 PI 15:3;O_18:1 PI 12:2;O_18:2 PIP 18:1_18:1	PI 9:1;O_18:0 PI 11:1;O_18:1 PI 18:1_15:1 PI 18:3;O_18:1 PI 15:3;O_18:1	PI 24:0_18:1 PI 22:0_18:1

Supplementary Data: List of identified lipids
https://analyticalsciencejournals.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Fjssc.202300165&file=jssc8022-sup-0002-SuppTable.docx

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Chapter Five:

Conclusions and Future Directions

Concluding Remarks

The distributions of PI species reported throughout the literature are generally sparse in number despite the considerable interest associated with these molecules and their derivatives. In addition to the complications that arise from their low natural abundance, the shallowness of these characterisations is perhaps due to the use of generalised and insufficient LC-MS/MS methods, which are not conducive to their analysis.

In Chapter Three, a re-evaluation of previously optimised analysis conditions revealed this to be the case, and the results indicated that commonly used generalised analysis conditions only served to suppress the detection of phosphatidylinositol species. Rather, a buffer consisting of 0.25 mM ammonium fluoride was found to significantly enhance the ionisation efficiency of the phosphatidylinositols, improving their signal-to-noise ratio, on average, by 38-fold, compared to the conditions used by previous methods. The manuscript associated with this work is currently cited in 7 other publications. Beyond the utility of ammonium fluoride for the analysis of the phosphatidylinositols, it is also likely that these analysis conditions served to enhance the ionisation efficiency of the numerous other lipid classes based on the appearance of other peaks.

However, the analysis conditions remained unsuitable for the PI derivatives, such as the lyso-phosphatidylinositols and phosphatidylinositol phosphates, as they remained unobserved. Simultaneously, numerous peaks attributed to phosphatidylinositol species in Chapter Three produced fragmentation patterns corresponding to more than a single structure; thus, whilst these newly developed conditions enhanced the detectability of the phosphatidylinositols, they also remained unsuitable for their characterisation. Based on the considerable polarity exhibited by the phosphatidylinositols and their derivatives, it was likely that a weakening of the chromatographic elution solvents would permit their increased retention and address both of these complications; although, additional considerations were necessary to account for the unique chemical properties exhibited by these molecules.

In Chapter Four, an additional set of analysis conditions based on an ammonium carbonate buffer and acetonitrile-based solvents permitted the sensitive and simultaneous analysis of PI, PIP and LPI species in a variety of canola extracts, to the extent that particular cultivars could be differentiated based solely on the unique distributions of these molecular species. These conditions also permitted chromatographic resolution between numerous isomers, revealing a complexity within these minor lipids, which was not observed in the prior ammonium fluoride experiments. However, in these experiments, it was noted that the ammonium fluoride buffer had performed roughly equivalent to that of ammonium bicarbonate and ammonium acetate

in terms of ionisation efficiency, suggesting that the suppression experienced in the generalised methods may arise due to the presence of isopropanol in the mobile phase solvents. In addition to these classes above, the newly developed chromatographic conditions produced in Chapter Four also revealed the presence of an unanticipated class of oxidised PIs, which appeared to contain an array of unique fatty acid species. In addition to their low abundance, it is suspected that the oxidation exhibited by these species previously interfered with their chromatography, as they appeared to exhibit some peak diffusion, similar to the PIPs. Thus, it seemed that the ammonium carbonate buffer was simultaneously suited to preventing non-specific interaction in their case as well. These conditions may also be suitable for the analysis of other anionic lipid classes, such as PAs, PGs and PSs, in addition to their lyso-counterparts, due to their similarities to the PIs. However, they were not evaluated here.

The increase in identifications brought about by the improved chromatographic conditions was only made possible due to the *in-silico* generated spectral library produced as a result of the Lipid Spectrum Generator software introduced in Chapter Two. The software proved its utility in accurately predicting the masses and fragmentation patterns for thousands of molecular species, enabling, in total, the identification of 148 unique lipids in Chapter Four. The major species for these lipids and their relative proportions are provided in Figure 38.

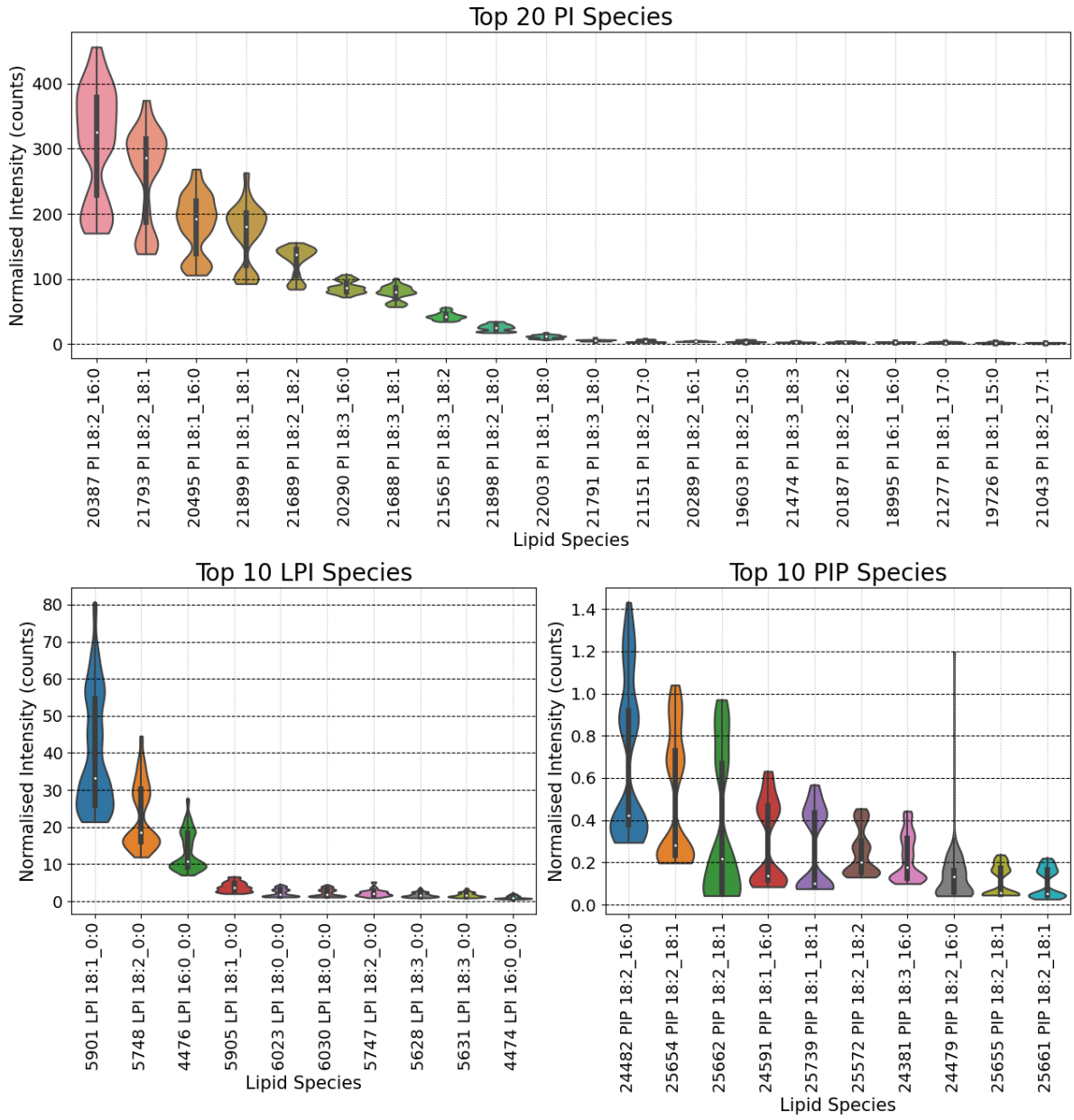


Figure 38 – The major PI, LPI and PIP species present in the previously evaluated cultivars of canola. Although the distribution of species different between the cultivars, the major species predominantly remained in similar proportions. For these species, intensity was normalised to that of an internal standard (PI 14:1_17:0).

Future Directions

- Perform PI-Lipidome characterisations on soy lecithin, infant formulae, and breast milk with the newly developed analysis conditions.

Although the PI lipids observed here comprise only a tiny fraction of the entire canola lipidome, their impressive bioactivities may allow them to induce physiological responses when ingested. Consequently, the proportions at which these compounds are present in the diet may have severe implications for the quality of food products. Due to the favourable lipid profile of canola and its non-allergenic properties, it is a likely candidate to replace soy lecithin as the basis for infant formula. As infants have demonstrated sensitivity to the precise distribution and isomerism of consumed lipid species,[59] the profile of PI lipids may be critical to infant health in this early period. Thus, understanding canola's PI profile, compared to soy lecithin, infant formulae, and breast milk, may indicate its nutritive quality.

Based on the PI profiles observed in literature characterisations, the distribution of molecular species characterised here in canola varied only slightly from those of soy lecithins and infant formulae,[67] though it did vary significantly from the distributions in several recent human milk characterisations.[7, 9, 10, 12] However, these comparisons are considerably limited as these studies provide only a brief list of PI species for comparison. The distribution of LPI species is difficult to evaluate due to only a few studies reporting species of this class. Furthermore, no recent studies have reported the observation of any PIP or oxidised-PI species in human milk; thus, no comparisons may be made. In general, the current understanding of lipid species is considerably lacking in matrices such as breast milk, and further characterisations of these matrices with analytical conditions such as those developed in the previous chapters are warranted. Of these species requiring further evaluation, the Lyso-PIs are perhaps a significant contributing factor to the health of infants as they are understood to influence bone growth and adipose tissue generation.[85] Thus, special consideration should be given to these molecules in further characterisations.

- Explore additional avenues of method development, with specific consideration to enhancing chromatographic performance, via:
    1. Increasing mobile phase pH
    2. Varying aqueous content of mobile phase initial conditions and sample

Whilst the currently developed analytical conditions provided the most comprehensive evaluation of phosphatidylinositol species reported, numerous potential avenues for further

development remain. To further enhance chromatographic performance, increasing the mobile phase pH by adding excess ammonia may improve the peak shapes observed for the PIPs and related compounds. This improvement in peak shapes is anticipated as higher pH mobile phases have not only demonstrated to correspond to a reduction in non-specific interactions on stainless steel,[139] but will also produce a higher proportion of divalent carbonate ions, which may improve their ability to shield active sites via bidentate chelation, as opposed to monodentate chelation with bicarbonate. However, the addition of excess ammonia is simultaneously expected to increase the retention of compounds further, as demonstrated in Chapter Four.[282] This may result in undesirable consequences, as the peak shapes for the PIPs are expected to worsen under less aqueous conditions.[143]

Furthermore, an excess of ammonium and carbonate ions may result in the rapid degradation of silica-based stationary phases, especially at higher temperatures [283], and thus both the pH and analysis temperature must be considered. Alternatively, the sample's aqueous content may be controlled to improve the peak shape and chromatographic performance of PIPs, as they have also been shown to be influenced by the water content in the sample solvent.[143] However, this approach has considerable limitations; if the sample is too aqueous, the hydrophobic PIs will not dissolve.

- Pursue method and software development to enable the differentiation of additional isomeric species, via:
    1. Development of isomeric fragmentation templates using descriptive ions
    2. Generation of alternative fragmentation spectra through chemical modification
    3. Hyphenation to high-resolution / multipass ion-mobility spectrometers
    4. Observation of unique adducts

From the perspective of lipid identification, the lipids characterised in the canola extracts did not have their fatty acid *sn*-isomerism determined, although this isomerism could be evaluated manually based on the ratios of fragment ions, such as those indicated in Figure 39. The *in-silico* spectrum generation software, LSG, could be upgraded in the future to aid in the automated identification of these isomeric species through the prediction of ion ratios, at the expense of doubling the library's size. However, simply considering the ratios of fatty acid ions is likely to result in the incorrect identification of molecular species, as the intensity of these ions is also subject to the influence of chain length and desaturation, and the fragments produced through the neutral loss are perhaps more descriptive. In addition to the ambiguity surrounding *sn*-isomerism, the precise locations of oxidation and desaturation also remained undetermined, as the fragmentation spectra for the observed peaks lacked sufficient

information to make this distinction. The generation of reactive oxygen species post-chromatography may permit the in-line modification of unsaturated molecular species and thus enable the generation of new and descriptive fragment ions.[278, 284] Similar protocols have been implemented that take advantage of the reactive species generated by acetone when exposed to ultraviolet light,[285] though this may plausibly be substituted for a boron-doped diamond electrode as demonstrated elsewhere.[286, 287] In either case, due to the alteration of fragmentation patterns, previous spectral libraries would need to be updated to account for the mass shifts and the generation of new fragment ions.

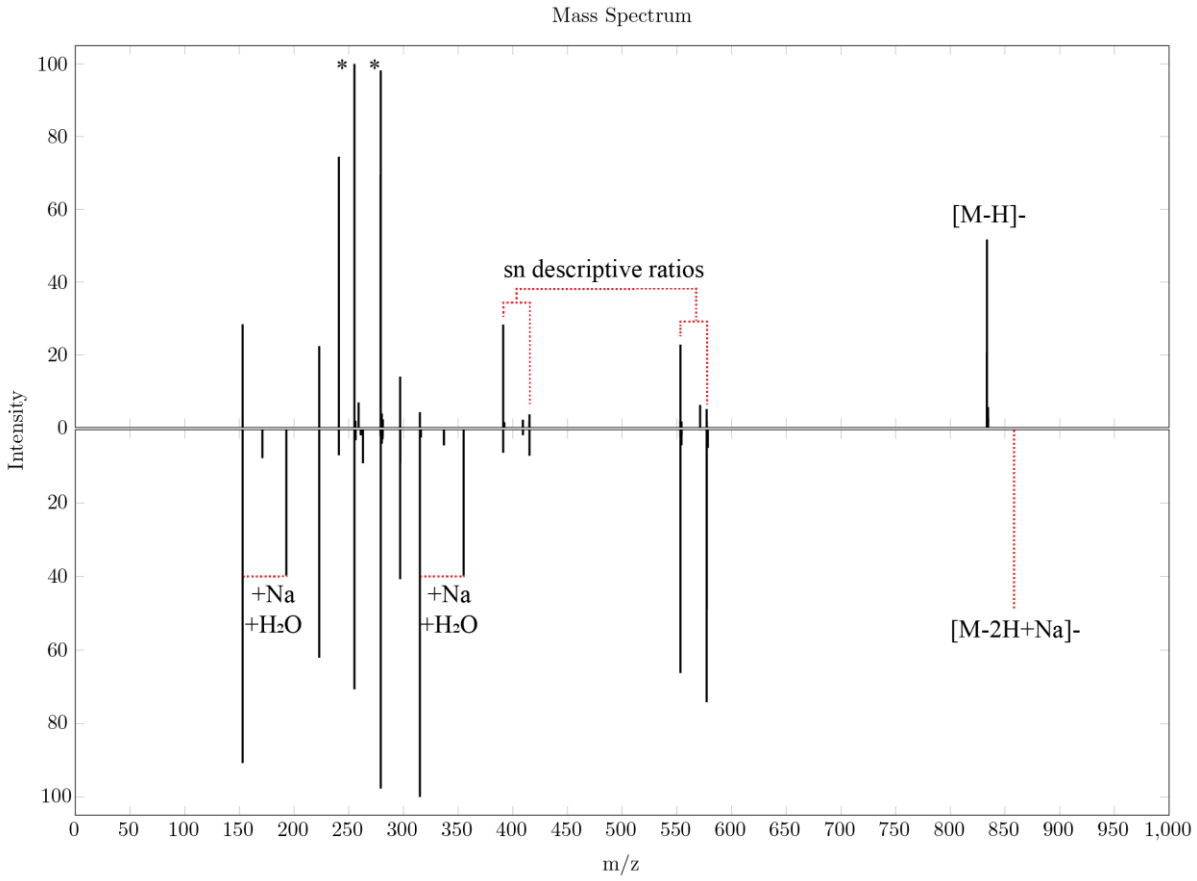
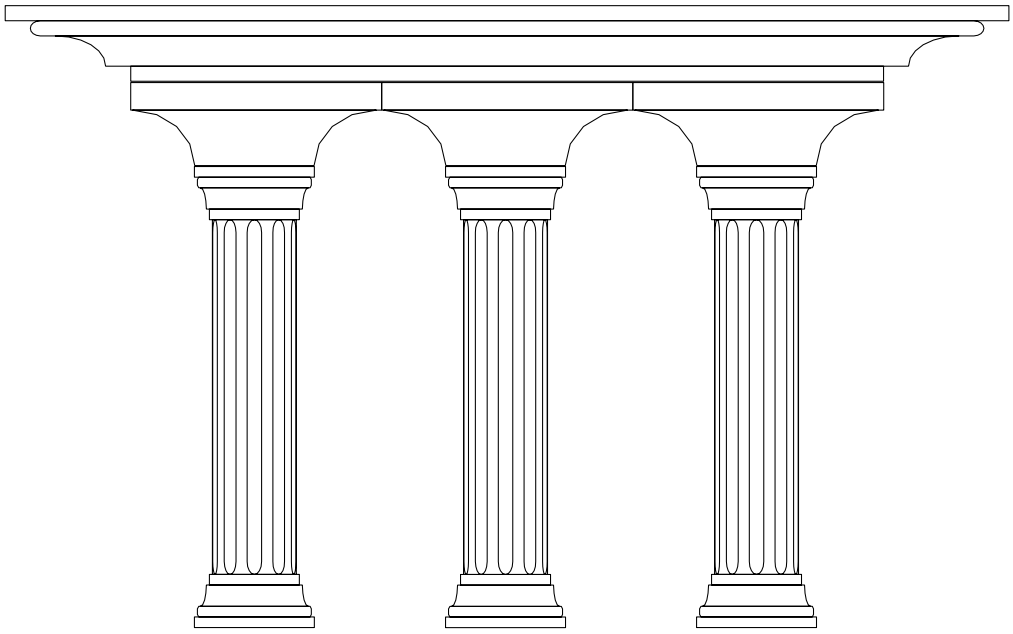


Figure 39 – TOP: an example fragmentation spectrum for a PI [M-H]$^-$. Fragments corresponding to a fatty acid ion are marked with an asterisk (*). Fragments corresponding to the neutral loss of fatty acid are indicated as *sn* descriptive, according to their ratio. The fragment ion presenting in greater intensity is suggested to belong to the neutral loss of the *sn-2* fatty acid. Accordingly, the isomerism with respect to fatty acid positioning may be determined through inspection of this ratio. Two sets of these ions are observed, as the pair with lower mass also corresponds to the simultaneous loss of the headgroup moiety. BOTTOM: The fragmentation pattern of a peak congruent to the PI [M-H]$^-$ species shown above. This ion displayed a near-identical fragmentation pattern in terms of masses, although it differed in ratio. The mass of the precursor ion, in addition to the similarity of the fragmentation spectra, suggested that the spectra corresponded to a [M-2H+Na]$^-$ adduct of the same species. The identification of this adduct was not expected, as PI species contains only a single exchangeable hydrogen. In addition to this, the singular charge state on this ion suggests that another hydrogen on the structure had been replaced by a sodium ion. This unique adduct may enable the resolution of isomeric species via ion mobility spectrometry, as the inclusion of the sodium ion may exacerbate the conformational changes in the gas-phase structure produced as a result of precise fatty acid structure.

Additional information about the precise structure of these lipids could otherwise be achieved through orthogonal separation methods, such as ion mobility hyphenated mass spectrometry. These techniques are able to distinguish particular molecular structures by determining their collisional cross-section values; however, in many earlier implementations, the mobilograms for these lipids are superimposed to a significant extent, and the fragmentation patterns corresponding to particular isomers cannot be fully resolved. Resolution of isomeric species may necessitate a specialised technique, such as through the use of a targeted ion-mobility separation,[288] long drift tube or multi-pass ion-mobility separation,[289, 290] or the induced formation of unique silver adducts to enhance resolution.[277] In the case of the latter, a novel adduct, as suggested in Figure 39, may be further investigated for this purpose.

GVTTA CAVAT LAPIDEM



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