Development of a Shotgun Lipidomics Approach for Analysis of Lipids in Perennial Ryegrass

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Thomas David Featonby

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Abstract

Mass Spectrometry is a powerful analytical tool which is used for identification and quantitation of compounds within samples for a variety of sample matrices. One example of this is to look into the lipid profile (lipidome) of perennial ryegrass (*lolium perenne*). The lipid profile tells us many things about the inner workings of rye grass which can lead to better understanding of mechanisms behind desirable traits (such as lipid quantity and composition). Traditional high performance liquid chromatography (HPLC) is the most widely used chromatographic technique when researching into the lipidome of different plants (Burgos et al., 2011; Chen, Markham, & Cahoon, 2012; Degenkolbe et al., 2012). Shotgun lipidomics applies major principles of the traditional methods but differs in the delivery of the sample to the Mass Spectrometer and data analysis; providing considerable advantages, disadvantages and challenges.

A shotgun lipidomics method for analysing the lipids in perennial ryegrass has been developed. This involved first determining the most efficient extraction protocol and then establishing a methodology (based on one found in the literature for animal samples) for shotgun lipidomic analysis of perennial ryegrass. The shotgun data was problematic to analyse using traditional methods so LCMS data was investigated and the results were transferred to the shotgun data. Investigation was conducted to find the limiting factor for the analysis of the shotgun data. This limiting factor was found to be pheophytin a and other chlorophyll derivatives. The high abundance and ion suppression effects attributed to pheophytin a and other chlorophyll derivatives

contributed to unfavourable conditions for analysing the lipidome of perennial ryegrass. The major outcomes of this study are the annotation of 118 lipids in perennial ryegrass using LCMS, with 27 of those being found in the shotgun data also and also the understanding of the limitations of using shotgun techniques for perennial ryegrass. With this understanding further research can be conducted to enhance the methodologies detailed herein.

Acknowledgements

I would like thank my two supervisors, Dr. David Harding and Dr. Karl Fraser, without whom I would not have been able to complete this thesis. You have both been amazingly supportive and encouraging throughout the process and I really thank you both for that. It has been quite a challenging time but your confidence in me really helped to get it done.

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List of Abbreviations

BP	Before present
C	Choline
CID	Collision-induced dissociation
CLA	Conjugated linoleic acid
CVD	Cardiovascular disease
d_6-DMBNHS	d_6-*S,S'*-Dimethylthiobutanoylhydroxysuccinimide ester
dd-MS2	Data dependent MS2
DG	Diglycerol
DGDG	Digalactosyldiacylglycerol
DIA	Direct injection analysis
DMF	Dimethyl formamide
E	Ethylamine
EIC	Extracted ion chromatogram
ESI	Electrospray ionisation
Fmoc-Cl	Fmoc chloride
GC	Gas chromatography
HESI	Heated electrospray ionisation
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
HPLC-MS	High performance liquid chromatography mass spectrometry
I	Inositol
IPA	Isopropyl alcohol
LCMS	Liquid chromatography mass spectrometry
LMSD	LIPID MAPS Structure Database
LPC	Lysophosphotidylcholine

m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption ionisation
MG	Monoglyceride
MGMG	Monogalactosylmonoacylglycerol
MS	Mass spectrometry
MS2	Fragmentation of an ion or ions. MS1 is a Full MS scan, where MS2 is the fragmented spectra of ions, which can be targeted or non-targeted depending on experiment and mass spectrometer used.
MTBE	Methyl tert-butyl ether
MUFA	Monounsaturated fatty acid
NPLC	Normal phase liquid chromatography
P680	Chlorophyll P680
PA	Phosphatidic acid
PC	Phosphocholine
PE	Phosphoenthanolamine
PG	Phosphoglycerol
PI	Phosphoinositol
PUFA	Polyunsaturated fatty acid
PS	phosphoserine
PS II	Photosystem II
RPLC	Reverse phase liquid chromatography
S	Serine
SFA	Saturated fatty acid
TEA	Triethylamine
TLC	Thin layer chromatography
TOF	Time of flight
UCD	University of California, Davis

1 Introduction

1.1 Lipids

1.1.1 The dairy industry and milk lipids

A major part of the New Zealand economy relies on milk lipids and the dairy industry in particular. So it is very important to understand how milk lipids are produced and furthermore how they can be increased or manipulated for positive outcomes.

10,500 years before present (BP) is the oldest known evidence of human beings utilising the milk of other animals for their own benefit. Scientists using Mass Spectrometry investigating both clay vessels found at different sites and skeletal remains came to this conclusion. Mass Spectrometry was used by first extracting lipids from clay vessels and then loading them onto a Gas Chromatography Mass Spectrometer. The investigation of skeletal remains determined the age and sex distribution of animals at death. As expected and is still a current practice, young male cattle were generally killed off very early on due to being of very little value while females were kept due to their milk production potential. (Gerbault, Roffet-Salque, Evershed, & Thomas, 2013)

Nutritional value of foods is becoming increasingly important as the consumer becomes more informed of the nutritional effects of what they eat. As outlined by the New

Zealand Ministry of Health (2003) the recommended daily intake of dairy products for a 'healthy adult' is two servings per day (Ministry of Health, 2003). They also suggest that low fat options are preferential. When the words 'low fat' are used in this sort of document, they are referring to low saturated fat percentages. The ideal nutritional milk fat would contain less than 8% saturated fatty acids, less than 10% polyunsaturated fatty acids with greater than 82% monounsaturated fatty acids, as postulated by attendees of the Wisconsin Milk Board 1988 Milk Fat Roundtable (O'Donnell, 1989). Seen below in figure 1.1 are the ideal values compared to typical values, there is a clear discrepancy and although this would be an ideal composition in terms of nutritional value it is unachievable through modification of diet alone. As stated by Grummer (1991), the total monounsaturated fatty acid or C18:1 content can only realistically be increased by 50-80% giving an approximate high end total of 50% of milk fatty acids by feeding the cows lipids rich in 18 carbon long fatty acids. The degree of unsaturation is irrelevant due to ruminal hydrogenation and desaturase activity in both the intestines and mammary. (Grummer, 1991)

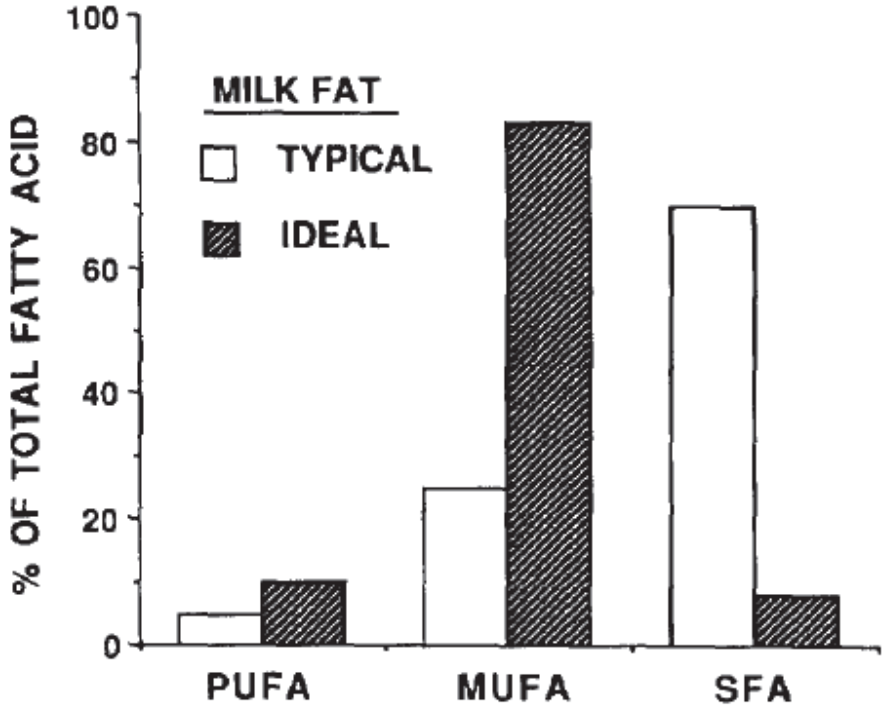

Figure 1.1 – Ideal values of fatty acids as determined by the Wisconsin Milk Board 1988 Milk Fat Roundtable compared to typical values where PUFA is polyunsaturated fatty acids, MUFA is monounsaturated fatty acids and SFA is saturated fatty acids. (Grummer, 1991)

In order to better understand milk lipids and how nutritional value of milk can be increased it is important to investigate what is actually happening in the rumen to the main contributors of poly unsaturated fatty acids. The two most predominant poly unsaturated fatty acids are linoleic acid (major component of dietary supplements discussed further below) and linolenic acid (from forage crops). The first stage of lipid metabolism is hydrolysis of the ester linkages found in triglycerides, phospholipids and glycolipids by hydrolases produced by rumen bacteria. The second major transformation is the biohydrogenation of unsaturated fatty acids. The two main reactions happening in the second stage are poly unsaturated fats being hydrogenated

into trans C18:1 fatty acids by Group A bacteria and then trans C18:1 fatty acids are further hydrogenated to form stearic acid (C18:0) by Group B bacteria as seen in the figure below (figure 1.2). Not all steps in the reactions observed in the rumen are complete, intermediates are seen further down the milk lipid production pathway. (Lock & Bauman, 2004)

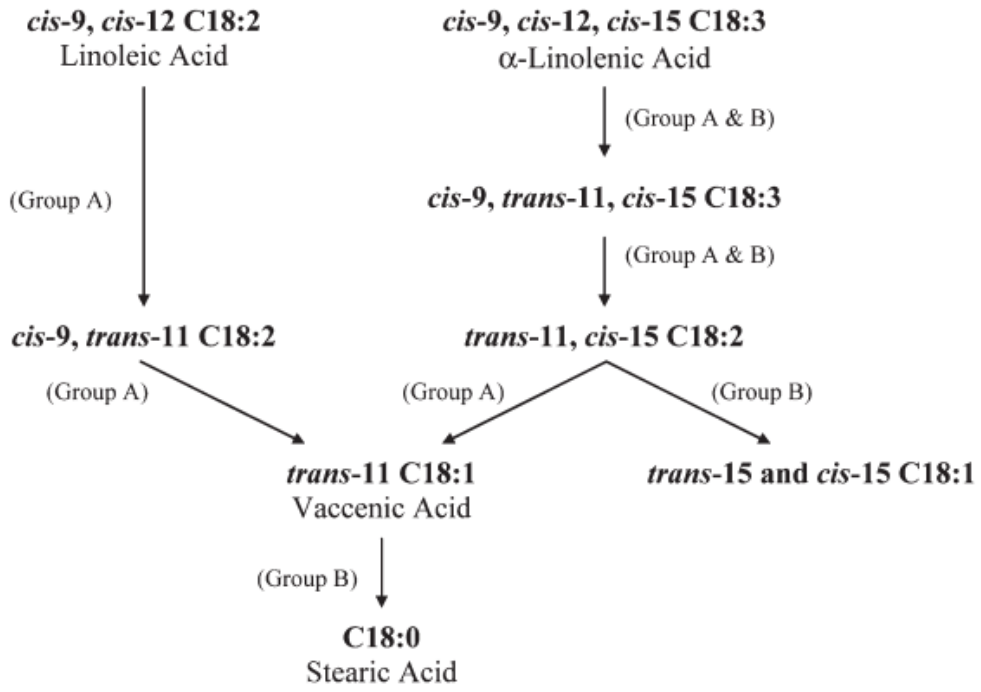

Figure 1.2 – Chemical pathway of Biohydrogenation of linoleic and linolenic acid in the rumen. (Lock & Bauman, 2004)

There have been many studies focussed on the alteration of the fatty acid profile of milk through modification of diet. Several different academic groups (Dewhurst, Shingfield, Lee, & Scollan, 2006; Lourenço, Van Ranst, Vlaeminck, De Smet, & Fievez, 2008; Vanhatalo, Kuoppala, Toivonen, & Shingfield, 2007) have studied the effects of replacing grass silage with red clover silage. They all observed an increase in

C18:2(*n*-6) and C18:3(*n*-3) in milk and also a reduction in C4 to C18 saturated fatty acids due to the difference in lipid composition between red clover and grass silages. It has also been found that milk from grazing ruminants is lower in saturated fatty acids and has higher concentrations of *trans*-11 C18:1, *cis*-9, *trans*-11 conjugated linoleic acid (CLA), C18:2(*n*-6) and C18:3(*n*-3). This has all been attributed to the higher energy intake through pasture foraging compared to silage fed animals. (Shingfield, Bonnet, & Scollan, 2013)

Dietary supplements are another way of impacting the fatty acid composition of milk. Plant oil or oilseed supplements are generally used and are a known way of providing positive results (Chilliard et al., 2007; Glasser, Ferlay, & Chilliard, 2008; Shingfield, Chilliard, Toivonen, Kairenius, & Givens, 2008). There are several factors that alter the efficacy of dietary supplements. Shingfield et al. (2013) lists these factors as: "amount of oil included in the diet, fatty acid profile of the lipid supplement, form of lipid in the diet, and/or processing of oilseeds and the composition of the basal diet." They go on to note that the concentrations of 10- to 16-carbon fatty acids is greatly decreased dependant directly on dosage of plant oils or oilseeds enriched with *cis*-9 C18:1, C18:2(*n*-6) and C18(*n*-3) with very little difference in medium-chain saturated fatty acid concentration. Various studies have produced very modest gains in C20:5(*n*-3) and C22:6(*n*-3) in ruminant milk through the supplementation of fish oil, fishmeal and marine algae. Although there are some positive results in dietary supplements, the gains are moderate compared to the relative ease of forage grazing animals. (Shingfield et al., 2013)

1.1.2 Sheep and beef meat lipids

New Zealand's agricultural industry is a very important part of the nation, both economically and as it forms a big part of our identity as New Zealanders. The lamb and beef exports from New Zealand are considered very desirable and of high quality. There are many risks and benefits associated with red meat consumption. The two main risks are cardiovascular disease (CVD) and cancer. Diet has been identified as one of the modifiable risk factors for CVD. Although there have been many studies looking at the effect red meat consumption has on CVD, McAfee, McSorley et al. (2010) have observed that findings are conflicting and are difficult to compare due to experimental conditions. It has however been assumed that the fat and fatty acid composition of red meat consumed is a contributing factor to CVD. There is some validity to this assumption, as consumption of red meat does increase saturated fatty acid intake, which increases cholesterol and therefore CVD risk. But there are also studies that indicate that trimming red meat of excess fat mitigates this risk considerably. (D. Li, Sirithon, Wahlqvist, Mann, & Sinclair, 2005)

Lean red meat (low in saturated fatty acids) is a key part of a healthy diet. It provides a single source for a multitude of essential minerals, amino acids and vitamins. One vitamin essential for good health is vitamin B12. Red meat is the only major dietary source of this vitamin, providing over two thirds of the daily recommended intake in a single 100 g serving. Vitamin B12 is responsible for keeping down levels of homocysteine which reduces the risk of CVD and stroke. Another major advantage of eating lean red meat is the level of poly unsaturated fats. Increasing levels of poly unsaturated fatty acids in the diet has been linked to a reduction in total cholesterol

levels found in the blood of test subjects. Likewise, *n*-3 poly unsaturated fatty acids in particular have been shown to improve platelet aggregation, vasodilation and thrombotic tendency and also has beneficial effects to the central nervous system, retinal function and the inflammatory response. (McAfee et al., 2010)

1.1.3 Perennial ryegrass lipids

Perennial ryegrass (*lolium perenne*), as seen in figure 1.3, is a fibrous, rooted perennial. It has vegetative tillers which can range in number from low to high. The sheaths are quite flat and generally reddish at the base. The leaves are relatively long and narrow and are glossy on the underside but dull above with fairly prominent ribs. Perennial ryegrass is commonly seen in paddocks while driving in the New Zealand countryside. It generally looks at its best (green and luscious) during Autumn and Spring when there is plenty of rain and sunshine for good growing conditions but is fairly sensitive to drought and browns off in the hotter months.

Figure 1.3 – Visual diagram of perennial ryegrass. (Anderton, 2009)

Lolium perenne is the forage of choice for most farmers with cultivatable land in New Zealand. It grows very well in New Zealand which makes it ideal for farmers looking for a relatively cheap, fast growing, high yield source of protein for livestock. It is also very important to most farming situations that lipid content is optimal, not only in lipid composition but also total lipid content of the forage. Specifically in dairy farms, dairy

cows typically consume between 15 and 20 kg of dry matter per day so the forage they are consuming is very important for the quality and quantity of the final product. (Elgersma et al., 2003)

One of the preferred ways to alter lipid composition is to selectively breed different *lolium perenne* cultivars after thorough trials and analytical testing. These trials can target many different environmental and genetic variables. Some of these variables are as follows: alteration of nitrogen supply (Foito et al., 2013), effect of bruising and environmental conditions (Khan, Cone, Fievez, & Hendriks, 2011), cultivar and cutting date (seasonal effects) (Van Ranst, Fievez, Vandewalle, De Riek, & Van Bockstaele, 2009). One of the major time and cost consuming steps in these approaches is analysing the samples. Of the three examples given above, all three use gas chromatography for analysis of fatty acids. This chromatographic separation takes time which adds cost to analysis.

There is a definite need for a quick and relatively cheap lipid analysis method in the area of plant breeding and especially in *lolium perenne* breeding programs. Using shotgun (direct infusion) delivery into a high resolution mass spectrometer along with various techniques for analysing the data, a quick screening tool could be developed.

1.2 Mass Spectrometry

1.2.1 Introduction

In the most simple of terms, a mass spectrometer is an instrument that creates gas-phase ions, separates these ions based on mass/charge ratio and then counts the number of these ions. In reality it is much more complex than that. Mass spectrometry is an immensely powerful analytical tool that allows the user to deduce structure and abundance of compounds within samples with relative ease and high reproducibility. The major components of a mass spectrometer are the sample delivery system, ion source, mass analyzer and detector.

1.2.2 Sample delivery system

The main delivery systems are gas chromatography (GC), liquid chromatography (LC or high performance liquid chromatography/HPLC) and direct infusion/shotgun. Direct infusion/shotgun is a very simple sample delivery system, the sample is drawn into a syringe, the syringe is attached to the source (explanation of sources below) and then the sample is injected (either by hand or using a syringe pump at a controlled flow rate). Gas and liquid chromatography are very different to direct infusion/shotgun. The major difference is the chromatographic separation.

1.2.3 Chromatography

The main purpose of chromatography is to separate compounds within a sample. There are two main components of any chromatographic system, the stationary phase and the

mobile phase. The stationary phase is contained inside a column varying in diameter and length depending on the instrument and separating power required (e.g. 100 um to 40 mm internal diameter, 5 cm to 100 m long). The column consists of a packing (e.g. HPLC columns) or coating on the inside of the column (e.g. GC capillary columns) that is the stationary phase. The compounds in the sample interact with the stationary phase to create separation. The two most common separation parameters are based on size and polarity. Unlike LC, the mobile phase in GC, other than its speed, does not contribute to any interactions between analytes and the stationary phase.

Liquid Chromatography

Liquid chromatography uses liquid as its mobile phase to elute compounds off the column that have interacted with the stationary phase. The liquid can be just one type for the whole analysis but most applications will have two solvents being fed into the column by a high pressure pumping system which also includes a mixing valve before being pumped through the column as depicted by figure 1.4. This mixing of solvents is called the gradient. Gradients are most commonly used when trying to separate different types of compounds where running just one solvent does not elute all compounds correctly or in a timely fashion. The gradient is created by having solvents differing in properties such as polarity or ionic strength, e.g. one aqueous and the other organic. The most basic gradients start off at 100% of one of the solvents and then slowly move to being 100% of the other. This helps to make sure that the majority of compounds have eluted off the column and that very little carry over is present for the next sample that is run. There will always be compounds stuck to the column stationary phase even at 100% organic solvent. If just organic or just aqueous solvents were used then some

compounds would not elute off the column, which reduces the efficacy of the column resulting in poor separation of the target analytes.

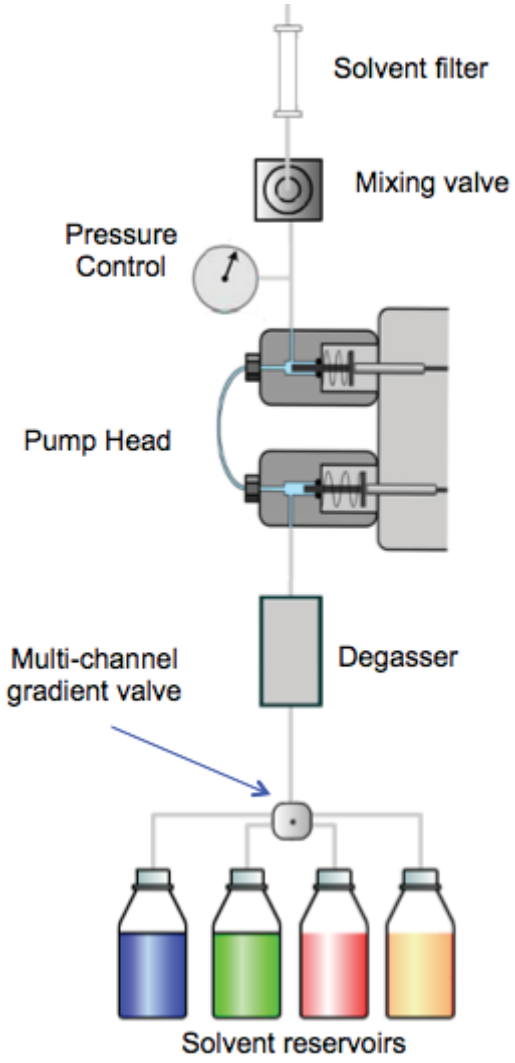

Figure 1.4 – Basic diagram of a liquid chromatography pump system. (Crawford Scientific, 2012)

Gas chromatography

Gas chromatography uses gas as its mobile phase. The basic principles are the same as liquid chromatography. The compounds in the sample interact with the column (in this case a capillary column) with different affinities, generally based on a combination of

both the volatility of the compound and its interaction with the stationary phase. Where liquid chromatography uses solvent mixtures to create a gradient to separate compounds, gas chromatography uses temperature of the column as the gas stays constant (usually helium, hydrogen or nitrogen). The temperature gradient profile is programmed depending on what column is used and what analytes are being targeted.

1.2.4 Source

Electrospray Ionisation (ESI)

The source as mentioned in this context is the source of the ions. The source most commonly used in HPLC-MS (high performance liquid chromatography mass spectrometry) and lipidomics research is electrospray ionisation (ESI). ESI generates ions by first taking the liquid sample and forming charged droplets. ESI ionises samples by using a nebuliser gas and an extremely small diameter electrically charged capillary (as seen in figure 1.5 below). The droplets that are initially formed are further reduced in size as a combination of the nitrogen drying gas (for desolvation) evaporating the droplet and the Coulombic forces between the charged ions fissuring the droplets. It is this later event that releases charged gas phase ions that are then directed into the capillary of the mass spectrometer for analysis. The sheath gas as pictured keeps the non-charged particles away from the entrance of the mass spectrometer while the ions are attracted electrically. (Awad, Khamis, & El-Aneed, 2014)

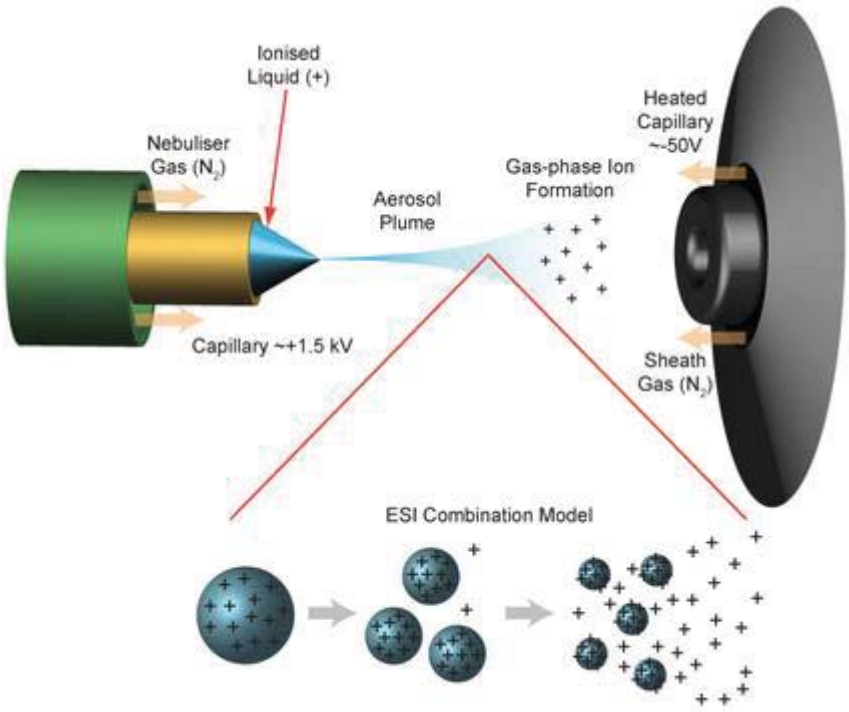

Figure 1.5 – Diagram of electrospray ionisation. (Lamond Laboratory, 2010)

Matrix effects

A major limitation of the ESI source is matrix effects. This is where other compounds in the sample matrix are competing for charge with your target. Some compounds take on charge much easier than some specific target compounds and swamp the target signal. This is especially an issue where the delivery system used is direct infusion. In direct infusion if there are significant matrix effects then it can be difficult to observe the compounds of interest over highly abundant, or readily ionisable, non-target compounds that preferentially take the charge (for example, caffeine provides challenges to researchers of tea leaves).

Mass Analyser

The quadrupole found in the Q Exactive (mass spectrometer used in this thesis project) is an ion filter. The quadrupole gets its name, in part, from its physical appearance as it has four stainless steel rods in parallel formation, as seen in figure 1.6 below. The quadrupole has two main modes. The first mode is to let all ions through and the second mode is to select ions dependent on *m/z* ratios. This selection is achieved through applying voltage and radio frequency potentials across opposite (not neighbouring) poles. As expected, positive ions will be attracted to the negative pole/s and positive ions will be attracted to the positive pole/s. Dependent on the mass targeted, different potentials can be applied and polarities can be switched to manipulate the trajectories of the ions so that just the targeted mass will get through.

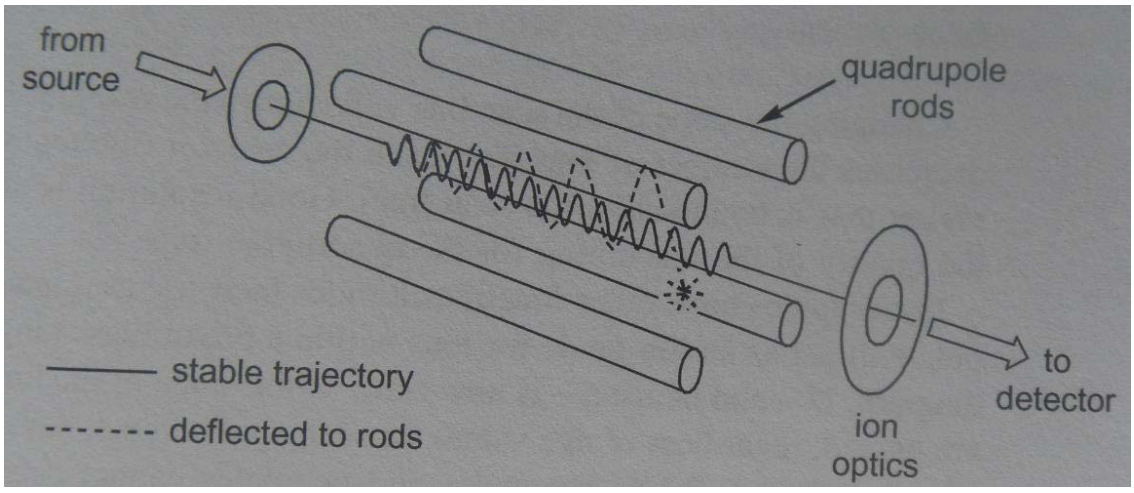

Figure 1.6 - Expanded diagram of a quadrupole. True form of the quadrupole has the rods very close together (opposite poles are closer together than the diameter of a single rod) and rods must be exactly parallel. (Henderson and McIndoe, 2005)

Matrix-assisted laser desorption ionisation (MALDI)

The first step in MALDI ionisation is to prepare the sample plate. The plate is loaded with sample which has been mixed with a matrix. The laser blasts the surface of the plate which then vaporises the matrix and sample. It is believed that during both the initial blast of the laser and in the vapour plume that the matrix protonates the sample, therefore ionising it. MALDI is a 'soft ionisation' technique (as is ESI), which means that larger molecules are not destroyed or broken up in the ionisation process. This 'soft ionisation' allows the analysis of compounds such as proteins, DNA and lipids. Another major advantage of MALDI over ESI is the ability to sample intact tissue samples directly on the plate allowing for mapping of cells etc. There are however, a few disadvantages of using MALDI. The main disadvantages are that it is highly matrix dependent, has a low mass cutoff of approximately 500 Daltons, it cannot employ chromatography of any kind into the system so therefore suffers from an extra matrix effect (the matrix used to spot the sample onto the plate) and it is very hard to quantify compounds using the MALDI. (Knochenmuss, 2006)

1.2.5 Detector

Time of flight (TOF)

Time of flight detectors measure the time it takes ions to reach a detector. The theory behind this is that ions of differing mass will travel through a drift path faster or slower than each other based on their masses. As the acceleration energy is constant this means that lighter ions will travel faster and reach the detector in less time than heavier ions.

Measurement of these flight times enables calculation of the mass/charge (*m/z*) ratio. (IONTOF, n.d.)

Orbitrap

The Orbitrap works by trapping ions in a rotation or orbit around the central, axial (in figure 1.7 below) electrode. Mass/charge ratios are measured by how they interact with the electric field created between the inner axial electrode and the outer coaxial, jacket-like electrode. Specifically the frequency that the ions oscillate around the inner electrode is proportional to $(z/m)^{1/2}$. This signal is then transformed into a spectrum through Fourier Transform. The Orbitrap is a highly accurate mass analyser with extremely low noise (noise is the wobble seen in the baseline of a mass spectrum, high noise makes it difficult to determine if a peak is 'real' or if it is just part of the baseline) (Hu et al., 2005)

Figure 1.7 – Thermo Scientific's Orbitrap. (Thermo Fisher Scientific, 2015)

Comparison

The TOF and Orbitrap both have the potential for accurate mass (4dp) at high resolutions. The major differences are based on the physical differences. The TOF is greatly influenced by the length of the flight path and the quality of the electronics that are timing the flight. These two factors have a considerable effect on resolution. In contrast, the Orbitrap's resolution is affected mainly by the time the ions spend in the trap. The longer the ions are in the trap, the higher the resolution. The main limit to resolution is the trade-off between having higher resolution or more points across the peak in the chromatogram.

1.2.6 Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Used in this Masters project)

The internal workings of the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) are briefly described below (bold letters in brackets correspond to key components depicted in figure 1.8), as this was the key instrument used during this Masters project.

After ionisation occurs (by the ESI source) the ions are focused into a beam by the S-lens. The S-lens (**A**) is a stack of metal discs which has a voltage potential put across them to focus the ions. From here the ions are directed around the corner by the bent flatapole with neutral ions going straight into the wall. These ions then enter the quadrupole (**B**) where precursor ions can be selected (if MS2 scan events are needed, MS2 is the fragmentation of an ion or ions) or for full MS all ions are allowed to pass through the quadrupole filter. The ions are then sent through to the C-trap (**C**). In the

full MS scanning mode the C-trap is set to let everything through to the Orbitrap (**E**). While in dd-MS2 mode the C-trap is set to send precursor ions through to the HCD (higher energy collisionally activated dissociation) collision cell (**D**) where the ions are fragmented and then sent back to the C-trap and sent through to the Orbitrap for detection.

Figure 1.8 – Thermo Scientific Q Exactive (top). Bottom shows inner working of the instrument. From the right hand side following the path of the ions is the S-lens (A), the quadrupole (B), the C-trap (C), the HCD collision cell (D) and finally the Orbitrap (E). (Thermo Fisher Scientific, 2011)

Scanning modes

The Thermo Scientific Q Exactive mass spectrometer has many different scanning modes (full MS, selected ion monitoring, MS/MS, all ion fragmentation, source fragmentation, positive/negative ion switching, data dependent MS2 and timed selected ion monitoring). Full MS and data dependent MS2 are both very important when analysing lipids within samples. The full MS scanning mode is the most basic scanning mode but provides a lot of information. Using the full MS scanning mode on the Q Exactive provides accurate masses for all compounds within the sample. These masses are accurate to four decimal places with an accuracy of 1-3 ppm. This high accuracy allows for formulas to be deduced through the use of the Thermo software which can then be searched in the online lipid databases an example of which is the LIPID MAPS Structure Database (LMSD) (Sud et al, 2007). (Thermo Fisher Scientific, 2011)

The full MS scan is also a vital partner for the data dependent MS2 (dd-MS2) scan mode. The dd-MS2 scan mode essentially employs the full MS scan mode to provide a list of ions for the instrument to fragment, yielding MS2 spectra of those selected ions. In more detailed terms, the mass spectrometer performs a full MS scan, from that scan the computer selects *n* (user defined) the number of the most abundant ions to perform a MS2 scan event on, those ions are then put on an exclusion list for a set amount of time (generally a few seconds so that it is not producing multiple MS2 scans of the same ion within a single peak on the chromatogram) and the loop continues with another full MS scan. This method is perfect for experiments where targets are of unknown mass or there are too many targets to create a targeted method for. (Thermo Fisher Scientific, 2011)

1.2.7 Fragmentation

The ability of mass spectrometers to fragment compounds makes them an extremely useful analytical tool. Much can be learnt about a sample through the understanding of fragmentation patterns of target compounds. This fragmentation mainly occurs through a process known as collision-induced dissociation (CID). Henderson and McIndoe (2005) state that, "Collision-induced dissociation of ions occurs when some of the translational energy of an accelerated ion is converted into internal energy upon collision with a residual gas (typically nitrogen or one of the noble gases, helium, argon, xenon). The increase in internal energy can induce decomposition (fragmentation) of the ion." The aim for the CID segment of the scan is to have 10% of the original abundance of the parent ion (the ion being fragmented) remaining in the MS2 spectra. This is achieved through adjusting the collision energy. Some ions need more energy put into the CID to fragment, while others take very little energy to fragment. When the dd-MS2 scanning mode is being used, a mean or most commonly needed collision energy is settled on. Fragmentation patterns are a fingerprint for molecules or classes of molecules. Once key fragments are known of a molecule or class of molecules then this makes identification of unknowns relatively easy.

1.3 Lipidomics

1.3.1 Introduction

Lipiomics as first described in the European Journal of Lipid Science and Technology is, "the full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including

gene regulation." (Spener, Lagarde, Géloên, & Record, 2003). Lipidomics has grown significantly since it first branched off from metabolomics in 2003. It is a very popular area of research with new applications being uncovered regularly. To understand lipidomics, we must first understand lipids.

1.3.2 Lipids

Lipids are extremely important as they play many critical roles in cells (some of which are depicted in figure 1.9). They have roles such as comprising a major component of cellular membranes bilayer, providing structural integrity along with creating intracellular environments (organelles), providing the hydrophobic environment for membrane proteins, participating in cell growth, multiplication and death, regulation of signal transduction, and control of membrane trafficking and activation of signalling pathways. (Bou Khalil et al., 2010; M. Li, Yang, Bai, & Liu, 2014)

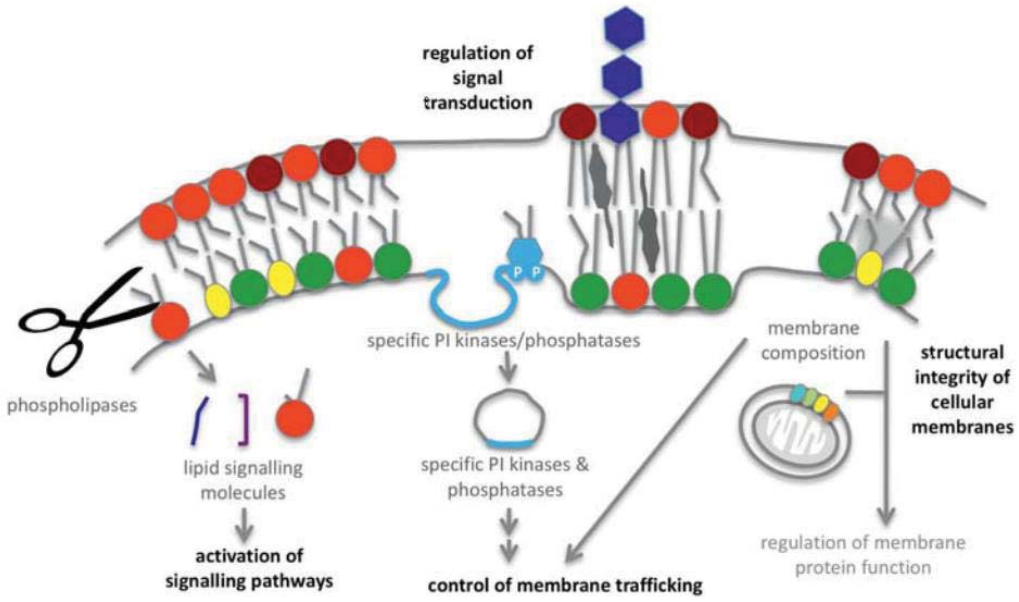

Figure 1.9 – Biological functions of lipids in the cell. (Bou Khalil et al., 2010)

Lipid nomenclature is very important for distinguishing between the thousands of possible lipids present in any one sample. It is also very important to have a universal naming convention that is consistent, clear and relatively simple. It was the purpose of Fahy et al, (2005) to create such a system. The features of their lipid nomenclature scheme are a) Using the stereoscopic numbering (*sn*) method to describe glycerolipids and glycerophospholipids. b) Sphinganine and sphing-4-enine are the core structures for the sphingolipid class. Where there is deviation from this the full systematic names are to be used. c) Core names such as cholestane and estrane are to be used for sterols. d) Adherence to the IUPAC-IUBMB recommendations for naming fatty acids and acyl chains. e) Condensed text for the glycan portion of lipids. f) *E/Z* is to be used to denote double bond geometry. g) *R/S* is to be used to define stereochemistry. h) The term 'lyso' is only to be included as a synonym when denoting a glycerolipid or glycerophospholipid lacking a radyl group. i) Carbons participating in the cyclopentane ring closure are defined and a consistent chain-numbering scheme is proposed when naming prostaglandins, isoprostanes, neuroprostanes and related compounds. j) When naming sphingolipids in shorthand refer to 1,3-dihydroxy and 1,3,4-trihydroxy long-chain bases as "d" and "t" respectively. (Fahy et al., 2005)

One example of the lipid nomenclature rules is LPC(16:0) as detailed further in section 3.4.4. This shorthand describes a lysophosphotidylcholine with a C16:0 carbon chain. C16:0 describes a 16 long hydrocarbon chain with zero double bonds. In the case of the two important milk lipids, C18:2(*n*-6) and C18:3(*n*-3), they both have 18 carbon long hydrocarbon chains with 2 and 3 double bonds respectively. The (*n*-6) and (*n*-3) refer to the placement of the double bonds in the chains. The start of the conjugation of the lipid

is numbered from the end of the carbon chain. For (*n*-6), the conjugation starts at carbon number 6 for example.

The term 'lipid' is a very broad term describing a wide variety of compounds. These compounds can be organised into groups based on common structural elements called lipid classes. The most common lipid classes are fatty acids, glycerolipids, glycerophospholipids, sphingolipids and sterol lipids. Examples of the most common lipid classes are seen in figure 1.10 below. These most common lipid classes are also split into sub classes with each sub class then having many possibilities of hydrocarbon chain lengths.

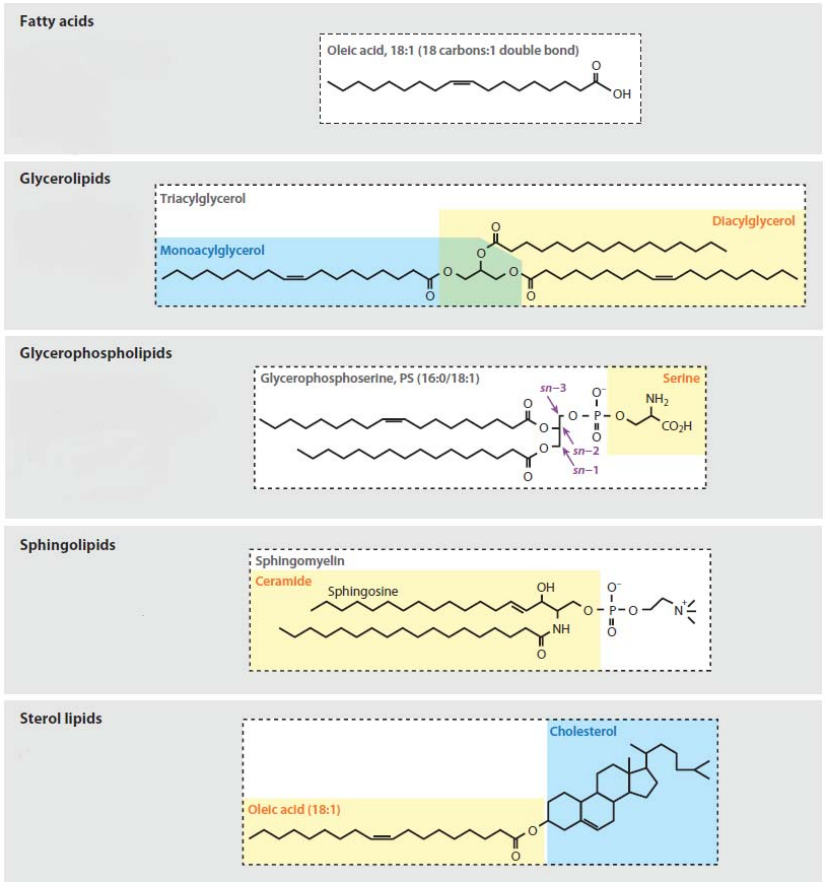

Figure 1.10 – Examples of the most common lipid classes. (Blanksby & Mitchell, 2010)

Fatty acids, glycerolipids and glycerophospholipids are all observed in perennial ryegrass in fairly high abundance. Fatty acids are the fundamental building block of complex lipids. There are 14 sub classes of fatty acids as depicted in figure 1.11 below with a further 17 sub sub classes of fatty acids and conjugates alone. This complexity is not unique to the fatty acid lipid class but is an extreme example. Fatty acids comprised of a carboxylic acid head group create a region of polarity and hydrophilicity attached to a hydrophobic unbranched hydrocarbon chain (tail) which can be saturated or unsaturated. As covered in the earlier sections poly unsaturated fatty acids are highly sort after by farmers for numerous reasons. The majority of fatty acids found in nature contain even hydrocarbon chain lengths. Some odd numbered chains are seen in some plants, bacteria and even less commonly in animals but these are far less abundant so need to be targeted to be observed. The major fatty acid chain lengths of interest when investigating perennial ryegrass are C16 and C18. These are most commonly seen as basic fatty acids or A in the figure below (figure 1.11) with C16 or C18 chains with differing degrees of unsaturation. (Bou Khalil et al., 2010)

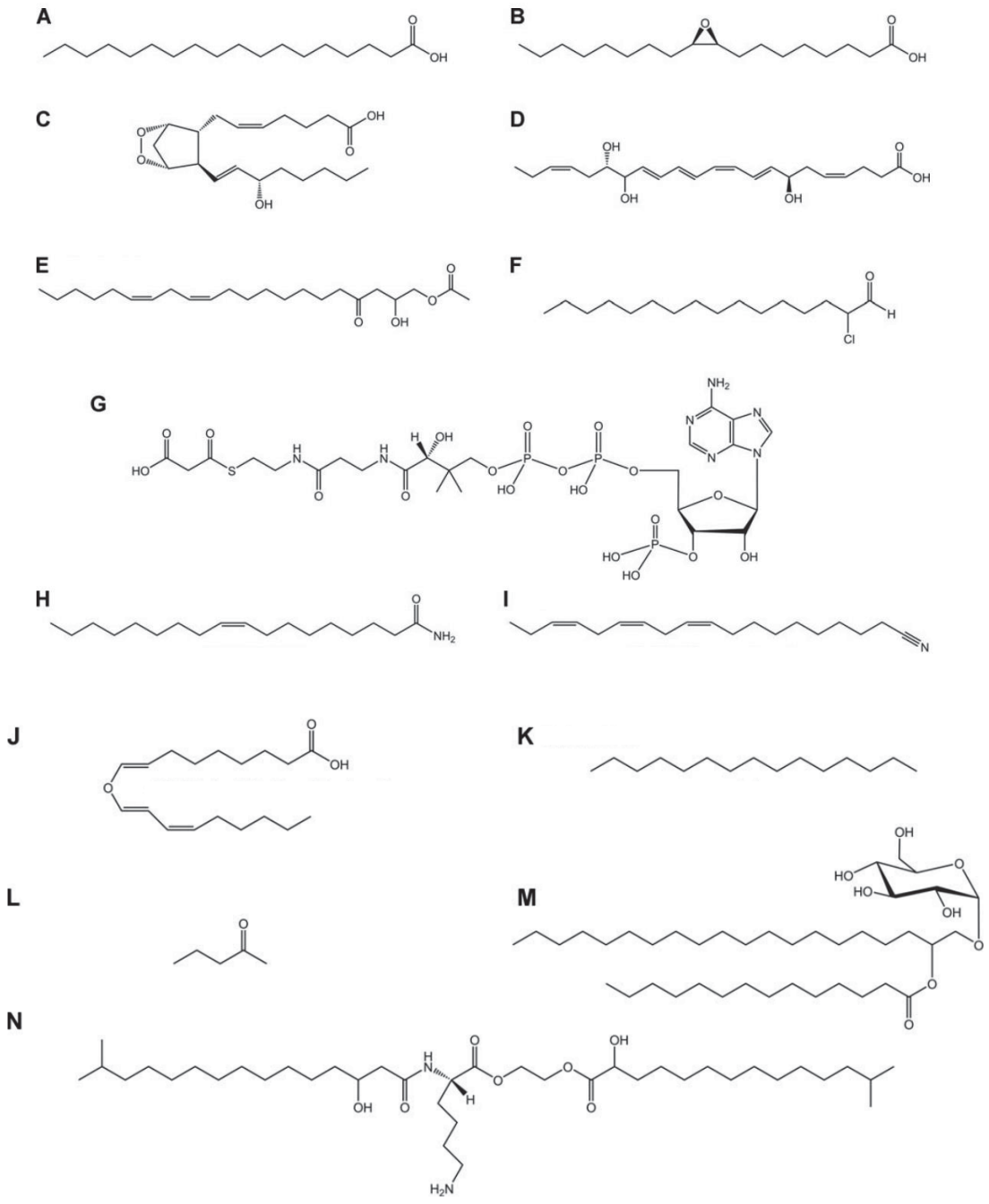

Figure 1.11 – Examples of the 14 sub classes of fatty acids. A-Fatty acids and conjugates. B-Octadecanoids. C-Eicosanoids. D-Docosanoids. E-Fatty alcohols. F-Fatty aldehydes. G-Fatty esters. H-Fatty amides. I-Fatty nitriles. J-Fatty ethers. K-Hydrocarbons. L-Oxygenated hydrocarbons. M-Fatty acyl glycosides. N-Other fatty acyls. (Bou Khalil et al., 2010)

Glycerolipids and glycerophospholipids are relatively simple compared to fatty acids. The glycerolipid class is only split into three sub classes (mono-, di- and tri-acylglycerols) where there is a glycerol backbone with either one (mono), two (di) or three (tri) hydrocarbon chains attached to it. This is depicted in figure 1.10 above. Glycerophospholipids are very similar to fatty acids in that there is a region of hydrophilicity (also known as the headgroup) and a region of hydrophobicity (also known as the tail). In figure 1.10 the headgroup depicted and highlighted is serine, this is one of the major sub classes of glycerophospholipids, the other major sub classes are shown in figure 1.12.

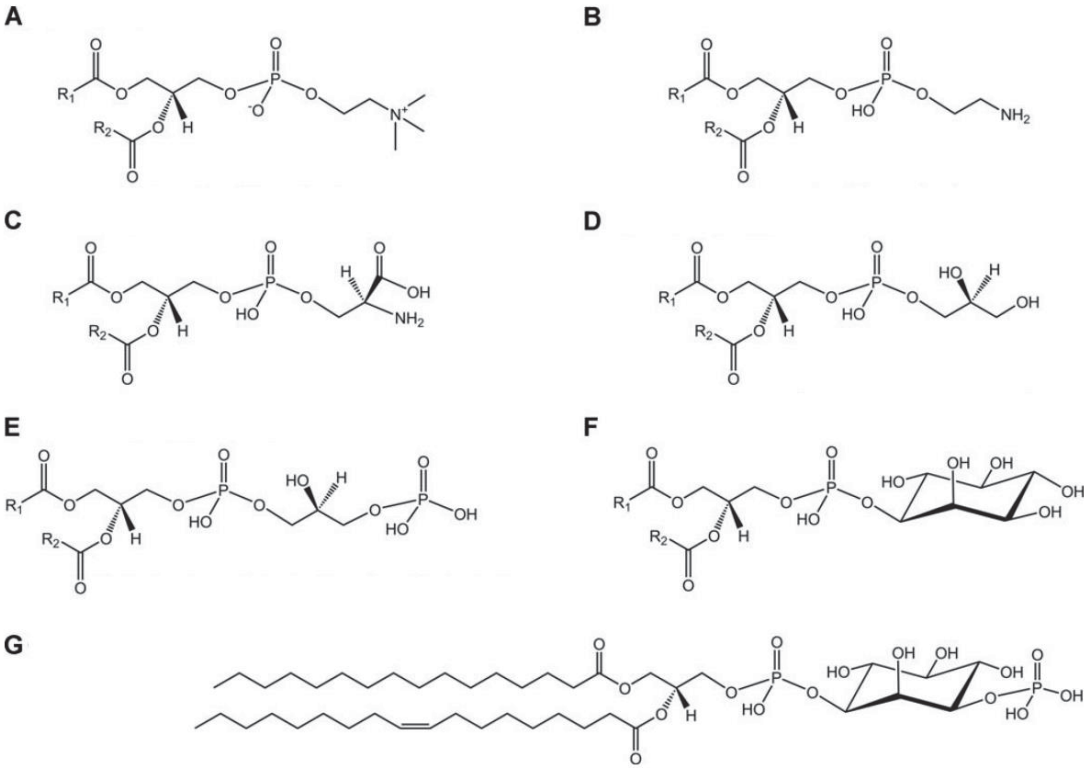

Figure 1.12 – The major sub classes of glycerophospholipids where R_1 and R_2 are hydrocarbon chains. A-Glycerophosphocholine. B- Glycerophosphoethanolamine. C-Glycerophosphoserine. D-Glycerophosphoglycerol.
E-Glycerophosphoglycerophosphate. F-Glycerophosphoinositol.
G- Glycerophosphoinositol monophosphate. (Bou Khalil et al., 2010)

1.3.3 Instrumentation

High performance liquid chromatography (HPLC)

The three most important types of liquid chromatography when researching lipids are reverse phase liquid chromatography (RPLC), normal phase liquid chromatography (NPLC) and hydrophilic interaction chromatography (HILIC). The way lipids interact in RPLC is through lipophilicity, where carbon chain length and the number of double bonds governs how the lipids interact with the column. The longer the chain, the longer the lipid species takes to elute and inversely, the higher the degree of polyunsaturation (more double bonds) the faster the lipid elutes. NPLC and HILIC work by interacting with the polar (or hydrophilic) head group of the lipids (as described below). So lipids are separated according to their head group and not the carbon chain using NPLC and HILIC. RPLC is the most widely used setup for lipid investigation, however if phosphotidic acid lipids are targeted then NPLC is best suited. (Cajka & Fiehn, 2014)

Gas chromatography

Gas chromatography is perfectly suited for identification and quantification of low molecular weight lipids including fatty acids and sterols. (Blanksby & Mitchell, 2010) This is due to the compounds being run using GC needing to be volatile. When using GCMS if a target compound or class of compounds is not volatile then it is derivatised to become volatile. In the case of lipids this only works to a certain extent, in the derivatisation process most structural information of lipids is lost due to the nature of the chemicals used. To get around this issue preseparation using HPLC or thin layer

chromatography (TLC) has been used prior to derivatisation. This yielded good results for sensitively detecting most lipid classes. (M. Li et al., 2014)

1.3.4 Fragmentation patterns of lipids

Lipids fragment in fairly predictable patterns. As detailed earlier, lipids are mainly comprised of a head group (polar, hydrophilic section) and a hydrocarbon chain (non-polar, hydrophobic section). This allows for assumptions to be made about fragmentation within the lipids depending on class. For instance, as seen below (figure 1.13), the red dashed lines depict possible fragmented bonds within a phosphoethanolamine (PE) lipid. The most likely fragments seen in the MS2 spectra are the result of the bond indicated with the star symbol breaking. This bond fragmenting will produce the full head group loss (observing the rest of the lipid in the spectrum) and/or the fragment on its own. Another of the major fragments will be the head group subclass detachment, in this case the ethanolamine will fragment away from the phospho group depicted by the dash red line indicated with a circle. The last fragments and losses possibly seen will be the loss of one or both of the chains (R_1 and R_2) and possibly fragments on their own.

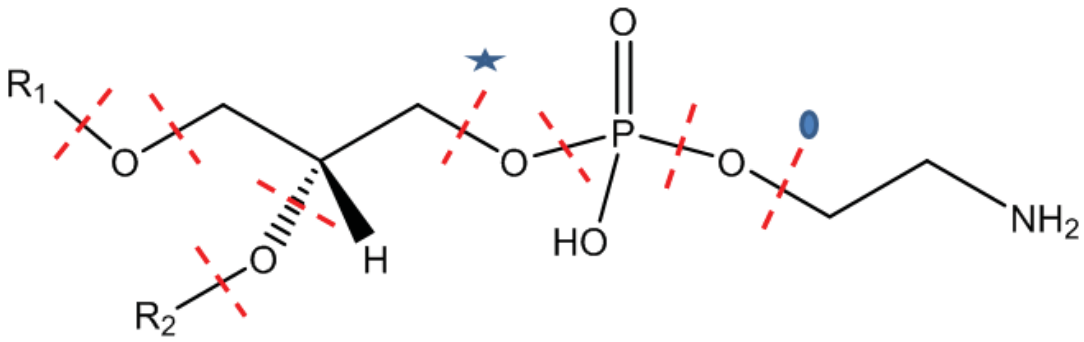

Figure 1.13 – PE class lipid and possible points of fragmentation depicted using dashed lines.

1.3.5 Applications

There are many different applications for lipidomics. Two common areas of research using lipidomics based approaches are lipidomics of cancers and lipidomics of plants. Several different studies as outlined in a review by Li et al. (2015) have utilised lipidomics to search for biomarkers for different cancers. It is postulated that body fluids have the potential to reflect not only different cancers but also may reveal the efficacy and toxicity of anti-cancer treatments. This research is still in its infancy but early results of small test groups are very promising. With the continual development of new technologies in mass spectrometry biomarker identification will become easier which will benefit this research greatly. (M. Li et al., 2014)

As previously outlined, lipids are very important in plants. They are involved in a myriad of roles internally within the cell and contribute towards New Zealand's income (due to exports) as part of the cost effective agricultural production of milk and meat.

No extensive studies of the lipids within perennial ryegrass were able to be found during extensive literature search. There has however been extensive lipidomics research on *Arabidopsis thaliana.* Examples of which include studies focussing on lipidome changes due to different temperatures and light (Burgos et al., 2011) and also looking at lipid markers for freezing tolerance (Degenkolbe et al., 2012). There is the potential to replicate these kinds of studies for perennial ryegrass to further understand and utilise the properties that make it such a good forage around the world.

1.4 Aim of thesis

To investigate the potential of a shotgun lipidomics approach for analysing the lipid profile of perennial ryegrass and to compare this to current LCMS protocols.

2 Materials & Methods

2.1 Materials

Optima LC/MS grade isopropyl alcohol (IPA), acetonitrile, methanol and formic acid and HPLC grade methyl tert-butyl ether (MTBE) and chloroform were all purchased from Fischer Scientific. Dimethyl formamide (DMF), 99.5% minimum and toluene, AnalaR grade, were both purchased from BDH Chemicals LTD. HPLC grade ammonium formate, triethylamine (TEA) and Fmoc chloride (Fmoc-Cl) were purchased from Sigma-Aldrich. Deionised water was obtained from a Millipore Gradient Milli-Q system and is referred to as Milli-Q water/H_2O.

d_6-S,S'-Dimethylthiobutanoylhydroxysuccinimide ester (d_6-DMBNHS) was kindly donated by Associate Professor Gavin Reid of Michigan State University at the time, now Professor at The University of Melbourne.

Ryegrass bulk sample is a pooled perennial ryegrass sample (that has been freeze dried and ground into a fine powder), routinely used in the Metabolomics lab at AgResearch, Palmerston North as a control sample when running metabolomics research.

2.2 Methods

2.2.1 Lipid extraction test

Three different lipid extraction protocols were tested and compared. The three protocols were the Folch (Folch, Lees, & Stanley, 1957), UCD (University of California, Davis) which is a modified version of the Matyash protocol (Matyash, Liebisch, Kurzchalia, Shevchenko, & Schwudke, 2008) and IPA (Isopropyl alcohol) (Sarafian et al., 2014). These three protocols were applied to both the perennial ryegrass bulk sample (as described above in the materials section) and also to a sub sample of bovine plasma samples. After lipid extraction, all samples were run through an Acquity CSH C18, 1.7 µm column and analysed using a Thermo Fischer Q-Exactive mass spectrometer (using the HPLC-MS settings as described below).

Folch

A modified version of the Folch method for lipid extraction was used. The original Folch method uses a 2:1 chloroform to methanol ratio, this was modified and a 3:1 chloroform to methanol ratio was used along with 0.1% H_2O as the extraction solvent. Previous in-house studies had shown that the amount of lipids extracted from ryegrass was increased by increasing the chloroform content slightly from the original Folch extraction solvent, this modified version was used henceforth. For ryegrass lipid extraction 50 mg of bulk ryegrass sample was weighed into a 2 mL micro centrifuge tube. To this tube a ceramic bead (for mixing) and 1 mL of extraction solvent was added. This was then shaken horizontally on a Qiagen Tissuelyser II for 1 minute and centrifuged at 14,000 G for 6 minutes. The supernatant liquid was then transferred to a 2

mL vial for analysis. The same process was followed while analysing plasma except that 50 µL of plasma was extracted with 1 mL of solvent.

UCD

The Matyash MTBE (methyl tert-butyl ether) protocol has been modified by Cajka et al. (2014) and was applied to rye grass as such (Cajka, 2014). 20 mg of bulk rye grass sample was weighed into a 2 mL microcentrifuge tube. To this 225 µL of ice-cold methanol was added and the mixture was vortexed for 10 seconds. 750 µL of ice-cold MTBE was added, the resulting mixture was vortexed for another 10 seconds and was the shaken for 6 minutes at 4°C. Once shaken, 188 µL of Milli-Q H_2O was added and after a further 20 seconds of vortexing, it was centrifuged at 14,000 g for 2 minutes. The top phase was then transferred into two separate tubes, one was analysed as such and the other was dried down and reconstituted in 65 µL of 9:1 methanol:toluene. The two samples were then analysed on the mass spectrometer. 20 µL of plasma was analysed using the same protocol.

IPA

50 mg of bulk rye grass (50 µL of plasma) was weighed into a 2 mL microcentrifuge tube. To this, 150 µL of ice cold isopropyl alcohol (IPA) was added. This was then vortexed for 20 seconds and left in a -20°C freezer overnight. The next morning the mixture was centrifuged at 14,000 g for 3 minutes and the supernatant was transferred to a vial for analysis.

2.2.2 Mass shifting

Two mass shifting techniques were analysed using both the Folch and IPA lipid extraction methods. The two mass shifting techniques used were Fmoc (Han, Yang, Cheng, Fikes, & Gross, 2005) and d_6-S,S'-dimethylthiobutanoylhydroxysuccinimide ester (d_6-DMBNHS) (Fhaner, Liu, Ji, Simpson, & Reid, 2012).

Fmoc

100 µL of lipid extract was added to a 2 mL microcentrifuge tube and dried down completely under nitrogen. 100 µL of 10 nM Fmoc-Cl in chloroform was added to the tube, this was then flushed with nitrogen gas, capped and vortexed. The tube was then covered in tin foil and left at room temperature for 30 minutes with occasional vortexing. 2 mL of 1:1 chloroform:methanol was then added to stop the reaction and the resulting solution was analysed.

***d_6*-DMBNHS**

25 µL of lipid extract was dried down in a 2 mL microcentrifuge tube under nitrogen and then reconstituted in 40 µL in 39:1.1 chloroform containing 0.0125 M triethylamine. This was then vortexed for 30 seconds. To this solution 10 µL of 0.00125 M d_6-DMBNHS in DMF was added and then vortexed for a further 30 minutes. The reaction was then stopped by drying down under nitrogen. This was then reconstituted in 4:2:1 isopropyl alcohol:methanol:chloroform containing 20 mM ammonium formate and then was analysed.

2.2.3 Direct Infusion/Shotgun

Three sets of samples were analysed by direct infusion: Original IPA and Folch extracts, Fmoc mass shifted IPA and Folch extracts and d_6-DMBNHS mass shifted IPA and Folch extracts. The samples were drawn up into a Hamilton 250 µL syringe and injected into the source at 5 µL/min flow rate. The syringe and peak tubing was rinsed 3 times between each sample with iso-propyl alcohol. Each sample was measured two different ways using the Q-Exactive mass spectrometer; DIA (Direct Injection Analysis) and dd-MS2 (data dependent MS2).

2.2.4 HPLC-MS

As described in the lipid extraction test, a modified version of the Folch method was used to extract lipids from a sample of bulk rye grass. This lipid extract was then run through a Waters Aquity CSH C18, 100x2.1 mm, 1.7 µm column and analysed by a Q Exactive mass spectrometer (with a Thermo Accela 1250 HPLC pump and a Thermo PAL autosampler).

2.2.5 Mass Spectrometer settings

HPLC-MS

Tune options: Spray voltage: 4000 V. Sheath Gas: 40.00. Aux Gas: 10.00 Sweep Gas: 5.00 S-Lens: 50 V. Ion Source: HESI (heated electrospray ionisation).

Method settings: Fulll MS - Resolution: 35,000. Scan range: 200 to 2000.

dd-MS^2- Resolution: 35,000. Loop count: 2. Isolation window: 1.5. NCE: 30.0. Dynamic exclusion: 7.0 s

HPLC settings: Solvent A: 60:40 acetonitrile:water + 10 mM ammonium formate + 0.1% formic acid. Solvent B: 90:10 isopropyl alcohol:acetonitrile + 10 mM ammonium formate + 0.1% formic acid. Flow rate: 600 uL/min. Gradient: Percentage of Solvent A over time is graphed where percentage Solvent B is 100% - Solvent A percent at any given time.

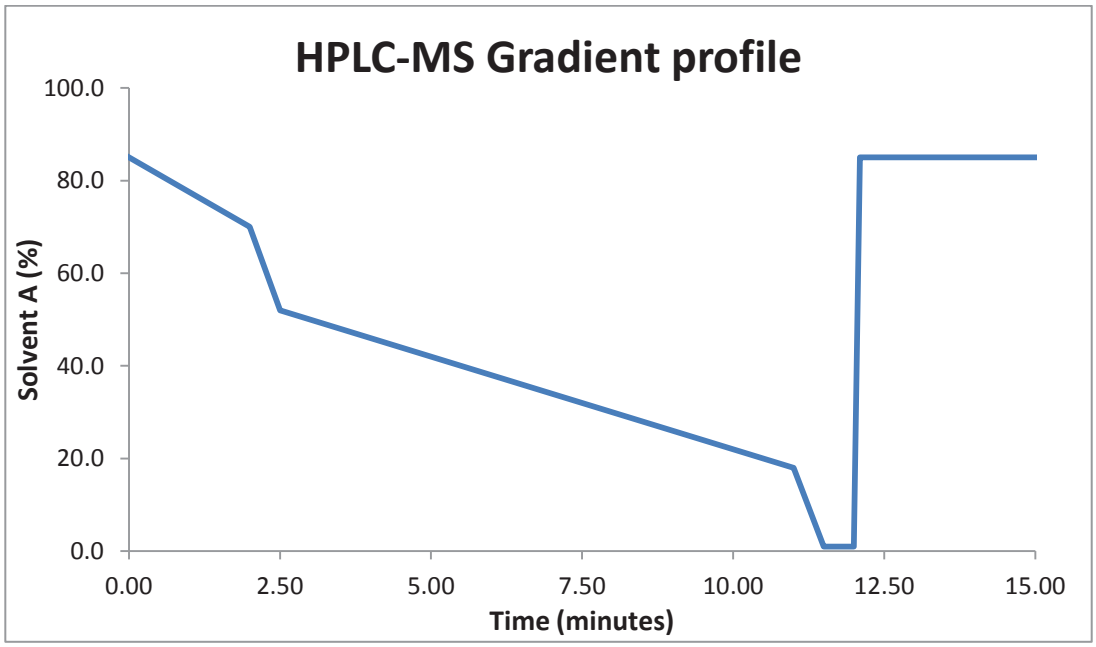

Figure 2.1 – Solvent gradient profile used in the HPLC-MS experiment.

Shotgun dd-MS2

Tune options: Spray Voltage: 4000 V. Sheath Gas: 10.00. Aux Gas: 0.00. Sweep Gas: 0.00. S-Lens: 50. Ion Source: HESI

Method settings: Full MS - Resolution: 140,000. Scan range: 200 to 1250.

dd-MS2- Resolution: 17,500. Loop count: 100. Isolation window: 1.0s. NCE: 30.0.

Syringe pump settings: Syringe type: Hamilton. Flow rate: 5.000uL/min

Shotgun DIA

Tune options: Spray Voltage: 4000 V. Sheath Gas: 10.00. Aux Gas: 0.00. Sweep Gas: 0.00. S-Lens: 50 Ion Source: HESI

Method settings: Full MS - Resolution: 35,000. Inclusion list: 200.00 to 1250.00 in 1 *m/z* increments. Loop count: 1. NCE: 30.00

Syringe pump settings: Syringe type: Hamilton. Flow rate: 10.00 uL/min

2.2.6 Data analysis

LMSD search

Accurate mass MS1 data was compared to the LMSD and searched using a basic R script developed in house and not published. These tentative annotations were then checked manually.

Steps involved:

1- Calculate head group and chain masses.
2- Check for head group fragment or neutral loss.
3- Search for loss of chains or fragments.

A tentative annotation was only 'confirmed' (no full confirmation can be made without standards) after observing presence of headgroup and chain/s.

LipidSearch

Lipid spectra were searched for within the samples using LipidSearch (version 4.1, ThermoFisher). Briefly, the software was tasked with searching through Q-Exactive MS2 fragmentation spectra with a parent mass tolerance of 5 ppm and a product mass tolerance of 8 ppm. Searching for parent ions of M+H, $M+NH_4$ and M+Na in positive ionisation mode and M-H, M-H+HCOO and $M-H+CH_3COO$ in negative ionisation mode. Once a list was created of possible matches, these were confirmed or denied using the same steps as used in checking the LMSD data.

3 Results & Discussion

3.1 Lipid extraction test

The ‘Lipid extraction test’ was run to optimise testing conditions for the rest of the experiments. In order to obtain the best possible results, lipid extraction methods must first be analysed. As described in the methods section the three methods compared were the Folch (Folch et al., 1957), UCD (University of California, Davis) which is a modified version of the Matyash protocol (Matyash et al., 2008)and IPA (Isopropyl alcohol)(Sarafian et al., 2014).

3.2 Laboratory procedures

The Folch extraction is a tried and true protocol. It has been the most popular lipid extraction method (has been cited 48,589 times as at 06/03/2015) since it was first published in 1957. It is a straight forward protocol that includes weighing of sample, addition of solvent, shaking, spinning and removal of supernatant liquid for analysis. The main way the Folch and IPA methods differ is that the IPA method does not require solvent preparation which cuts down on preparation time, but not by much. In stark contrast the UCD method is complicated. It not only contains the steps similar to those of the Folch and IPA methods, but after the first steps, the initial supernatant is dried down and reconstituted twice to give two different aliquots for testing.

3.3 Interpretation of chromatographic results

The main factors for comparing the methods when looking at the mass spectral results are number of visual chromatographic features, separation and shape of peaks and intensity. Although all four chromatograms look very similar at first look, as seen in figure 3.1, a closer inspection of the region between 5.5-7.5 minutes reveals the most dramatic differences. In this region it is quite easy to see that the UCD and Folch extracts have more peaks with good separation and shape compared to the IPA extract. The Folch and UCD extracts are very similar in the aspects scrutinised. The main differences seen between the three are the non-Gaussian peak at approximately 8.8 minutes in the UCD reconstituted extract compared to the same peak seen at approximately that time in the UCD original and Folch extracts. Also, the higher level of overall intensity of the UCD reconstituted extract is another major difference. Overall the Folch and UCD original extracts satisfy the criteria to the greatest extent.

Taking into consideration the ease of laboratory procedure and also the quality of the resulting spectra, the Folch lipid extraction protocol was used for the rest of the experimental work.

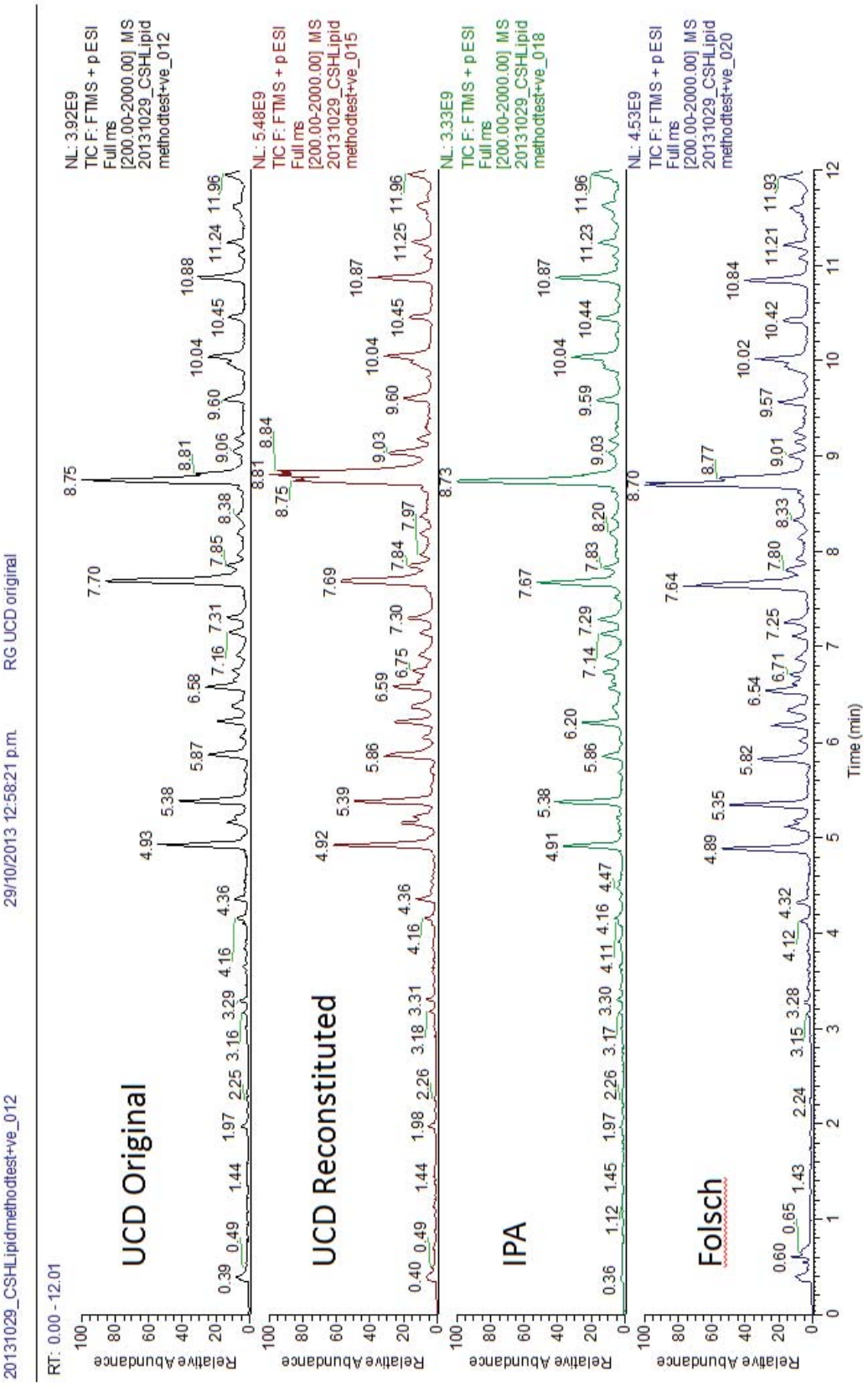

Figure 3.1 – Total Ion Chromatograms of the four samples compared in the lipid extraction test.

3.4 Shotgun method development and analysis

3.4.1 Mass shifting

The main theory behind mass shifting is that you can shift your target masses away from regions of high mass competition to an area where there are less competing masses. This is done through the use of specifically chosen or synthesised compounds attaching to selected functional groups of the target compound which adds mass to the target. This added mass can be calculated and theoretical mass shifts are investigated. As shotgun does not have a separating step this technique is essential to separate masses of interest to ease annotation. Both methods used for acquiring mass spectral data have fairly narrow windows for selecting masses for MS2 events but if a competing mass is within the range of that window then the competing mass would also contribute to the MS2 spectra. This is very unfavourable, especially when the competing mass 'drowns out' the mass of interest due to being in much higher abundance than the target mass. This can either make it impossible to annotate the target mass or can lead to incorrect annotations due to assigning fragment masses that do not originate from the target.

Fmoc

Fmoc is a commonly used protecting group attaching itself to free amino groups in certain syntheses to be readily cleaved later in the process to retain free amino functionality (Carpino & Han, 1972). This use of Fmoc is utilised in part to mass shift a specific class of lipid. The class of lipid mass shifted through the use of Fmoc is phosphoenthanolamines (PE). PEs have a free amino group as part of their head-group as seen in figure 3.2 below. From our knowledge of the mass of our target and how

Fmoc attaches to the target we can calculate the theoretical mass shift. This shift should be an approximate gain of *m/z* 222.2.

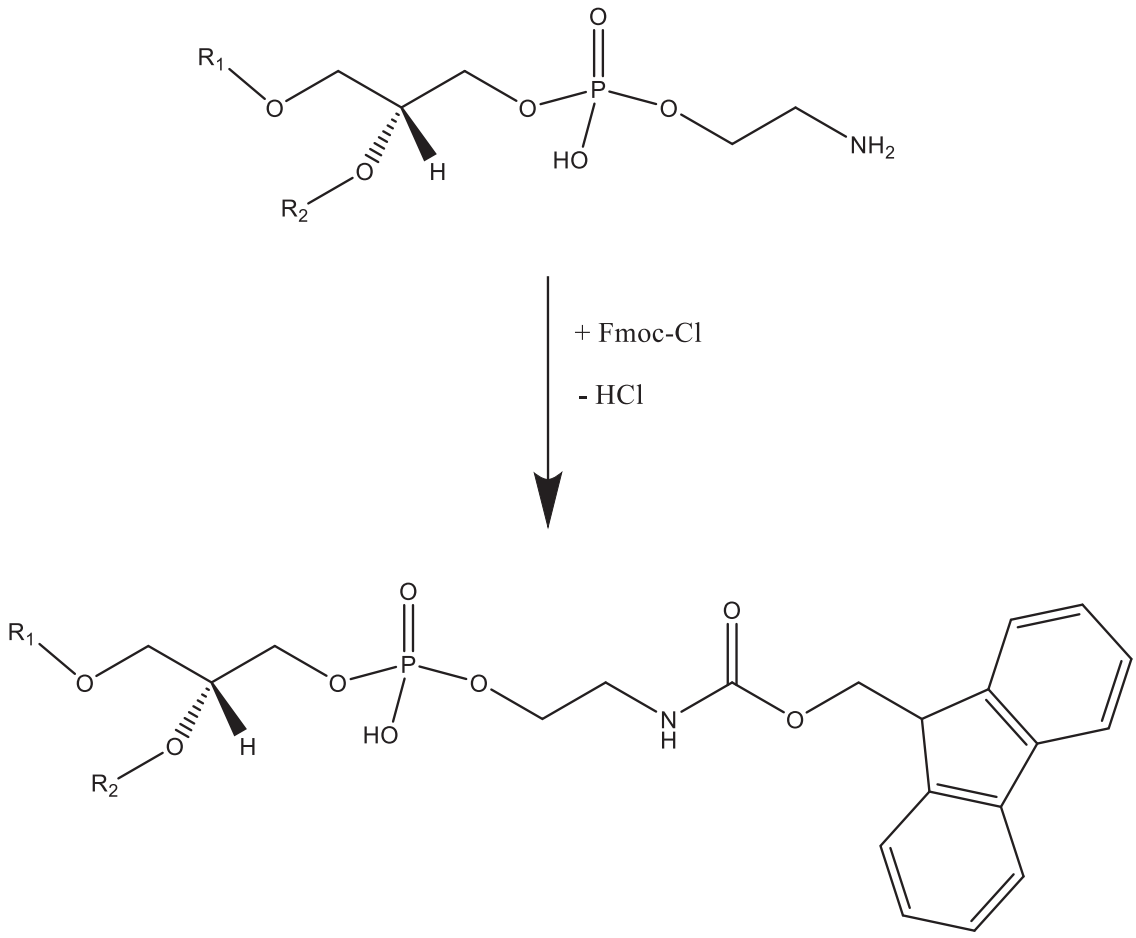

Figure 3.2 – PE class lipid and the theorised amino group protection reaction using Fmoc.

The procedure for validating if the mass shift was a success or not was to first find a PE in the original extract. The most common fragment seen in mass spectra for PE class lipids that is assigned to the head group is *m/z* 140. Performing an extracted ion chromatogram on the MS2 data collected selecting just to view MS2 spectra containing

the *m/z* 140 fragment is the best way to narrow down PE class lipids. Once this was performed the potential PE class lipids were investigated further and those confirmed to be PEs had 222.2 added to their *m/z*. Unfortunately the masses corresponding to the PE lipids + 222.2 *m/z* were not found in the mass shifted control samples.

d_6-DMBNHS ester

The d_6-*S,S'*-Dimethylthiobutanoylhydroxysuccinimide ester (d_6-DMBNHS) is a compound purely synthesised for the purpose of mass shifting. It was designed and synthesised by Prof. Gavin Reid and his team at Michigan State University (Fhaner et al., 2012). Its main purpose is to mass shift PE lipids and PS (phosphoserine) lipids. The overall action of the ester is the same as F-moc. It binds to the free amino groups in both the PE and PS lipids (theoretical reaction with PS class lipids seen below in Figure 3.3). It is seen to bind more efficiently to PS lipids because it is not as bulky as F-moc so can possibly physically 'fit' in the available space.

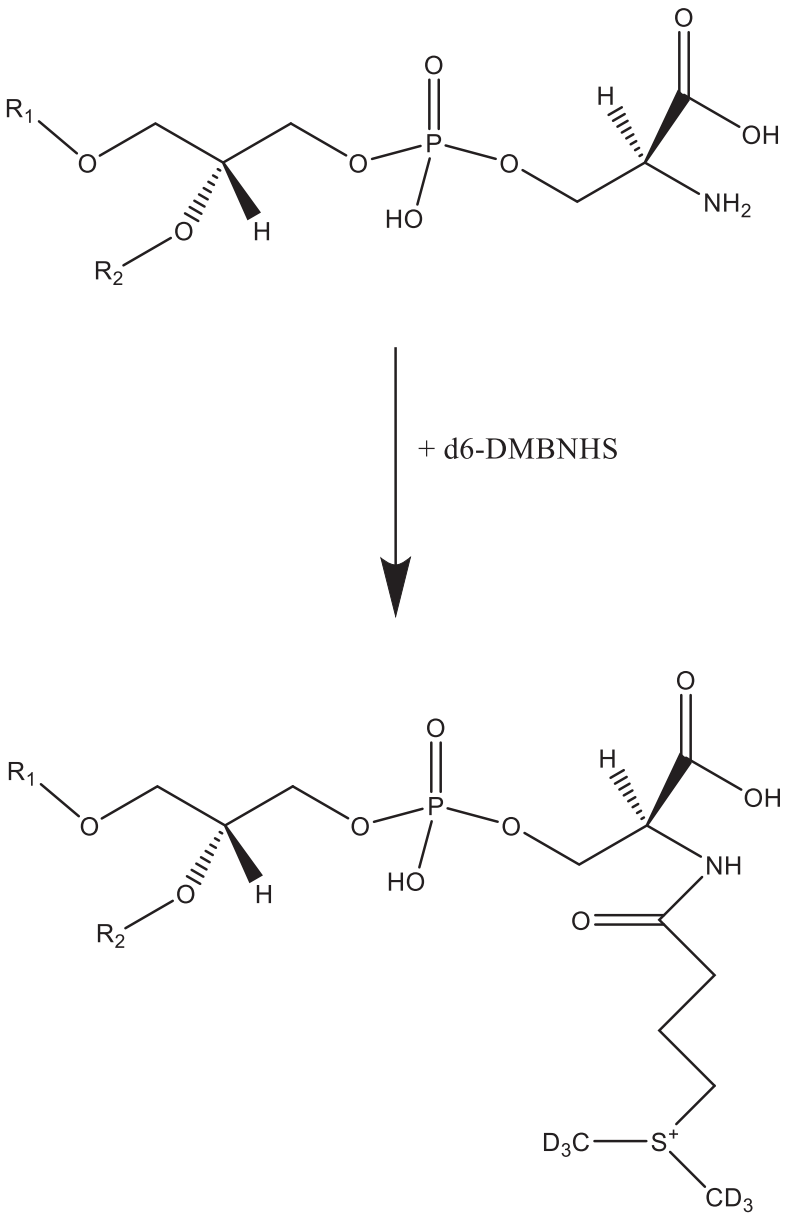

Figure 3.3 - PS class lipid and the theorised amino group protection reaction using d_6-DMBNHS ester.

The validating technique used to make sure the mass shift was a success is exactly the same as that used for validating Fmoc binding. The same PE class lipids that were identified when looking at Fmoc were also investigated here along with identifying PS class lipids. PS class lipids are easily identified through the *m/z* 184 fragment attributed to its head group. The d_6-DMBNHS ester should give a mass shift of *m/z* 136.0825. The

result reflected those of Fmoc where no mass shifted PE or PS class lipids were observed.

There are a number of different factors that could have contributed to the mass shifting techniques used not being successful. These factors include: reagents, concentrations and matrix type. The two main reagents used were one opened bottle of Fmoc chloride, opened 6 months earlier (which is very reactive to water, both in the atmosphere and in the samples, which can render the reagent inactive) and one vessel containing d_6-DMBNHS ester which flew at room temperature from Michigan, United States of America to Palmerston North, New Zealand. Unfortunately the easiest way to test if either of these two reagents was still active is to use them. Concentration could have also been a factor but was not considered when setting up the initial experiment where the protocols followed had good results using the concentrations outlined. Matrix type is the most probable cause of the failed mass shifting. The matrices used in the protocols followed were both animal based whereas in this study the matrix is plant based. This is the fundamental difference between experiments and could be the major cause of negative results in the mass shifting experiment.

3.4.2 Direct infusion/shotgun

When analysing the shotgun data the first obstacle is the lack of any chromatographic separation. ESI without chromatographic separation means that all analytes are in the droplet at the same time, competing for charge. Those that ionise best will be more abundantly observed in the spectrum and effectively suppress other less ionisable

compounds. This provides the most challenging aspect of this thesis project, shotgun data analysis.

3.4.3 Automated LMSD Full MS search

An R script was written by Dr Mingshu Cao (AgResearch, Palmerston North, New Zealand) to take the accurate masses from the full MS scan and to then compare those with the database. Where there were matches a tentative annotation was awarded. This resulted in a spreadsheet of accurate masses found in the full MS scan with possible matches beside them. In the positive mode scan there were 284 possible matches to check. The best way to confirm or deny these matches was to check them by hand by analysing their MS2 spectra.

The first step of confirmation of matches was to create a table for quick and easy reference that included common lipid headgroup mass losses and common hydrocarbon chain length losses as neutral masses. This resulted in the table below (Table 3.1).

Table 3.1 - Indicative losses calculated or seen in lipid extracts using mass spectral techniques.

Short Name	Lipid Class	Molecular Formula	Mass
C6:0		$C_6H_{12}O_2$	116
C10:0		$C_{10}H_{20}O_2$	172
C12:0		$C_{12}H_{24}O_2$	200
C12:1		$C_{12}H_{22}O_2$	198
C14:0		$C_{14}H_{28}O_2$	228
C14:1		$C_{14}H_{26}O_2$	226
C14:2		$C_{14}H_{24}O_2$	224
C16:0		$C_{16}H_{32}O_2$	256
C16:1		$C_{16}H_{30}O_2$	254
C16:2		$C_{16}H_{28}O_2$	252
C16:3		$C_{16}H_{26}O_2$	250
C18:0		$C_{18}H_{36}O_2$	284
C18:1		$C_{18}H_{34}O_2$	282
C18:2		$C_{18}H_{32}O_2$	280
C18:3		$C_{18}H_{30}O_2$	278
C18:4		$C_{18}H_{28}O_2$	276
C20:0		$C_{20}H_{40}O_2$	316
C20:5		$C_{20}H_{30}O_2$	306
C22:0		$C_{22}H_{43}O_2$	339
C22:1		$C_{22}H_{41}O_2$	337
C22:6		$C_{22}H_{29}O_2$	327
Phosphate (2H)		H_3PO_4	98
Ethyl Amine	E	C_2H_7N	45
Diglycerol	DG	$C_3H_8O_3$	92
Diglycerol without 2xO	DG	C_3H_6O	58
Sulfoquinovose		$C_6H_{12}SO_8$	244
Phosphocholine	PC	$C_5H_{14}O_4NP$	184
Choline	C	$C_5H_{14}N$	88
Digalactosyldiacylglycerols	DGDG	$C_{12}H_{22}O_{11}$	342
Serine w/o OH	S	$C_3H_7NO_3$	105
Phosphoserine	PS	$C_3H_7NO_6P$	184
Inositol	I	$C_6H_{11}O_5$	163
Phosphoinositol	PI	$C_6H_{12}O_9P$	259
Monogalactosylmonoacylglycerol	MGMG	$C_6H_{12}O_6$	180
	MGMG w/o H_2O	$C_6H_{10}O_5$	162
Phosphotidic acids	PA	$C_3H_5O_4P$	136
Phosphoethanolamine	PE	$C_2H_7O_4NP$	140
Phosphoglycerol	PG	$C_3H_8O_6P$	171
Phosphoglycerol w/o H_2O	PG w/o H_2O	$C_3H_6O_5P$	153
Monoglyceride	MG	$C_3H_8O_3$	92

The second and most time consuming step was to search the LMSD to find what the R script had assigned to the mass and to then confirm or deny. If the mass was matched to a relatively simple lipid such as a phosphorylated lipid it was quite quick to check against the created table. On the other hand if the match was more complicated then it took much longer to check the validity of the match. This involved first calculating possible fragment masses based on the structure given by the LMSD search and then checking for the masses as both neutral losses and fragments in the MS2 spectra captured.

This was a fairly simple task but it was also extremely time consuming. Out of the 284 possible matches found through this technique 150 were checked by hand. This took a total of approximately 160 hours to complete. From the 150 that were checked, 11 were confirmed matches. 11 out of 150 is an unacceptable success rate. With this information the remainder of possible matches in the positive mode data were left unchecked and the negative mode data was also abandoned. The time needed to check the remainder of matches was disproportionate to the gain in information. From here, other avenues were explored.

3.4.4 LipidSearch

LipidSearch is a very powerful tool for searching mass spectral data for lipids. The basis behind LipidSearch is that MS1 masses are first searched against a database and then the MS2 spectra are further investigated looking for specific fragments or losses. In essence LipidSearch is an automated version of the painstaking technique used to

confirm the matches given using the R script above. There is, however, one major downfall of LipidSearch. It currently does not support shotgun data. Fortunately in this case, the lipid extraction test data is compatible with LipidSearch. So although the software cannot handle shotgun data, the same sample run through a column can be analysed.

After analysis through the LipidSearch software a table is created. 122 possible matches were found in positive ionisation mode (listed in Appendix 1) but confirmation was still needed. The same confirmation process was employed as with the previous attempt to annotate the shotgun data, an example of which is shown in Figure 3.4 below. This figure shows the steps taken to confirm the annotation assigned to mass *m/z* 496.34 in positive ionisation mode of LPC(16:0). The abundant fragment masses of *m/z* 104.1075 and *m/z* 184.0739 are indicative of a phosphotidylcholine headgroup and the loss of 256 indicates a loss of a C16:0 chain length. The table created with headgroups and common chain length losses and fragments was utilised to confirm or deny the possible annotations. Out of the 122 matches found by LipidSearch in the positive ionisation mode LCMS data, 97 were tentatively confirmed, with only 25 being definitively incorrect (mostly due to uneven chain lengths not being present in rye grass). The negative ionisation mode LCMS data was also run through LipidSearch resulting in 24 possible matches (listed in Appendix 1). Of these 24 matches, 21 were tentatively confirmed.

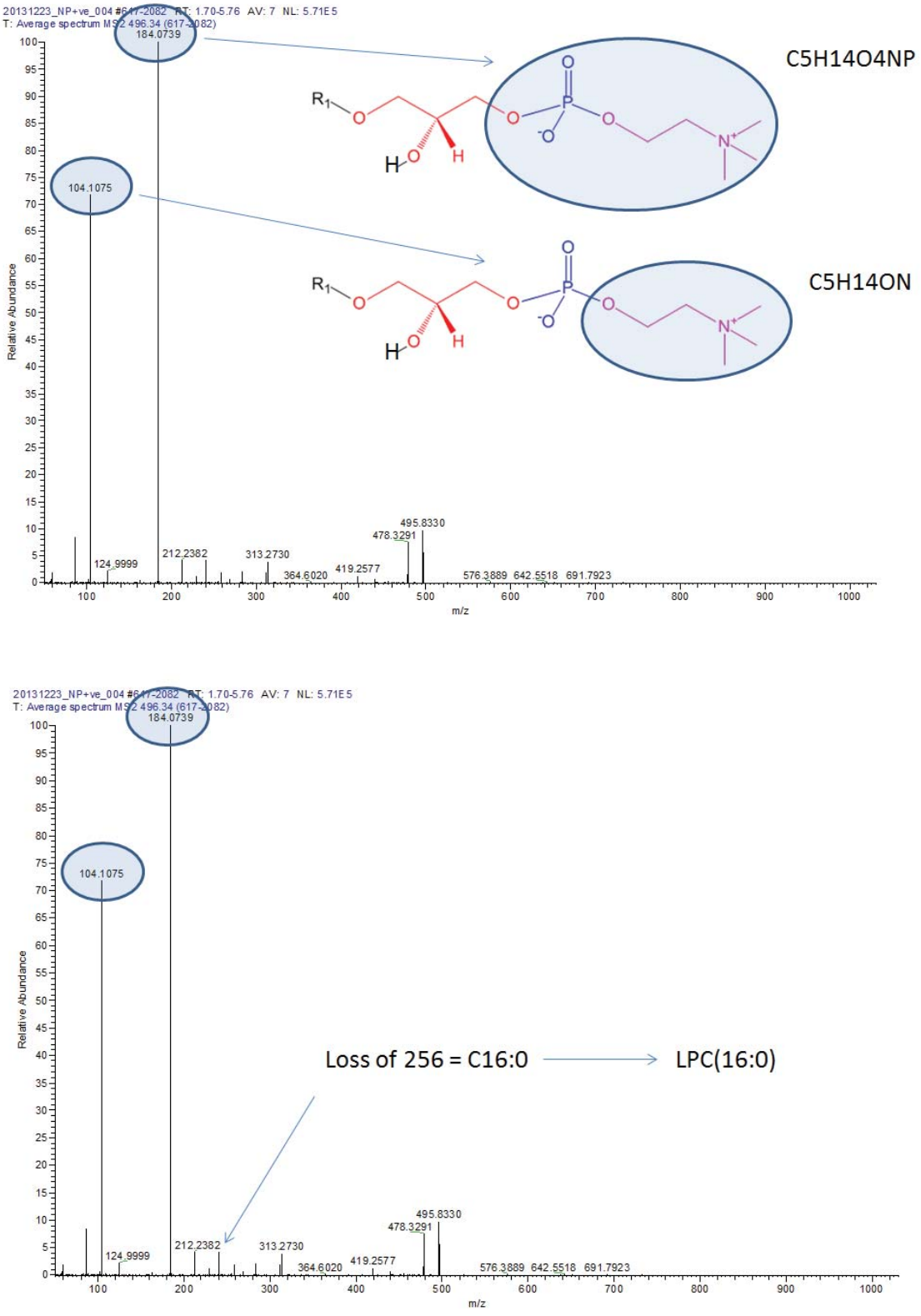

Figure 3.4 – Visual representation of steps taken to confirm an annotation in both the R script and LipidSearch Results. Above shows lysophosphotidylcholine(16:0). Top shows the typical headgroup fragments and bottom shows the loss of a C16:0 hydrocarbon chain.

3.4.5 Comparing LipidSearch results to Shotgun data

It was very interesting to note when first comparing the LipidSearch results to the shotgun mass spectral data. The main point of interest was that the major peak (highest abundance) in both the shotgun data and the chromatographic data was unaccounted for in the LipidSearch results. This provided a major hole in the tentative annotations as was the next focus of investigation once the comparison was completed. Besides the lack of annotation for the most abundant peak the other main finding was that out of the 97 tentative annotations found in the positive mode LCMS data, 27 of those were found in the shotgun data. Unfortunately those 27 were all minor peaks and so did not account for any of the most abundant peaks. The highest peak annotated in the shotgun data was the 37th most abundant mass.

The nature of shotgun data and the obvious matrix difference to that found in the literature followed are the two main contributors to the downfall of the annotation attempt. While many different strategies were implemented to try to annotate the major peaks within the shotgun data, these strategies were generally unsuccessful. The nature of shotgun data means that any attempt to annotate masses is very difficult. The main source of issues is mass overlap. This overlap causes doubt and confusion when looking at fragment masses as it is unclear which fragments belong to the target mass. The mass shifting techniques would have provided a possible solution when targeting specific lipid classes but even if it was a success, there were still various other lipids that would have been left unclear. Unfortunately the matrix effect of the rye grass provided these difficulties. The major peak in the positive ionisation mode data is *m/z* 871.57. This mass drowns out the majority of the other masses and is unaccounted for in the

LipidSearch results. Annotation of this mass is key for understanding the sample and also understanding what is contributing to so many of the issues faced in this study.

3.4.6 Annotation of *m/z* 871.57

m/z 871.57 in positive ionisation mode is the main mass in the shotgun data (upper panel) and the main peak in the chromatogram in the LCMS data (lower panel) as seen in Figure 3.5 below. It is postulated that this peak is one of the major contributing factors towards the unsuccessful annotation of peaks in the shotgun data so it is very important to gain a better understanding of it. The first step in the investigation is to look at the fragmentation pattern (also known as MS2 spectra) of the peak. As seen in Figure 3.6, the ion breaks into two major fragments. It loses 278 to give a *m/z* 593.28 peak and also loses 338 to give a peak at *m/z* 533.26 with a difference of 60 mass units between them. The first conclusion made was that it is a PC class lipid as they have a characteristic headgroup loss of 278. This does not fit with the other fragments and losses seen however so a tentative annotation cannot be assigned based on this alone. The loss of 338 and the difference between the two major fragment peaks of 60 mass units is unaccounted for based on it being a PC class lipid so it cannot be a PC class lipid.

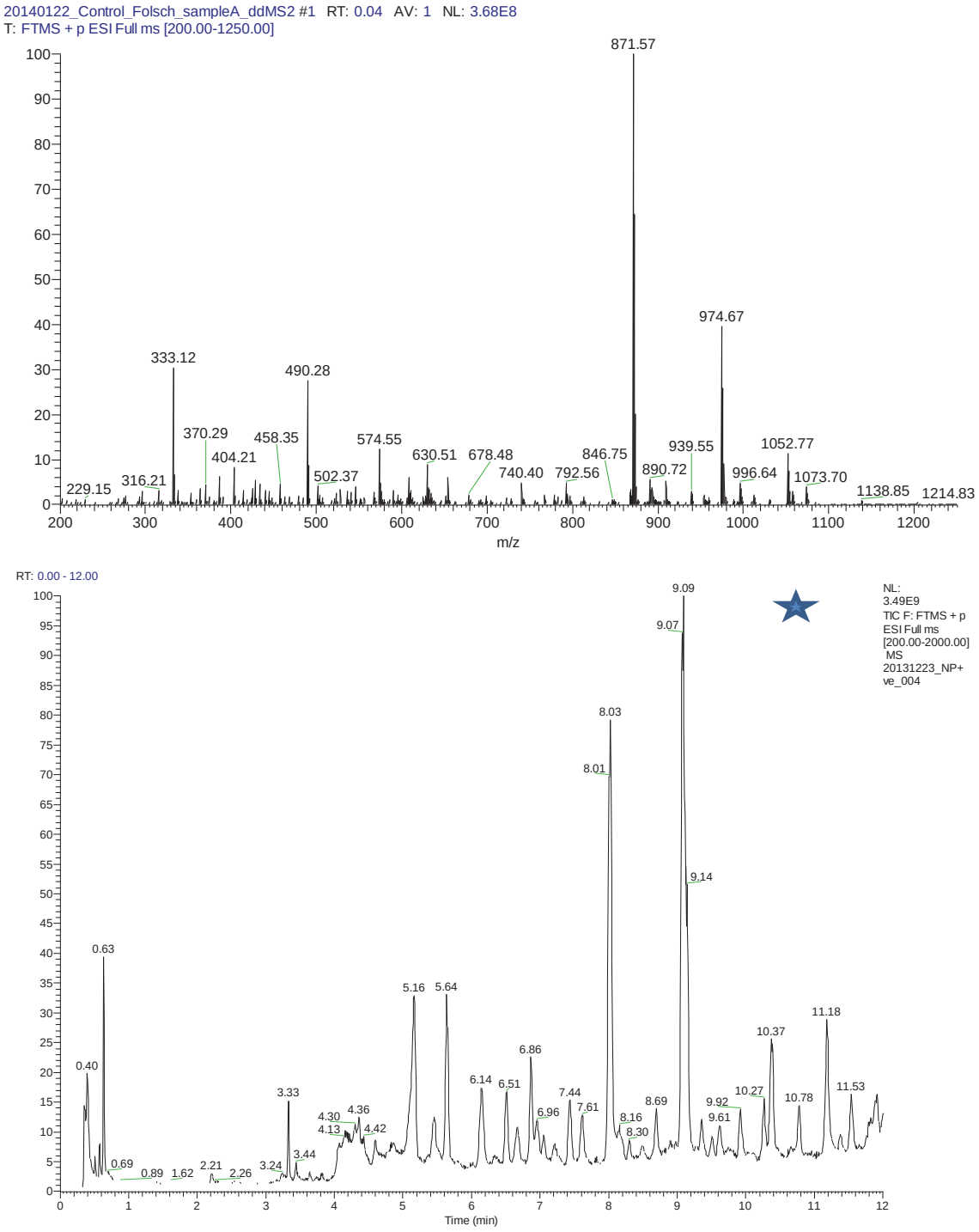

Figure 3.5 – Top spectra is the Full MS scan of the shotgun data showing the high abundance of *m/z* 871.57. Bottom chromatogram shows (as indicated with a star symbol) the peak at 9.09 minutes which is the *m/z* 871.57 peak.

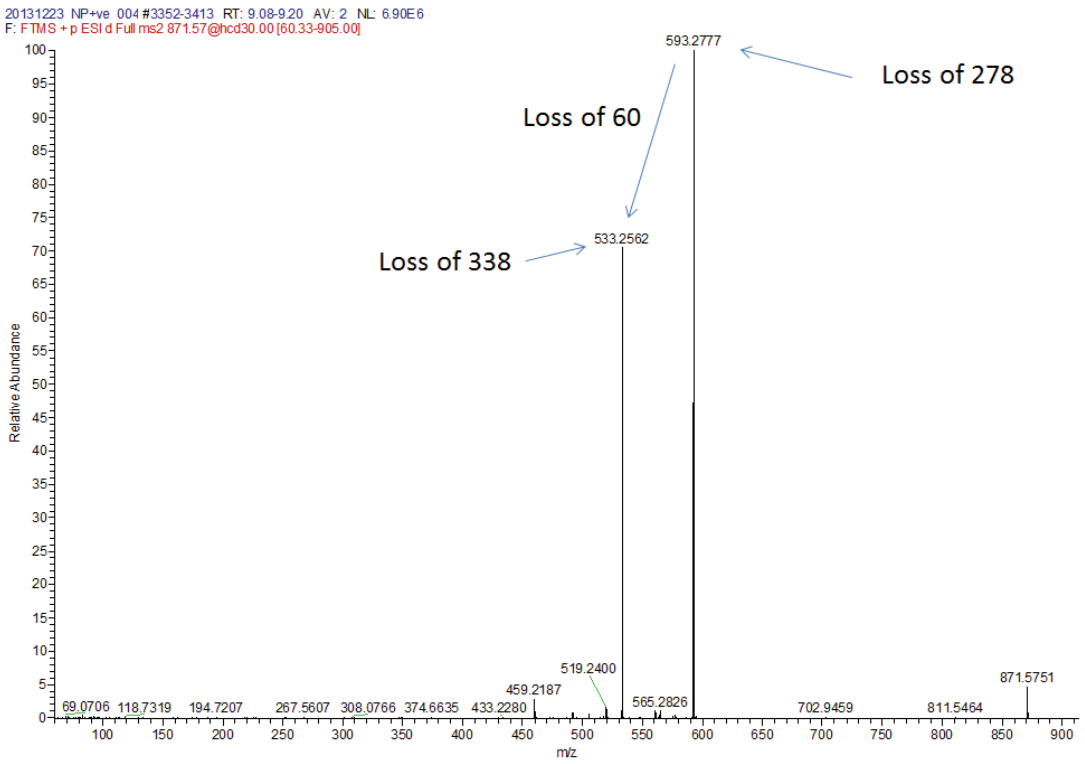

Figure 3.6 – Fragmentation pattern (MS2 spectra) of *m/z* 871.57 in the LCMS experiment. Note: MS2 spectra of the 871.57 mass using the shotgun data was almost identical, but did have a noisier baseline so the 'cleaner' spectra was used.

The second step was to use an online tool called MZedDB (Draper, 2008). This tool provides users with not only a molecular formula generator but also provides calculated isotope patterns. It uses complex algorithms which can include Feihn's seven golden rules (which in this case have been employed), as detailed in the Introduction section, in the background along with a tentative guess of what atoms are in your compound to create a list of possible matches. In this case the tentative guess was that the compound contained 45-65 carbons, 60-150 hydrogens, 0-15 nitrogens, 0-30 oxygens, 0-2 phosphorus atoms and 0-2 sulfurs. The mass tolerance was set at 0.2mmu and the mass entered for the calculation was 870.566 (due to taking off a proton to search for the

neutral form). Fortunately this calculation yielded only two results. The two formula generated were $C_{47}H_{88}N_2O_4P_2S_2$ and $C_{55}H_{74}N_4O_5$.

In order to validate the calculated formulae the approach used was to compare calculated isotope patterns with the experimental isotope pattern collected from the chromatographic data. Table 3.2 shows the calculated relative intensities of the isotope peaks where the masses are all minus a proton compared to the experimental isotope pattern as seen in Figure 3.7. The individual intensities of each whole mass unit (i.e. 871) are added together to give the expected experimental peak for that mass (plus a proton from the positive ionisation mode spectra). In order to evaluate the overall validity of the calculated isotopes, the calculated intensities are compared directly to those in the experimental spectra. The deviation from the experimental value is then calculated per peak and then an average deviation percentage is calculated. These results are tabulated in Table 3.3.

Table 3.2 – Isotope intensities of both search results for mass 871.57 *m/z* in neutral form.

Results for C47H88N2O4P2S2				Results for C55H74N4O5		
Acurate Mass	Probability	Relative Intensity		Acurate Mass	Probability	Relative Intensity
870.566082	5.21E-01	100		870.565921	5.23E-01	100
871.565468	8.33E-03	1.60		871.562955	7.77E-03	1.49
871.569436	2.75E-01	52.8		871.569275	3.23E-01	61.7
872.561872	4.62E-02	8.88		872.570166	5.24E-03	1.009
872.57279	7.09E-02	13.6		872.572629	9.79E-02	18.79
873.565226	2.44E-02	4.69		873.575983	1.94E-02	3.719
873.576144	1.19E-02	2.29				
874.56858	6.30E-03	1.21				

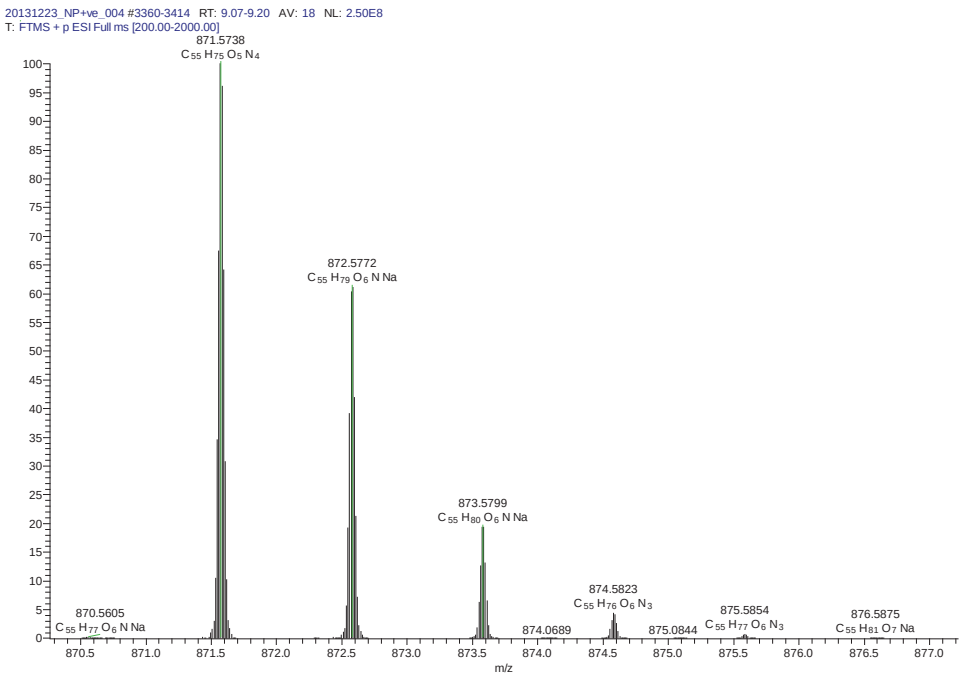

Figure 3.7 – Experimental isotope pattern of mass 871.57 as seen in rye grass lipid extract.

Table 3.3 – Isotope deviation calculations showing the deviation between the calculated and the experimental/observed isotope peaks.

Experimental accounting for +1 in mass			Results for C47H88N2O4P2S2				Results for C55H74N4O5		
Acurate Mass	Relative Intensity		Acurate Mass	Relative Intensity	Deviation to experimental (in %)		Acurate Mass	Relative Intensity	Deviation to experimental (in %)
870.57	100		870.57	100	0		870.57	100	0
871.57	61.5		871.57	54.4	11.6		871.57	63.2	2.72
872.57	20.0		872.57	22.5	11.3		872.57	19.7	1.19
873.58	4.25		873.57	6.98	39.1		873.58	3.71	12.7
874.57	0.95		874.57	1.21	21.5				
					Average deviation = 16.7				Average deviation = 4.15

The calculated average deviation from the experimental values observed show that the most likely molecular formula for the peak at *m/z* 871.57 in positive ionisation mode is $C_{55}H_{74}N_4O_5$. The difference between the two calculated deviations is fairly significant and the very low deviation of $C_{55}H_{74}N_4O_5$ from the experimental values indicates that it may be the 'real' formula.

The next step was to search for the formula in different databases and search engines. The first port of call was Google. The formula $C_{55}H_{74}N_4O_5$ registered 2,290 hits, the first of which was a page for a compound in the resource named ChemSpider which is hosted by the Royal Society of Chemistry. The webpage found through Google was for the compound named pheophytin a (figure 3.8). (Royal Society of Chemistry, 2014a)

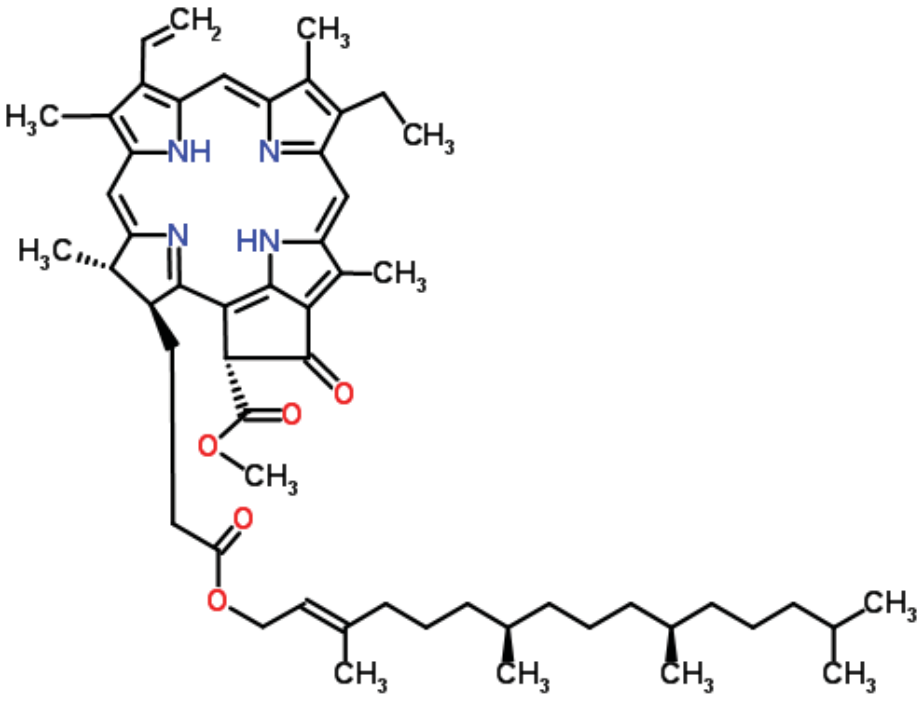

Figure 3.8 – Structural representation of pheophytin a. (Royal Society of Chemistry, 2014a)

Until fairly recently pheophytin a was considered a product of chlorophyll degradation in plant cells but research by Klimov in 2003 suggested otherwise. The paper in *Photosynthesis Research* contradicts previous thinking and proposes that pheophytin a is actually "the primary electron acceptor of PS II *(Photosystem II)* acting between P_{680} *(chlorophyll* P_{680}*)* and Q *(a special form of bound plastoquinone)*." (Klimov, 2003). The main point of interest for this research is based purely on its relationship to chlorophyll and how if the peak is pheophytin a, what other related compounds will also be present.

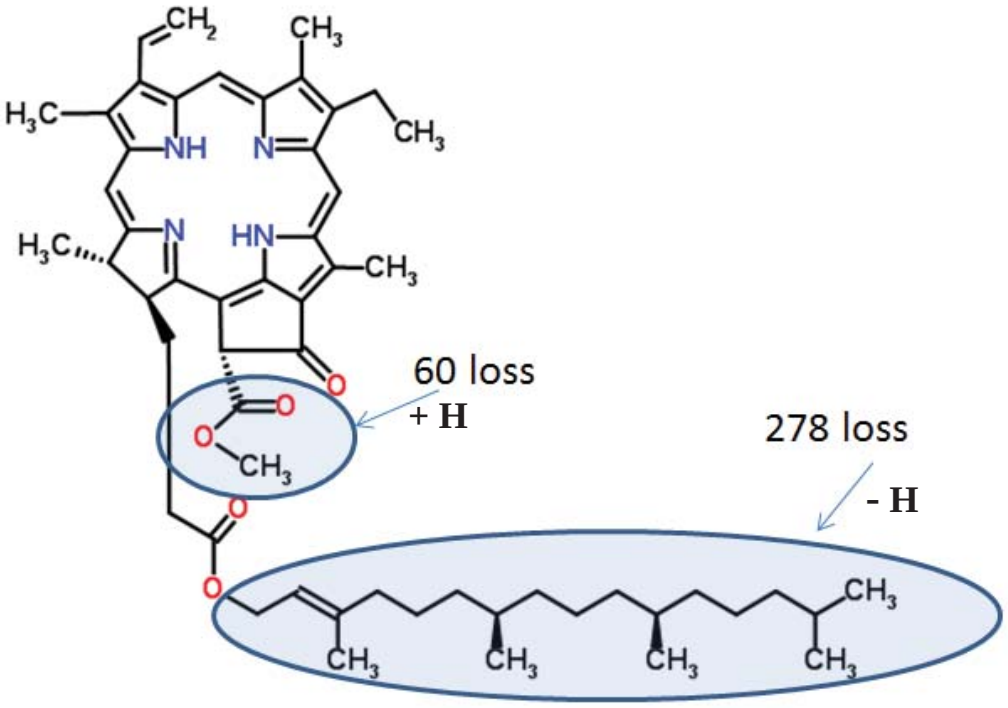

Figure 3.9 – Pheophytin a, highlighting fragment losses which align to MS2 results. (Royal Society of Chemistry, 2014a)

Revisiting Figure 3.6 along with the visual representation given above in Figure 3.9 provides a clear understanding as to how the mass at *m/z* 871.57 fragments and how pheophytin a fits that pattern. The initial loss of *m/z* 278 can be assigned to the loss of the hydrocarbon chain of pheophytin a. Although previous experience suggests that *m/z* 278 is the loss of a C18:3 chain, in this case the standard hydrocarbon chain is a good fit. When calculating possible molecular formula using the mass spectrometer vendor's software (Excalibur by Thermo), the loss is calculated as losing $C_{20}H_{38}$. This calculation also includes a ppm match for the possible formula that could account for the mass lost. In this case the ppm match is 0.335. This is extremely low and provides good confidence to the molecular formula. Using the same tool, the secondary loss of *m/z* 60

gives a formula of $C_2H_4O_2$ with a ppm match of 4.676 ppm which is just inside acceptable error of 5 ppm.

With the above information the peak at *m/z* 871.57 in positive ionisation mode can be tentatively annotated as pheophytin a. As with all mass spectral structural elucidations, without a chemical standard, assignments made can only be tentative. To confirm a tentative annotation requires further investigation but tentative annotations are widely published due to the robust nature of mass spectral data. Pheophytin a is a good fit for the peak. The main points to consider are: it is not present in animal plasma samples, it is highly abundant in grass lipid extracts due to its role in PS II, the fragments fit perfectly with probable losses when investigating the structure and molecular formula generated for both parent and daughter ions are within acceptable error range.

Another major piece of evidence for the annotation of pheophytin a at *m/z* 871.57 was found during a literature search on the compound. As outlined below in the following section, Goericke et al. (2000) performed LCMS on numerous chlorophyll derivatives and similar compounds. Below (figure 3.10) is the MS2 for *m/z* 871 found in their paper compared to our experimental results. The correlation between the two spectra gives good confidence to the annotation assigned. (Goericke et al., 2000)

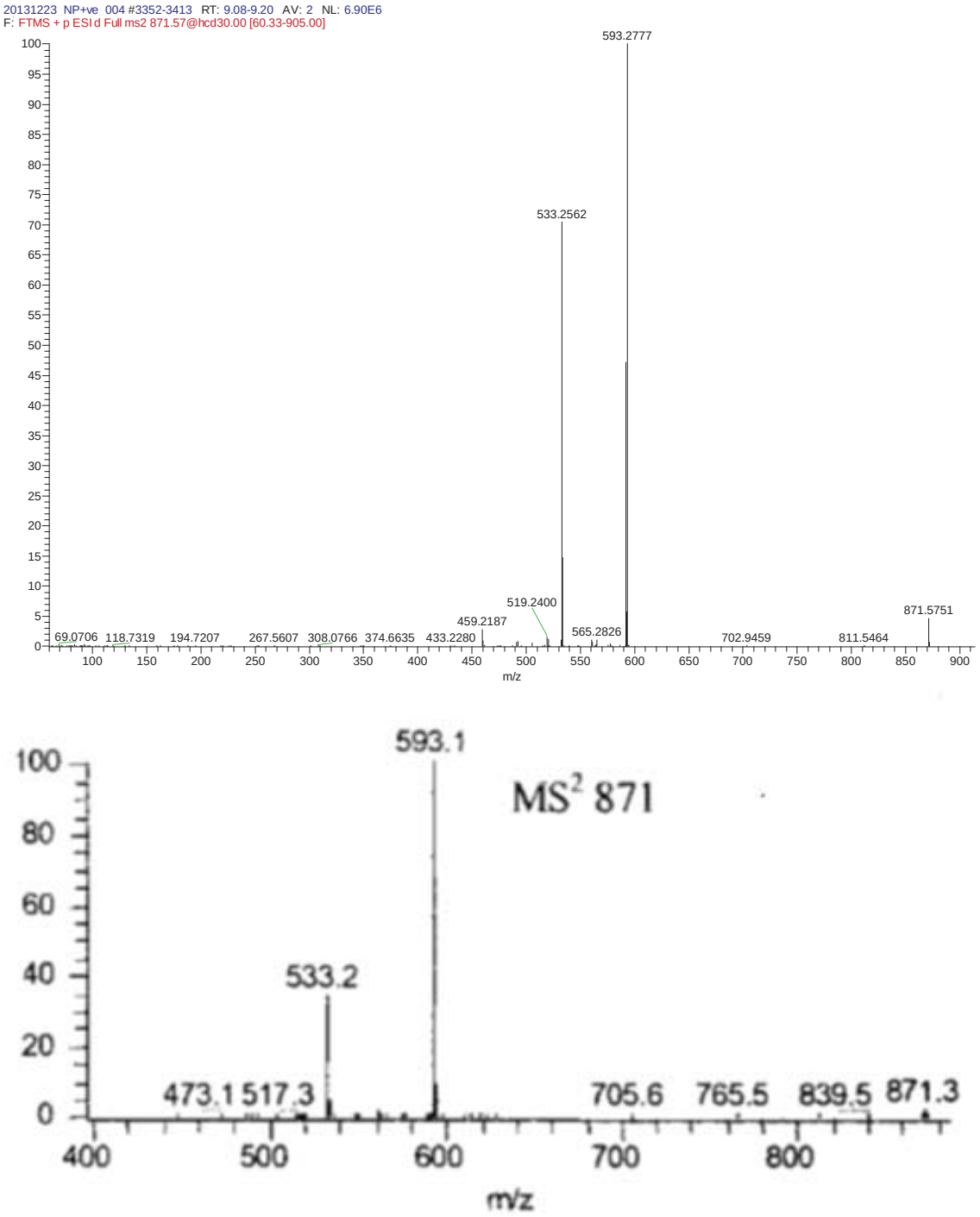

Figure 3.10 – Comparison of the MS2 fragmentation of mass *m/z* 871.57 experimental (top) and found in literature (Goericke et al., 2000) (bottom).

3.4.7 Investigation of pheophytin a like structures

Upon further investigation, pheophytin a is in a class of compounds called cyclic pheophorbides. The major other cyclic pheophorbide of interest is chlorophyll (a and b). Due to the green colour of the lipid extract of rye grass it is expected that chlorophyll is present and in high concentration. As seen in Figure 3.11 below, pheophytin a and chlorophyll a are very similar. The major difference seen in the structures is the bound magnesium metal in the active site of chlorophyll a. Besides this one major difference, the compounds are identical. (Goericke, Strom, & Bell, 2000)

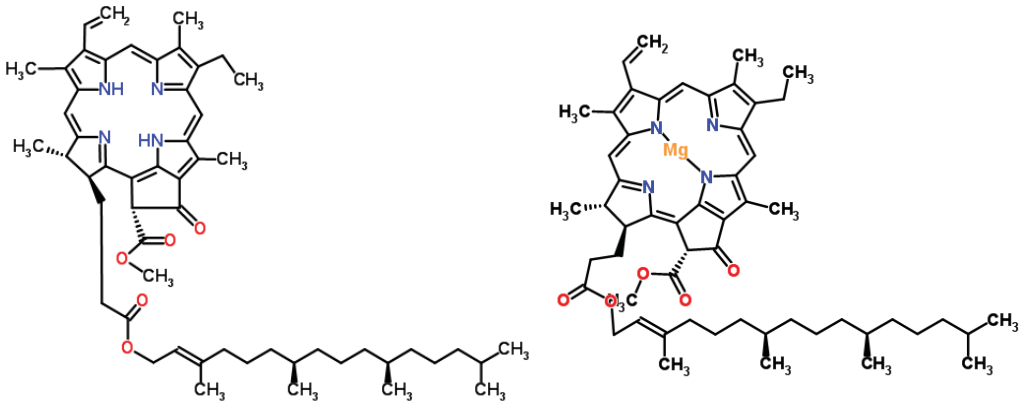

Figure 3.11 – Comparison of pheophorbides pheophytin a (left) and chlorophyll a (right). (Royal Society of Chemistry, 2014a and 2014b)

With this structural information an educated guess was formed about where we would observe chlorophyll a in the chromatogram (assuming chlorophyll a is present). We would expect chlorophyll a to be in close proximity to pheophytin a but would also expect to see it elute off the column slightly earlier. This is due to two things, chlorophyll a is slightly bulkier due to the magnesium atom so would expect it to not bind to the stationery phase as tightly and has also lost its active site in the middle of the ring.

As we know the mass of chlorophyll a (approximately 892.54) the approach to identification (or presence or absence) is in essence backwards to the elucidation of the pheophytin a peak. The first step was to use the software provided with the mass spectrometer (Excalibur by Thermo) to perform an extracted ion chromatogram (EIC) using *m/z* 893.54 (plus one mass unit due to being in positive ionisation mode) so that only peaks containing 893.54 *m/z* are shown. From here the MS2 spectra was studied of the most abundant peak in the EIC which was seen at 8.07 minutes. The first observation was that the MS2 spectra has the same two major losses as pheophytin a. These are the loss of the chain and then subsequent loss of $C_2H_4O_2$ as seen in the MS2 spectra below (Figure 3.12).

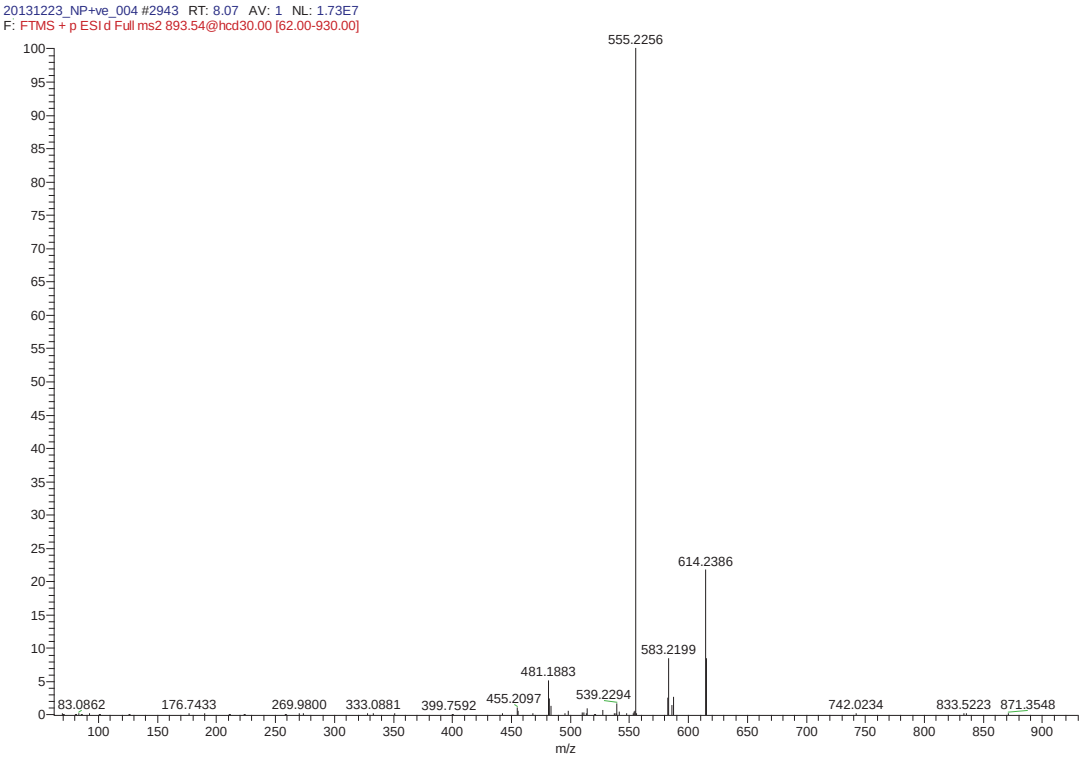

Figure 3.12 – MS2 spectra of mass *m/z* 893.54, tentatively annotated as chlorophyll a showing the familiar fragmentation pattern as that of *m/z* 871.57 but shifted to a higher mass.

Through tentatively confirming the presence of chlorophyll a the next step was to also investigate chlorophyll b. Chlorophyll b only differs from chlorophyll a by the addition of an aldehyde in b. Upon investigating chlorophyll b using the same steps as with a, chlorophyll b was also tentatively confirmed as being present. This is the peak at 6.93 minutes with a mass of *m/z* 907.52 in the positive ionisation mode LCMS data. Chlorophyll a and b are two of the main pigments in plant cells so it is no surprise (upon further reflection) that the dark green lipid extracts using the Folch extraction method were abundant in this class of compounds. The other pheophorbides outlined by Goericke et al. (2000) were all investigated but unfortunately no others were found.

However, this is not entirely unexpected, as the paper is focussed on pheophorbides in marine environments.

4 Conclusion

Lipidomics is currently an area of great interest for many research groups. The information that lipidomics can provide is vital for advancement in many areas of science. With the continuing advancement in mass spectrometer technology and the continuing demand for increased efficiency and productivity, lipidomics will continue to grow and advance also. There are however, two main downfalls for traditional LCMS based lipidomics; these are the length of time it takes to run each sample (approximately 30 minutes) and the large amount of data produced by each sample. These two downfalls are both very important when time is an issue as not only does it take a long time to run the samples, the data processing required is also time consuming. There is a definite need to alleviate these issues.

The main aim for this study was to develop a quick and effective screening tool to investigate the lipids in perennial ryegrass. With this aim, the stresses of instrumental analysis time and data processing time were targeted. By implementing a shotgun approach the instrumental analysis time was minimised by approximately ten times, however the data processing was much more time consuming. Where other groups had succeeded in developing shotgun methods for investigating the lipidome of animal samples, this study has not delivered a viable option compared to LCMS for perennial ryegrass. This is most probably due to one major factor, the presence of chlorophyll and chlorophyll derivatives (mainly pheophytin a). It is the most prominent difference between animal and plant samples and is the most likely source of the majority of the

issues that have surfaced during the course of this study. Chlorophylls make shotgun delivery impractical due to confusion caused by mass overlap and significant ion suppression effects.

Positive outcomes from this study include the positive, albeit tentative, annotation of 118 LCMS peaks total over both positive and negative ionisation modes and the tentative annotation of the major peak at *m/z* 871.57 of pheophytin a. This tentative annotation was a good step in the right direction towards creating a shotgun approach for studying the lipidome of perennial ryegrass. With this information, further studies can be conducted focussing on mitigating the negative effects of pheophytin a and other chlorophyll derivatives on lipid annotation attempts. One approach may be to isolate the lipids from the chlorophyll and chlorophyll derivatives but this approach may be too time consuming to be worthwhile. Another approach could be to utilise computing power to eliminate the nuisance peaks to just leave spectra containing the targeted lipids. Although this would create a better visual depiction of the lipids within perennial ryegrass it would not eliminate the matrix effects caused by the chlorophyll and chlorophyll derivatives so we may still see ion suppression effects.

While a shotgun lipidomics methodology for perennial ryegrass is still some way off being fully developed, this study has further advanced the understanding of both the lipid profile of perennial ryegrass and the chlorophyll products that are co-extracted with conventional lipid extraction techniques.

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Appendix 1 – LipidSearch results

Positive mode results including confirmation status

Note: ObzMz is observed *m/z*, Rt is retention time and confirmation is by hand (those not definitively 'Yes' or 'No' were classed as possible.

LipidIon	Formula	ObsMz	Rt	Confirmed?
MG(18:3)+H	C21 H37 O4	353.2694	2.505367	Yes
MG(18:3)+NH4	C21 H40 O4 N1	370.2964	2.492936	Possible
LPC(16:0)+H	C24 H51 O7 N1 P1	496.3411	2.203464	Yes
Cer(d16:0/16:0)+H	C32 H66 O3 N1	512.5048	5.5967	Yes
LPC(18:3)+H	C26 H49 O7 N1 P1	518.3258	1.287936	Yes
LPC(18:2)+H	C26 H51 O7 N1 P1	520.3411	1.325804	Yes
LPC(18:0)+H	C26 H55 O7 N1 P1	524.3721	3.272256	Yes
Cer(d18:0/16:0)+H	C34 H70 O3 N1	540.5364	6.326827	Yes
Cer(d16:0/18:0)+H	C34 H70 O3 N1	540.5364	6.453381	Yes
DG(16:0/14:0)+NH4	C33 H68 O5 N1	558.5108	7.927891	Yes
DG(15:0/18:2)+H	C36 H67 O5	579.4993	5.116153	No
DG(14:0/18:3)+NH4	C35 H66 O5 N1	580.4946	6.807703	Yes
DG(16:0/16:0)+NH4	C35 H72 O5 N1	586.5422	8.657949	Yes
TG(6:0/12:0/14:0)+NH4	C35 H70 O6 N1	600.5206	8.230286	Yes
DG(16:1/18:3)+NH4	C37 H68 O5 N1	606.5104	7.26497	Yes
DG(16:0/18:3)+NH4	C37 H70 O5 N1	608.5265	7.677787	Yes
DG(16:0/18:2)+NH4	C37 H72 O5 N1	610.5411	8.109663	Yes
DG(16:0/18:1)+NH4	C37 H74 O5 N1	612.5568	8.718707	Yes
DG(18:3/18:3)+H	C39 H65 O5	613.4833	5.165831	Yes
TG(6:0/12:0/15:0)+NH4	C36 H72 O6 N1	614.5355	5.101441	No
DG(18:0/16:0)+NH4	C37 H76 O5 N1	614.5733	9.383826	Yes
DG(18:3/18:2)+H	C39 H67 O5	615.4998	7.0724	Yes
DG(18:4/18:3)+NH4	C39 H66 O5 N1	628.4962	6.231382	Possible
TG(6:0/14:0/14:0)+NH4	C37 H74 O6 N1	628.5517	8.932158	Yes
TG(4:0/14:0/16:0)+NH4	C37 H74 O6 N1	628.5524	6.997518	Yes
DG(18:3/18:3)+NH4	C39 H68 O5 N1	630.511	6.586332	Yes
DG(18:3/18:2)+NH4	C39 H70 O5 N1	632.5266	7.009657	Yes
TG(6:0/14:0/14:0)+Na	C37 H70 O6 Na1	633.5078	8.962064	Yes
DG(18:2/18:2)+NH4	C39 H72 O5 N1	634.542	7.566929	Yes
DG(18:1/18:3)+NH4	C39 H72 O5 N1	634.5422	7.695154	Yes
DG(18:3/18:3)+Na	C39 H64 O5 Na1	635.4665	6.600413	Yes
DG(18:1/18:2)+NH4	C39 H74 O5 N1	636.5567	8.187826	Yes
DG(18:0/18:3)+NH4	C39 H74 O5 N1	636.5571	8.383218	Yes
DG(18:3/18:2)+Na	C39 H66 O5 Na1	637.4811	7.017204	No

DG(18:1/18:1)+NH4	C39 H76 O5 N1	638.5727	8.778327	Yes
DG(18:0/18:2)+NH4	C39 H76 O5 N1	638.573	8.90201	Yes
DG(18:0/18:1)+NH4	C39 H78 O5 N1	640.5891	9.467289	Yes
TG(6:0/14:0/15:0)+NH4	C38 H76 O6 N1	642.5682	5.108988	No
DG(18:0/18:0)+NH4	C39 H80 O5 N1	642.6048	10.04731	Yes
TG(12:0/12:0/12:0)+NH4	C39 H78 O6 N1	656.5827	9.503313	Yes
TG(6:0/14:0/16:0)+NH4	C39 H78 O6 N1	656.5833	9.678605	Yes
PE(16:0/18:3)+H	C39 H73 O8 N1 P1	714.5079	6.413665	Yes
PE(16:0/16:0)+Na	C37 H74 O8 N1 P1 Na1	714.5079	6.282787	Yes
PE(16:0/18:2)+H	C39 H75 O8 N1 P1	716.5233	6.952747	Yes
PC(10:0/22:3)+H	C40 H75 O8 N1 P1	728.5241	5.379382	Yes
PE(16:1/18:2)+Na	C39 H72 O8 N1 P1 Na1	736.4893	6.358643	No
PE(18:3/18:3)+H	C41 H71 O8 N1 P1	736.4918	5.265977	Yes
PE(18:3/18:2)+H	C41 H73 O8 N1 P1	738.5089	5.768994	Yes
PG(16:0/16:1)+NH4	C38 H77 O10 N1 P1	738.5298	6.448459	Yes
PE(18:2/18:2)+H	C41 H75 O8 N1 P1	740.5239	6.298348	No
PE(16:0/20:4)+H	C41 H75 O8 N1 P1	740.5241	6.421245	No
PG(16:0/16:0)+NH4	C38 H79 O10 N1 P1	740.5441	6.697253	Yes
PE(18:1/18:2)+H	C41 H77 O8 N1 P1	742.5389	6.938366	No
PC(15:1/18:2)+H	C41 H77 O8 N1 P1	742.5395	5.747991	No
PG(16:1/18:3)+H	C40 H72 O10 N0 P1	743.4868	5.464237	Yes
TG(15:0/14:0/14:0)+NH4	C46 H92 O6 N1	754.6942	11.50242	No
PC(16:1/18:2)+H	C42 H79 O8 N1 P1	756.5555	6.179759	Yes
PC(16:0/18:2)+H	C42 H81 O8 N1 P1	758.5708	6.612931	Yes
PG(16:1/18:3)+NH4	C40 H75 O10 N1 P1	760.513	5.447357	Yes
PC(16:0/18:1)+H	C42 H83 O8 N1 P1	760.5864	7.241842	Yes
PG(16:0/18:3)+NH4	C40 H77 O10 N1 P1	762.5297	5.728224	Yes
PG(16:1/18:2)+NH4	C40 H77 O10 N1 P1	762.5303	5.919768	Yes
PG(16:0/18:2)+NH4	C40 H79 O10 N1 P1	764.5446	6.150042	Yes
PG(16:1/18:2)+Na	C40 H73 O10 N0 P1 Na1	767.4847	5.704052	No
TG(16:0/14:0/14:0)+NH4	C47 H94 O6 N1	768.7085	11.76775	Yes
TG(15:0/14:0/15:0)+NH4	C47 H94 O6 N1	768.7092	11.64169	No
CerG1(d18:2/20:0+O)+H	C44 H84 O9 N1	770.6154	7.64158	Possible
MGDG(18:3/18:3)+H	C45 H75 O10	775.5329	6.660392	Yes
PC(16:1/20:5)+H	C44 H77 O8 N1 P1	778.5395	5.195145	Yes
PC(16:1/20:4)+H	C44 H79 O8 N1 P1	780.5549	5.686896	Yes
TG(16:1/14:0/15:0)+NH4	C48 H94 O6 N1	780.7091	11.61166	No
PG(18:4/18:3)+NH4	C42 H73 O10 N1 P1	782.4975	4.651116	Yes
PC(16:0/20:4)+H	C44 H81 O8 N1 P1	782.5706	6.208805	Yes
TG(16:0/14:0/15:0)+NH4	C48 H96 O6 N1	782.7236	11.88953	No
PG(18:3/18:3)+NH4	C42 H75 O10 N1 P1	784.5092	4.808877	Yes
PC(18:1/18:2)+H	C44 H83 O8 N1 P1	784.5854	6.683003	Yes
PC(18:0/18:2)+H	C44 H85 O8 N1 P1	786.6013	7.44829	Yes
TG(16:1/14:0/16:1)+NH4	C49 H94 O6 N1	792.7099	11.45471	Yes
TG(16:1/15:0/15:0)+NH4	C49 H96 O6 N1	794.7237	11.65655	No
TG(16:0/14:0/16:1)+NH4	C49 H96 O6 N1	794.7252	11.78257	Yes

MGDG(18:3/20:6)+H	C47 H73 O10	797.5186	5.716024	Yes
TG(16:1/15:0/16:1)+NH4	C50 H96 O6 N1	806.7252	11.69153	No
TG(16:0/15:0/16:1)+NH4	C50 H98 O6 N1	808.741	11.94788	No
TG(16:1/16:1/16:1)+NH4	C51 H96 O6 N1	818.7251	11.44246	Yes
TG(16:0/16:1/16:1)+NH4	C51 H98 O6 N1	820.7406	11.87177	Yes
TG(16:1/14:0/18:1)+NH4	C51 H98 O6 N1	820.7409	11.74864	Yes
TG(16:1/15:1/18:1)+NH4	C52 H98 O6 N1	832.7401	11.68396	No
TG(16:1/15:0/18:1)+NH4	C52 H100 O6 N1	834.7554	11.96997	No
PI(16:0/18:2)+H	C43 H80 O13 N0 P1	835.535	6.007574	Yes
TG(14:0/18:2/18:3)+NH4	C53 H96 O6 N1	842.723	11.0465	Yes
TG(16:0/16:1/18:3)+NH4	C53 H98 O6 N1	844.7409	11.44974	Yes
TG(16:1/16:1/18:1)+NH4	C53 H100 O6 N1	846.7558	11.80787	Yes
TG(16:0/16:0/18:3)+NH4	C53 H100 O6 N1	846.7561	11.93772	Yes
PI(16:0/18:3)+NH4	C43 H81 O13 N1 P1	850.5463	5.247166	Yes
PI(16:0/18:2)+NH4	C43 H83 O13 N1 P1	852.5615	5.968398	Yes
TG(15:1/18:3/18:3)+NH4	C54 H94 O6 N1	852.7087	10.52821	No
TG(15:1/18:2/18:3)+NH4	C54 H96 O6 N1	854.7256	10.91513	No
TG(15:1/18:2/18:2)+NH4	C54 H98 O6 N1	856.7389	11.31115	No
TG(16:0/17:1/18:3)+NH4	C54 H100 O6 N1	858.7568	11.71643	No
TG(16:1/18:3/18:3)+NH4	C55 H96 O6 N1	866.7243	10.93474	Yes
TG(16:1/18:2/18:3)+NH4	C55 H98 O6 N1	868.7385	11.31611	No
TG(16:0/18:3/18:3)+NH4	C55 H98 O6 N1	868.7403	11.1929	Yes
TG(16:0/18:2/18:3)+NH4	C55 H100 O6 N1	870.7569	11.61894	Yes
PI(18:3/18:3)+NH4	C45 H79 O13 N1 P1	872.5291	4.643567	Yes
TG(16:0/18:2/18:2)+NH4	C55 H102 O6 N1	872.772	11.83495	Yes
TG(16:0/18:1/18:3)+NH4	C55 H102 O6 N1	872.7725	11.96242	Yes
PI(16:1/20:4)+NH4	C45 H81 O13 N1 P1	874.5451	5.080684	Yes
TG(16:0/18:2/18:3)+Na	C55 H96 O6 Na1	875.7128	11.50737	Yes
TG(16:0/18:2/18:2)+Na	C55 H98 O6 Na1	877.7256	11.88691	Yes
TG(17:0/18:3/18:3)+NH4	C56 H100 O6 N1	882.7554	11.43491	No
TG(18:3/18:3/18:3)+NH4	C57 H96 O6 N1	890.7253	10.45368	Yes
TG(18:3/18:2/18:3)+NH4	C57 H98 O6 N1	892.7392	10.85594	Yes
TG(18:1/18:3/18:3)+NH4	C57 H100 O6 N1	894.7545	11.23786	Yes
TG(18:3/18:2/18:2)+NH4	C57 H100 O6 N1	894.7548	11.11516	Yes
TG(18:0/18:3/18:3)+NH4	C57 H102 O6 N1	896.7718	11.62391	Yes
TG(18:2/18:2/18:2)+NH4	C57 H102 O6 N1	896.7721	11.48493	Yes
TG(18:3/18:2/18:3)+Na	C57 H94 O6 Na1	897.6952	10.73952	Yes
TG(18:0/18:2/18:3)+NH4	C57 H104 O6 N1	898.7879	11.95979	Yes
TG(18:3/18:2/18:2)+Na	C57 H96 O6 Na1	899.7111	11.13004	Yes
TG(18:2/18:2/18:2)+Na	C57 H98 O6 Na1	901.7262	11.49979	Yes
TG(18:0/18:3/18:3)+Na	C57 H98 O6 Na1	901.7273	11.65392	Yes
PS(29:4/18:3)+Na	C53 H90 O10 N1 P1 Na1	954.6165	5.237259	No

Negative ionisation mode results

LipidIon	Formula	ObsMz	Rt	Confirmed?
MGMG(18:3)+HCOO	C28 H47 O11	559.3128	1.548598	Yes
PA(16:0/18:3)-H	C37 H66 O8 N0 P1	669.4487	7.974789	Yes
PA(18:3/18:3)-H	C39 H64 O8 N0 P1	691.4345	6.127729	Yes
PA(18:3/18:2)-H	C39 H66 O8 N0 P1	693.4497	6.66509	Yes
PE(16:0/18:3)-H	C39 H71 O8 N1 P1	712.4932	6.300083	Yes
PE(16:0/18:2)-H	C39 H73 O8 N1 P1	714.5081	6.832043	Yes
PE(18:3/18:3)-H	C41 H69 O8 N1 P1	734.4775	5.294164	Yes
PE(18:3/18:2)-H	C41 H71 O8 N1 P1	736.4941	5.786317	Yes
PE(18:2/18:2)-H	C41 H73 O8 N1 P1	738.5085	6.32632	Yes
PG(16:1/18:3)-H	C40 H70 O10 N0 P1	741.4713	5.499097	Yes
PG(16:0/18:3)-H	C40 H72 O10 N0 P1	743.4878	5.747612	Yes
PG(16:0/18:2)-H	C40 H74 O10 N0 P1	745.5034	6.146373	Yes
PG(18:3/22:5)-H	C46 H74 O10 N0 P1	817.5024	5.451331	No
MGDG(18:3/18:3)+HCOO	C46 H75 O12	819.5277	5.704712	Yes
MGDG(18:2/18:3)+HCOO	C46 H77 O12	821.5429	6.138632	Yes
PS(21:2/18:3)-H	C45 H77 O10 N1 P1	822.5292	5.122124	No
PS(21:1/18:2)-H	C45 H81 O10 N1 P1	826.5605	6.130898	No
PI(16:0/18:3)-H	C43 H76 O13 N0 P1	831.5044	5.556991	Yes
PI(16:0/18:2)-H	C43 H78 O13 N0 P1	833.5202	6.054707	Yes
PI(18:3/18:3)-H	C45 H74 O13 N0 P1	853.4867	4.614749	Yes
DGDG(16:0/18:3)+HCOO	C50 H87 O17	959.5959	6.208965	Yes
DGDG(16:0/18:2)+HCOO	C50 H89 O17	961.6119	6.650632	Yes
DGDG(18:3/18:3)+HCOO	C52 H85 O17	981.5787	5.216979	Yes
DGDG(18:2/18:3)+HCOO	C52 H87 O17	983.5949	5.621351	Yes