The effect of exercise and diet on rat skeletal muscle phospholipid molecular species profile: an electrospray ionisation mass spectrometric analysis

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THE EFFECT OF EXERCISE AND DIET ON RAT SKELETAL MUSCLE PHOSPHOLIPID MOLECULAR SPECIES PROFILE: AN ELECTROSPRAY IONISATION MASS SPECTROMETRIC ANALYSIS

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Declaration

I, Todd W. Mitchell declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biomedical Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Todd W Mitchell

6th October 2004

Dedication

This thesis is dedicated to my parents and my wife Tracy for their endless love and support and to my son Zane whose strength and determination to survive after a very early arrival has inspired me to realise my goals without losing sight of what is most important.

Abstract

Biological membranes separate cells from the external milieu and compartmentalise organelles within a cell, providing a specialised environment for many specific biochemical processes. They exist as bilayers of amphipathic lipids arranged with their hydrophobic moieties internalised and their hydrophilic regions directed to the membrane surfaces. Numerous proteins are also associated with membranes and are bound to the lipids by ionic or hydrophobic interactions. Phospholipids, however, are the major constituent of biological membranes and thus have a large influence upon the physical properties of the membrane and the many cellular functions membranes participate in.

To date our understanding of membrane lipid composition has been limited to phospholipid class or fatty acid analysis, primarily by thin layer chromatography, high performance liquid chromatography and gas chromatography. The results obtained by these techniques provide considerable evidence demonstrating an association between various metabolic disorders, such as insulin resistance and obesity and skeletal muscle phospholipid content. There is also a large pool of evidence confirming an effect of diet and exercise on the phospholipid fatty acid content of skeletal muscle membranes. Furthermore, these changes appear to have ameliorating effects upon the aforementioned metabolic disorders. An understanding of alterations in whole phospholipid molecular species induced by exercise and diet, however, is very limited. Recent advances in mass spectrometry allow the analysis of biological membranes at this whole molecule level.

In this thesis, a comparative analysis of skeletal muscle phospholipid molecular species profile between oxidative and glycolytic rat skeletal muscle and the effect of exercise and diet on these profiles have been performed using electrospray ionisation mass spectrometry (ESI-MS). Therefore, the primary aim of this thesis was to develop mass spectrometric techniques for analysing relative changes in phospholipid molecular species profile using a hybrid quadrupole time-of–flight (Q-ToF) mass spectrometer. To achieve this both total lipid and phospholipid extracts from various rat tissues such as brain, liver and skeletal muscle were obtained and used to (i) optimise instrument settings, (ii) ensure accurate identification of phospholipid molecular species, and (iii) ensure the

reproducibility of results. A normalisation procedure was then developed so that comparative analysis between groups could be performed. This was achieved by presenting the ion abundance of each phospholipid molecular species (after isotope corrections) as a percentage of the total ion abundance of all identified phospholipids within the *m/z* range analysed. The results obtained by the developed MS method were then compared to those attained by established GC methods and found to be in agreement, thus demonstrating the validity of the technique.

The methodology thus established was used to determine the effect of two exercise training intensities on the phospholipid profile of both glycolytic and oxidative muscle fibres of female Sprague-Dawley rats fed a standard laboratory chow diet. Animals were divided randomly into three training groups: control, which performed no exercise training; low intensity (8 m min^{-1}) treadmill running; or high intensity (28 m min^{-1}) treadmill running. All exercise-trained rats ran 1000 m $session^{-1}$, 4 days wk^{-1} for 4 wks and were killed 48 h after the last training bout. Exercise training was found to produce no novel phospholipid species but was associated with significant alterations in the relative abundance of a number of phospholipid molecular species. These changes were more prominent in glycolytic (white vastus lateralis) than in oxidative (red vastus lateralis) muscle fibres. The largest observed change was a decrease of approximately 20 % in the abundance of 1-stearoyl-2-docosahexaenoyl phosphatidylethanolamine [PE(18:0,22:6), $P<0.001$] ions in both the low and high intensity training regimes in glycolytic fibres. Increases in the abundance of 1-oleoyl-2-linoleoyl phopshatidic acid [PA(18:1,18:2), $P<0.001$] and 1-alkenylpalmitoyl-2-linoleoyl phosphatidylethanolamine [Plasmenyl PE(16:0,18:2), $P<0.005$] ions were also observed for both training regimes in glycolytic fibres.

The same exercise protocol was then performed by Sprague-Dawley rats fed a carbohydrate-free, high-fat diet and the skeletal muscle phospholipid molecular species profiles analysed. In agreement with the previous study, no novel molecular species were observed in the exercised rats yet significant changes in the relative abundance of various phospholipid molecular species were apparent. In contrast, however, the observed changes were more prominent in oxidative than glycolytic muscle fibres. The largest effect of exercise was found to be an increase of approximately 28 % in 1-

palmitoyl-2-linoleoyl phosphatidylcholine [PC(16:0,18:2), $P<0.05$] ions in oxidative muscle of rats in the low intensity training group when compared to the sedentary animals.

The phospholipid molecular species profile was found to be similar in both the oxidative and glycolytic muscles, however, a number of differences in the abundance of particular molecular species were observed. Of particular interest is the higher abundance of PE(18:0,22:6) in red vastus lateralis when compared to white vastus lateralis. In spite of the fact that the high-fat diet was completely deficient in n-3 polyunsaturated fatty acids the ratio of PE(18:0,22:6) in oxidative to glycolytic muscle was almost identical across both diet groups. For example, in the sedentary rats this ratio was 1.35 for the carbohydrate diet group and 1.32 for the fat diet group.

It is concluded that exercise training results in a significant level of membrane remodelling at the level of phospholipid molecular species and that traditional methods used to analyse phospholipids such as TLC and GC are not able to uncover these changes. Moreover, it is probable that the observed changes will have effects upon the activity of various membrane bound proteins and in turn cell function. At present an understanding of the role specific phospholipid molecular species play in membrane function is extremely limited and further correlative and manipulative studies are required to remedy this. It is likely that electrospray ionisation mass spectrometry will play a significant role in these future studies.

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thank my beautiful wife Tracy who has supported me financially and emotionally during my university career. You have sacrificed much for me over the eight years of our marriage and I could not have done it without you.

ABBREVIATIONS

ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionisation
BMR	Basal metabolic rate
CAD	Collision-activated dissociation
CI	Chemical ionisation
CID	Collision-induced dissociation
CRM	Charge residue model
DEFA	Deficient in essential fatty acids
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DPG	Diphosphatidylglycerol (Cardiolipin)
ED_{50}	Half-maximal glucose disposal
EDL	Extensor digitorum longus
EI	Electron ionisation
EPA	Eicosapentaenoic acid
ESI	Electrospray ionisation
ESI-MS	Electrospray ionisation mass spectrometry
FA	Fatty acid
FAB	Fast atom bombardment
GC	Gas chromatography
GLUT	Glucose transporter
HIGH	High intensity exercise training
HPLC	High performance liquid chromatography
IEM	Ion evaporation model
LC/MS	Liquid chromatography mass spectrometry
LOW	Low intensity exercise training
LSIMS	Liquid secondary ion mass spectrometry
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionisation
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MUFA	Monounsaturated fatty acid
oaToF	Orthogonal acceleration time-of-flight
PA	Phosphatic acid

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PS	Phosphatidylserine
PSD	Post source decay
PUFA	Polyunsaturated fatty acid
Q-ToF	Quadrupole time-of-flight
RF	Radio frequency
RVL	Red vastus lateralis
SED	Sedentary
SEM	Standard error of the mean
SFA	Saturated fatty acid
SIMS	Secondary ion mass spectrometry
SM	Sphingomyelin
TAG	Triacylglycerol
TLC	Thin layer chromatography
ToF	Time-of-flight
VO_{2max}	Maximal oxygen uptake
WVL	White vastus lateralis

PUBLICATIONS ARISING FROM WORK IN THIS THESIS TO DATE

Journal Articles

Turner N, Lee JS, Bruce CR, **Mitchell TW**, Else PL, Hulbert AJ & Hawley JA (2004) Greater effect of diet than exercise training on the fatty acid profile of rat skeletal muscle. *Journal of Applied Physiology* 96, 974-980.

Mitchell TW, Turner N, Hulbert AJ, Else PL, Hawley JA, Lee JS, Bruce CR & Blanksby SJ (2004) Exercise alters the profile of phospholipid molecular species in rat skeletal muscle. *Journal of Applied Physiology* 97, in press.

Abstracts

Morrissey B, Thomas MC, **Mitchell TW**, Ung AT, Pine SG & Blanksby SJ (2004) Negative ion phospholipid fragmentation: a mechanistic and regiochemical study. *Proc. LII American Society for Mass Spectrometry (Nashville).* ThPI - 148.

Mitchell TW, Blanksby SJ, Turner N, Else PL & Hulbert AJ (2003) Electrospray ionization mass spectrometric analysis of skeletal muscle lipid species: A powerful new tool in the exercise sciences. *Proc. Exercise, Muscle and Metabolism (Melbourne)*. p 49.

Mitchell TW, Hick LA, Blanksby SJ, Sheil MM, Turner N, Else PL & Hulbert AJ (2003) ESI-MS analysis of skeletal muscle phospholipids from sedentary and exercised rats. *Proc. XIX Australian and New Zealand Society for Mass Spectrometry (Lorne)*. p 106.

Turner N, Lee JS, Hawley JA, Blanksby SJ, Else PL, Hulbert AJ & **Mitchell TW** (2003) Effect of exercise on the fatty acid profile and molecular species composition of rat skeletal muscle phospholipids. *Proc. Exercise, Muscle and Metabolism (Melbourne)*. p 55.

Chapter 1

Membrane Phospholipids

1.1 The Structure of Biological Membranes

Biological membranes are structures that display both diverse structure and function. They separate cells from the external milieu and compartmentalise organelles within a cell, providing a specialised environment for many specific biochemical processes. Membranes exist as bilayers of amphipathic lipids arranged with their hydrophobic moieties internalised and their hydrophilic regions directed to the membrane surfaces. Numerous proteins are also associated with membranes and are bound to the lipids by ionic or hydrophobic interactions (Figure 1-1) (Gurr *et al.*, 2002). The mass ratio of lipid to protein in rat membranes varies from 3:1 in myelin to 1:3 in mitochondria and erythrocytes (Gurr *et al.*, 2002). Proteins may be bound to the surface of the membrane, peripheral proteins, or embedded in the membrane, integral proteins (Stryer, 1995) and perform a variety of functions. They may for example, function as ion channels, ATP-dependent or ATP-independent ion pumps, cell surface receptors, enzymes and energy transducers (Stryer, 1995). There is also a large diversity of lipid molecular species found in biological membranes. They can, however, be divided into three major classes; phospholipids, glycolipids and sterols. This thesis will focus on the most abundant of these membrane lipids, namely phospholipids.

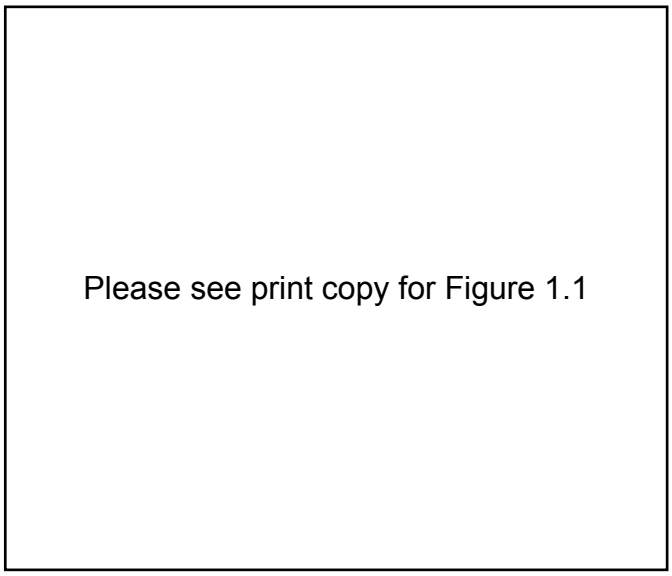


Figure 1-1: ***Model of a biological membrane.***
Taken from Purves et al. (1995)

1.1.1 Phospholipids

Phospholipids are the major constituent of biological membranes and thus have a large influence upon the physical properties of the membrane and the many cellular functions membranes participate in. There are two types of membrane phospholipids, the most common are phosphoglycerides, derived from glycerol and the other are sphingomyelins (SM), derived from sphingosine. The general structures of these two lipid classes are shown in Figure 1-2.

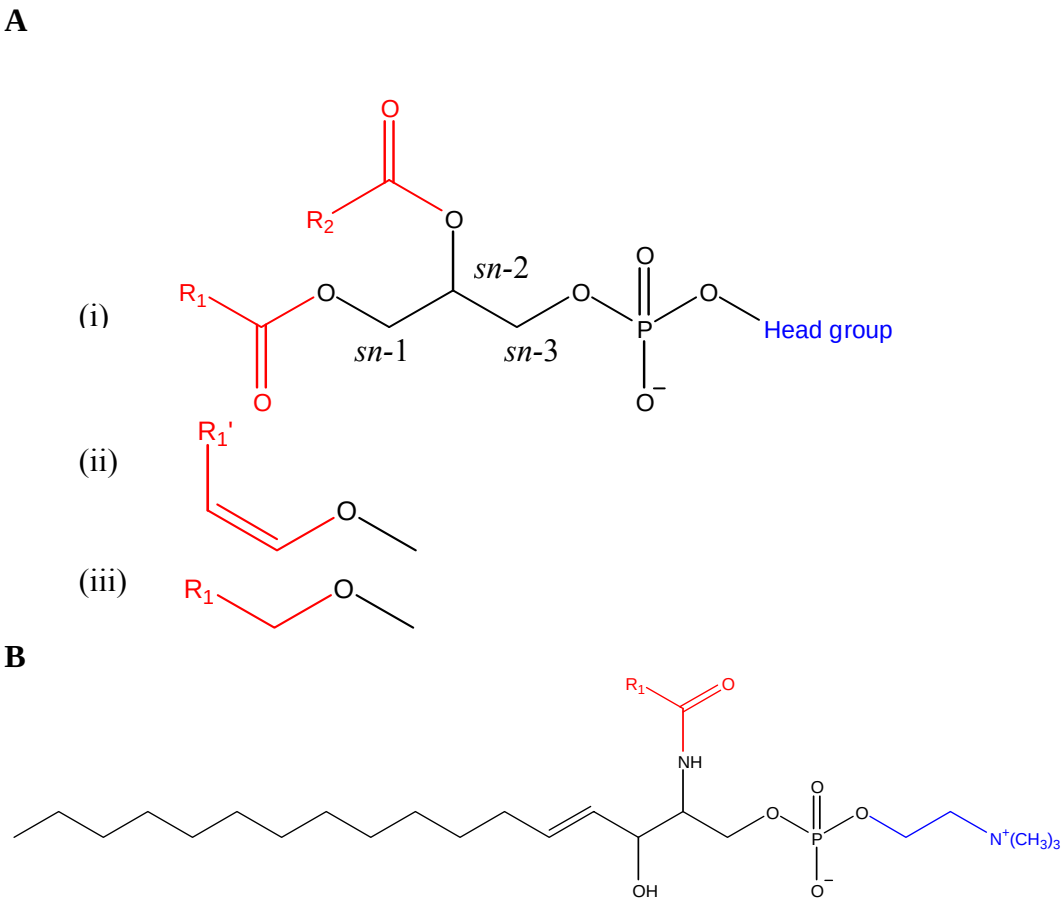


Figure 1-2: ***The general structure of (A) a phosphoglyceride showing the three types of bonds found at the sn-1 position (i) ester, (ii) vinyl ether (plasmalogen), (iii) alkyl ether and (B) a sphingomyelin.***

Phosphoglycerides contain a glycerol backbone to which are bound two fatty acids (FAs) at the *sn*-1 and *sn*-2 positions and a phosphate ester head group bound to the *sn*-3 position.[1] As shown in Figure 1-2 A, FAs may be bound by an ester, vinyl ether (plasmalogen species) or alkyl ether (ether species) linkage at the *sn*-1 position while the *sn*-2 linkage is always an ester (Pulfer & Murphy, 2003). Sphingomyelin consists of a sphingosine backbone with one FA attached by an amide linkage and a phosphate choline head group (Figure 1-2B). Sphingomyelin species are very similar in structure to phosphoglyceride molecules that contain a choline head group, as seen in Figure 1-3.

Phosphoglycerides can be further classified according to their unique head group; small organic bases, functionalised alcohols or amino acids bound to the phospholipid as phosphate esters. The structures of the five identified head groups, serine, ethanolamine, glycerol, inositol and choline are displayed in Figure 1-3. A sixth possible classification is phosphatidic acid where hydrogen is attached to the phosphate.

[1] *sn* refers to *stereospecifically numbered*, *i.e. sn*-1 = the first carbon atom in the glycerol backbone, by convention the phosphate ester is at the *sn*-3 position. This should not be confused with S_N1 and S_N2 reaction types from the lexicon of organic chemistry

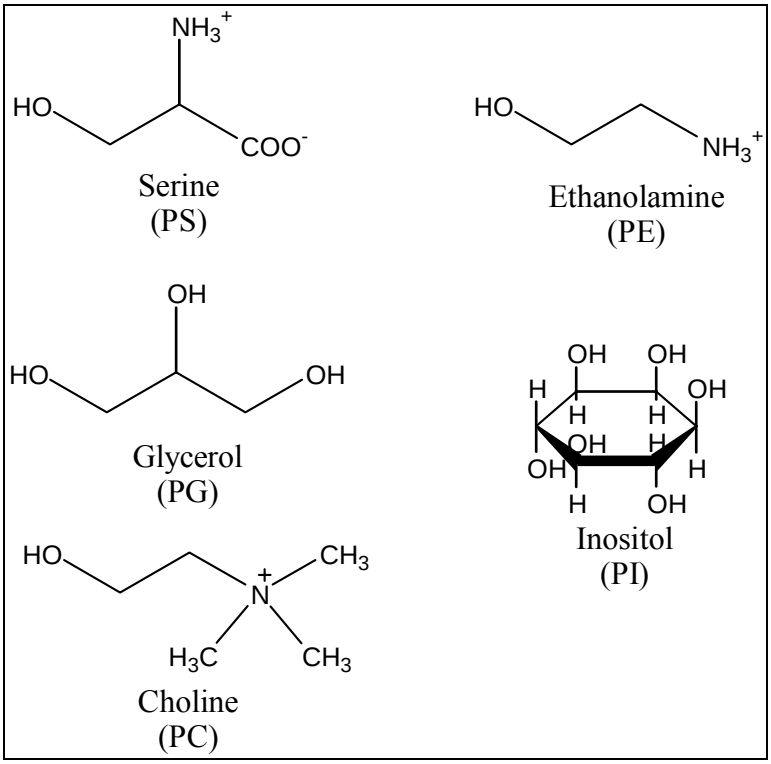


Figure 1-3: ***Phospholipid head groups***
The common abbreviation used to describe the phospholipid class associated with each head group is shown in brackets

Fatty acids are long chain carboxylic acids that have a number of major physiological roles. FAs are the building blocks for phospholipids and glycolipids, they are covalently attached to proteins for trafficking, act as hormones and intracellular messengers and are high energy fuel molecules (Stryer, 1995). The average chain length of FAs in vertebrate membranes is around 18 carbons and generally varies between 16 and 22 carbon atoms long (Hulbert & Else, 1999). The nomenclature of fatty acids refers to the number of carbons in the acyl chain followed by the number of double bonds, *i.e.* stearic acid, an 18 carbon saturated FA, is 18:0 and the polyunsaturated arachidonic acid, 20 carbons with 4 double bonds, is 20:4. The structures of these two FAs are displayed in Figure 1-4.

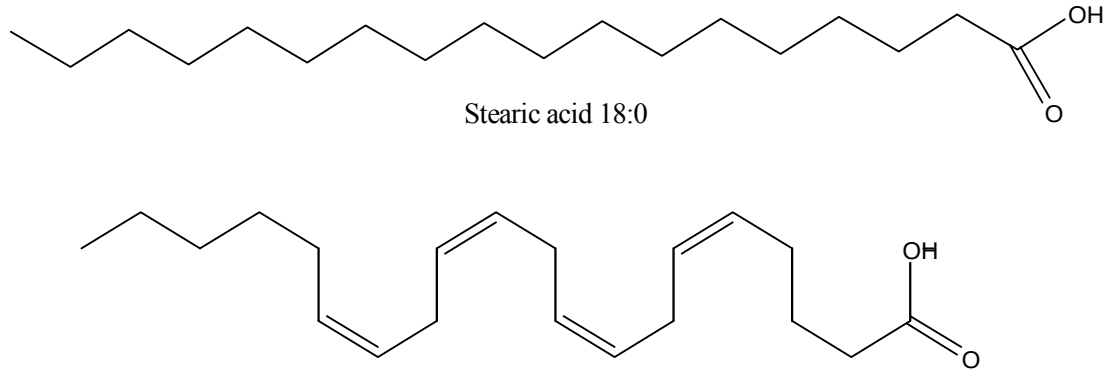


Figure 1-4: ***Structure and nomenclature of two common fatty acids, stearic acid (18:0) and arachidonic acid (20:4 n-6)***

FAs that contain solely single bonds between carbon atoms are referred to as saturated fatty acids (SFAs). Such an arrangement allows free rotation around each C-C bond resulting in a large number of possible conformations with an average structure resembling an extended straight chain. An alternative arrangement is for some carbon atoms to share electrons in double bonds. In eukaryotic membranes, FAs can have between one and six double bonds between carbon atoms, predominantly in the *cis* or Z configuration. Fatty acids with one double bond are termed monounsaturated fatty acids (MUFAs) and those with between two and six double bonds polyunsaturated fatty acids (PUFAs). The natural occurrence of *cis* rather than *trans* double bonds has a significant impact upon the structure of unsaturated FAs as they not only restrict motion around the C=C bond they also produce a kink in the acyl chain. This has an impact upon the packing of phospholipids in the membrane because they occupy a larger volume. The *cis* forms also have a lower intermolecular packing efficiency than the *trans* forms resulting in a melting point considerably lower than both saturated FAs and those containing *trans* double bonds. A more common form of isomerism found in unsaturated FAs is positional isomers, owing to different locations of double bonds. There are two methods used to

classify the position of the first double bond in the acyl chain. The first uses a Δ symbol to indicate its position from the carboxyl end and the second uses an n symbol to indicate its position from the methyl end. Arachidonic acid therefore may be expressed as either 20:4 Δ-5 or 20:4 n-6. In the nomenclature used to describe FAs the second notation is also used to classify unsaturated FAs into subtypes, namely n-9, n-7, n-6 and n-3.[2] This nomenclature, *i.e.* n-, will be used throughout this thesis. In the case of both n-6 and n-3 PUFAs, their presence in the vertebrate membranes is largely controlled by their supply in the diet (Hulbert *et al.*, 2004), as they cannot be synthesised *de novo* due to the lack of desaturase enzymes capable of removing hydrogens at these positions. Consequently these FAs are termed essential fatty acids. Some of the FA species commonly found in vertebrates and their various nomenclatures are listed in Table 1-1.

[2] This nomenclature replaces the old Greek system, *e.g.* ω-3 etc., which is still commonly used by some sections of the scientific community, food manufacturers and the media.

Table 1-1: ***Naturally occurring fatty acids commonly found in vertebrates***

Common name	Systematic name	Carbon chain length: No double bonds	FA class
Saturated acids			
Palmitic acid	Hexadecanoic acid	16:0	
Stearic acid	Octadecanoic acid	18:0	
Monounsaturated acids			
Oleic acid	9-Octadecanoic acid	18:1	n-9
Vaccenic acid	11- Octadecanoic acid	18:1	n-7
Polyunsaturated acids			
Linoleic acid	9,12-Octadecadienoic acid	18:2	n-6
γ-Linolenic acid	6,9,12-Octadecatrienoic acid	18:3	n-6
α-Linolenic acid	9,12,15-Octadecatrienoic acid	18:3	n-3
Arachidonic acid	5,8,11,14-Eicosatetraenoic acid	20:4	n-6
EPA[a] or Timnodonic acid	5,8,11,14,17-Eicosapentaenoic acid	20:5	n-3
DPA[a] or Clupanodonic acid	7,10,13,16,19-Docosapentaenoic acid	22:5	n-3
DHA[a] or Cervonic acid	4,7,10,13,16,19-Docosahexaenoic acid	22:6	n-3

[a]the more commonly used name.

With the possible combinations of the 6 different head groups and up to 20 different FAs, as well as sphingomyelin species, the phospholipid classification includes a vast number of molecules. Additionally, the phospholipid cardiolipin (or diphosphatidylglycerol, DPG Figure 1-4) involves the combination of a phosphatidylglycerol and phosphatidic acid molecule. Cardiolipin is a molecule found in great abundance in many bacteria but in eukaryotes is restricted to the inner mitochondrial membrane (Gurr *et al.*, 2002).

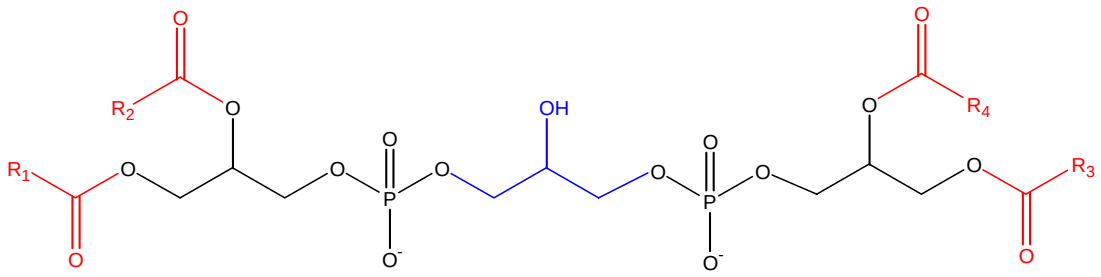


Figure 1-5: ***The general structure of cardiolipin***

In the scientific literature the nomenclature used to describe phospholipids may vary slightly from one source to another. In this thesis, phospholipid molecular species will be described by the two-letter acronym for the head group followed by the two FA chains (number of carbons: number of double bonds) in brackets. For example, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphatidylethanolamine will be expressed as PE(18:0,22:6)

1.1.2 The Fluid Membrane Bilayer

The first bilayer model of the membrane was proposed early in the last century after the surface area of extracted membrane lipid from a known number of erythrocytes was found to be twice that of the total cell surface area (Gorter & Grendel, 1925). Although this experiment did not correctly allow for packing density or include proteins,

fortuitously these two inaccuracies cancelled each other out allowing this data to form the concept of the lipid bilayer. In the early fifties (Davson & Danielli, 1952) and again independently in the mid sixties (Robertson, 1964) models of cell membranes were proposed where the phospholipid bilayer was described as being surrounded encased on both the exofacial and cytofacial surfaces by a layer of unfolded proteins (Figure 1-5a). This lipid sandwich model was soon extended to include models that suggested proteins were interspersed and intertwined with the phospholipids forming lipoprotein subunits (Figure 1-5b) (Benson, 1966) or discrete molecules partially embedded in or completely spanning the membrane (Figure 1-5c)(Lenard & Singer, 1966; Wallach & Zahler, 1966). The latter model, termed the 'lipid-protein mosaic model' was expanded upon until, in 1972 the 'fluid mosaic model' of cell membranes was described by Singer and Nicholson (Singer & Nicolson, 1972) (see Figure 1-1). Although this model has undergone further elaboration, it still serves as the basis for our current understanding of the structural assembly of cellular membranes.

Please see print copy for Figure 1.6

Figure 1-6: ***Schematic diagrams of the three membrane models proposed by 1966.***
(A) The Davson-Danielli-Robertson model surrounded by a monolayer of unfolded proteins. (B) The Benson model of lipoprotein subunits of intertwined proteins and phospholipids. (C) The Lenard-Singer-Wallach model of a lipid-protein mosaic. Taken from Singer (2004)

The fluidity, or inversely, the viscosity of biological membranes under standard environmental conditions is dependent on a number of factors. These include; (i) rotation around C-C bonds, (ii) movents throughout the hydrocarbon chain, (iii) lateral diffusion within the bilayer, and (iv) oscillations of the phospholipids between 'tall and thin' and 'short and wide' conformations (Robertson, 1983). Each of these factors is directly influenced by the number of carbon atoms and degree of unsaturation present in the FA chains. For example, DHA (22: 6, n-3) with six *cis* double bonds and 22 carbon atoms has an almost infinite number of structural configurations (Gawrisch *et al.*, 2003) that, on average, occupy a greater area of the membrane than a saturated FA (Feller *et al.*, 2002) and therefore reduces the lateral packing of membrane phospholipids (Cribier *et al.*, 1993).

An interesting feature of native phosphoglycerides is the distribution of saturated and unsaturated FAs. Convention dictates that an unsaturated FA is predominantly bound to the *sn*-2 position and a saturated FA is bound to the *sn*-1 position, however, the reverse may also occur (Stubbs & Smith, 1984). One possible explanation for this coupling of an unsaturated with a saturated FA is that it could prevent uncontrolled phase separation within the membrane. This may occur when areas of the membrane high in saturated FAs have a solid or gel state at mammalian body temperatures and other areas of the membrane containing more unsaturated FAs remain in a liquid-crystalline state (Stubbs & Smith, 1984; Hulbert & Else, 1999). There is a mounting body of evidence, however, to suggest that regulated phase separation may be an important functional requirement of naturally occurring membranes (for review see Brown & London, 1998). These gel phase areas of membrane, termed lipid rafts, however, are formed by the accumulation of lipid species with higher melting points such as cholesterol and sphingomyelin rather than the control of phospholipid regiochemistry at the *sn*-1 and *sn*-2 FAs (Simons & Ikonen, 1997).

Another important structural feature of biological membrane lipids is that they display a distinct compositional asymmetry across the bilayer. Studies, conducted predominantly on human erythrocytes have indicated a preference for PE, PS, PA and PI in the cytofacial leaflet and the choline containing phospholipids, PC and SM in the exofacial leaflet (Rothman & Lenard, 1977; Daleke, 2003). Such an arrangement has important structural significance, accommodating the difference in the radius of curvature required for each leaflet (Bergelson & Barsukov, 1977). It is also functionally significant as particular phospholipids involved in intracellular metabolic signalling pathways such as PI are preferentially localised in the cytofacial leaflet (Gurr *et al.*, 2002). Indeed, this asymmetry is so tightly regulated that the appearance of PS in the exofacial leaflet is known to target the cell for apoptotic removal (Fadok *et al.*, 1992) and initiates blood coagulation in platelets (Lentz, 2003).

1.1.3 Regulation of Membrane Phospholipid Composition

Mammalian glycerophospholipids are continually synthesised, remodelled, transported between intracellular organelles and catabolised by a range of enzymes working in concert. Work by Schmid *et al.* (1995) demonstrated that only four molecular species (16:0/18:2, 16:0/18:1, 16:0/22:6 and 18:1/18:2) of both PE and PC are synthesised *de novo* in rat hepatocytes. This is only a fraction of the PE and PC species that are present in the rat liver (Ramanadham *et al.*, 1998; DeLong *et al.*, 2001) with the remainder produced by remodelling of these four species (Schmid *et al.*, 1995). The remodelling of these species by the deacylation/reacylation of phospholipids is performed by phospholipases, acyltransferases and transacylases (Farooqui *et al.*, 2000). These processes are rapid as indicated by experiments where cultured cells were incubated with labelled FAs resulting in their incorporation into phospholipids within 2-10 minutes of their addition (Chakravarthy *et al.*, 1986).

Desaturase and elongase enzymes also act to remodel the acyl chains of membrane phospholipids (Stubbs & Smith, 1984). The desaturases are membrane-bound enzymes that have a higher specificity for FAs with double bonds closer to the methyl end of the chain, *i.e.* n-3 > n-6 > n-9 (Stubbs & Smith, 1984). Their activity is linked to membrane fluidity so that a decrease in fluidity triggers an increase in the production of MUFAs and PUFAs (Kates *et al.*, 1984). As mentioned in Section 1.1.1, n-3 and n-6 FAs must be obtained from external sources as vertebrates can only synthesise MUFAs *de novo*. Nevertheless, once obtained these FAs are rapidly incorporated and then remodelled by successive steps of elongation and desaturation to supply long chain n-3 and n-6 PUFAs (Gurr *et al.*, 2002).

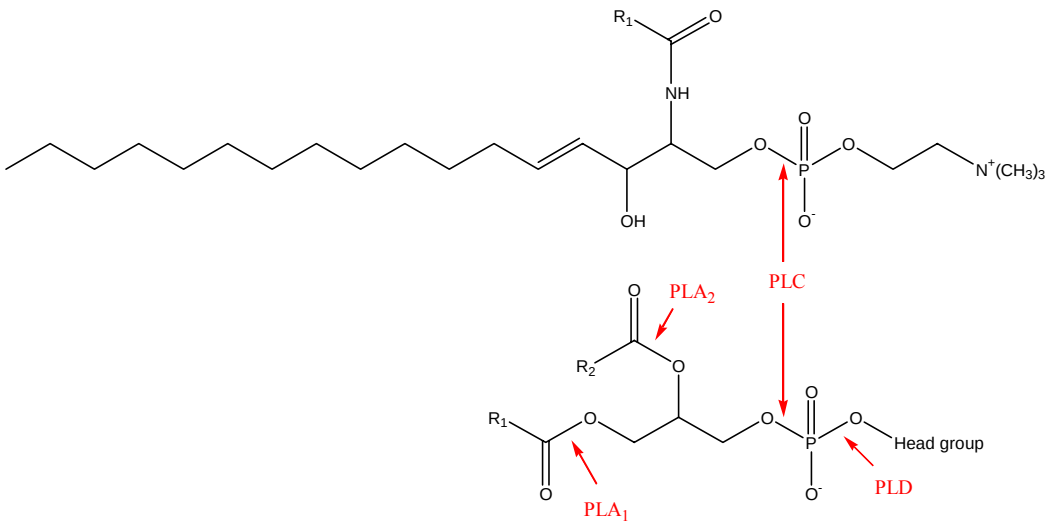


Figure 1-7: ***Specificity of phospholipases***

Phospholipases are a group of enzymes that cleave phospholipids at specific sites (Figure 1-7) and therefore play a significant role in determining membrane lipid composition. Phospholipases A (PLA) cleave the two FAs esterified to the glycerol backbone. There are two main groups of PLA based on the position of the FA they cleave, namely PLA_1 and PLA_2. PLA_1 cleaves FAs from the *sn*-1 position and in animals is found

predominantly in lysozymes while PLA_2 is more ubiquitous and deacylates at the *sn*-2 position (Gurr *et al.*, 2002). Three groups of phospholipase C (PLC) also participate in the regulation of membrane lipids by cleaving the phosophodiester bond at the *sn*-3 position of PI and PC forming 1,2-diacylglycerol or the equivalent position of SM species forming ceramide (Farooqui *et al.*, 2000; Gurr *et al.*, 2002). Phospholipase D (PLD) also cleaves the head group from glycerophospholipids to produce PA (Farooqui *et al.*, 2000; Gurr *et al.*, 2002). While the phospholipase enzymes affect membrane phospholipid composition, their main role is likely the production of molecules involved in various signal pathways (Gurr *et al.*, 2002).

The other major group of enzymes involved in the regulation of membrane phospholipid composition are those involved in the maintenance of bilayer asymmetry. There are three groups of enzymes involved in this regulation. They are; (i) cytofacially-directed, ATP-dependant 'flippases', (ii) exofacially-directed, ATP-dependant 'floppases', and (iii) bi-directional, ATP-independent 'scramblases' (Daleke, 2003). The most abundant flippase, aminophospholipid flippase, has a high affinity for PS and acts to keep these species sequestered from the cell surface as its appearance can act as a signal for various events such as platelet aggregation (Lentz, 2003) and apoptosis (Fadok *et al.*, 1992). The floppase ABCA1 is known to transport cholesterol to the cell surface for collection by high density lipoproteins (Daleke, 2003) and has been linked to disorders of cholesterol metabolism (Brooks-Wilson *et al.*, 1999). It is also involved in the transfer of PS to the exofacial leaflet during apoptosis (Hamon *et al.*, 2000; Hamon *et al.*, 2002). Another floppase, ABCB1 is selective for PC and helps to maintain its abundance in the exofacial membrane leaflet (Van Helvoort *et al.*, 1996). In contrast to the flippases and floppases, scramblases are relatively non-specific and they appear to be involved in the redistribution of newly synthesised lipids in the ER and Golgi membranes (Daleke, 2003).

1.2 Membrane Function

The most recognised function of biological membranes is their role in the partitioning of compartments to form cells and their membrane bound organelles. They provide an impermeable barrier for the passage of hydrophilic substances allowing their regulated uptake and release. Three decades ago data was published describing a 75% loss in glucose uptake by human erythrocytes and a decrease in the activity of the membrane bound Na^+K^+ATPase when PLA_2 was used to cleave *sn*-2 fatty acyl groups (Kahlenberg & Banjo, 1972). This experiment clearly demonstrated that biological membrane lipids are actively involved in influencing the activities of major biological processes.

Since that time it has been established that alterations in membrane phospholipid composition and cholesterol content can alter the properties of membranes and affect many cellular functions. They include, carrier mediated transport, properties of membrane-bound enzymes, receptor binding, phagocytosis, endocytosis and exocytosis, immunologic and chemotherapeutic cytotoxicity, prostaglandin production, and cell growth (Spector & Yorek, 1985). Phospholipids have also been shown to be important participants in the activation of reactions associated with blood coagulation (Hanahan & Nelson, 1984). Moreover, there is an increasing body of evidence that membrane lipid composition is linked to metabolic rate.

1.2.1 Membranes and Metabolic Rate

The rate of energy turnover associated with the maintenance of body processes and the performance of work constitutes the metabolic rate of an animal. The common measure of metabolic rate is termed basal metabolic rate (BMR) and is defined as the energy expenditure of a postabsorptive, resting adult in a thermoneutral environment. The BMR therefore, is a measure of energy required to maintain normal body function. Work conducted by Hulbert and Else over the last two decades has indicated a strong link

between membrane FA composition and basal metabolic rate (BMR). This finding has been based upon extensive comparative physiological and biochemical studies using both endothermic (mammals and birds) and ectothermic (reptiles, amphibians and fish) vertebrates. Substantial variations in metabolic rate have been demonstrated in comparisons between ectotherms and endotherms (Hulbert, 1980), during development (Bastin *et al.*, 1988; Else, 1991) and with body size, where up to a 100-fold difference in the rate of mass-specific metabolism is observed (Kleiber, 1961). In spite of these large differences in metabolic rate, it appears that the percentage contribution of various processes to BMR is constant (Else & Hulbert, 1987; Brand *et al.*, 1991; Porter & Brand, 1993; Couture & Hulbert, 1995b). A recent review by Rolfe and Brown suggests that this contribution is not only constant but is also dominated by a few processes that consume the majority of resting energy expenditure, as shown in Table 1-2 (Rolfe & Brown, 1997). Taken together these findings indicate that metabolism is composed of a series of linked processes that vary in concert to determine BMR. This suggests that a common factor may be influencing the activity of all these processes. Of interest is the fact that most of the major energy-consuming processes are directly (*e.g.*, the proton leak-pump cycle, sodium and calcium pumps) or indirectly (*e.g.*, the movement of amino acids into the cell for protein synthesis) associated with membranes.

Table 1-2: ***Contribution of energy consuming processes to basal metabolic rate***

Please see print copy for Table 1.2

BMR, basal metabolic rate

Adapted from Rolfe & Brown (1997)

Comparative studies of animals possessing a broad range of BMR have also uncovered a concurrent variation in phospholipid FA composition. This relationship between BMR and phospholipid FAs has been reported in comparisons between ectotherms and endotherms (Hulbert & Else, 1989; Brand *et al.*, 1991; Else & Wu, 1999; Wu *et al.*, 2001) and in allometric comparisons using various tissues (Couture & Hulbert, 1995a; Hulbert *et al.*, 2002) and mitochondria (Porter *et al.*, 1996; Portero-Otin *et al.*, 2001; Brand *et al.*, 2003). These comparisons suggest that animals with higher metabolic rates have increased levels of membrane PUFAs, particularly 22:6 n-3 and decreased levels of MUFAs than do their metabolically sluggish counterparts. Striking evidence for this link between BMR and membrane lipid composition was recently reported (Wu *et al.*, 2004). In this study, sodium pumps from crocodile kidney microsomes where delipidated and twice reconstituted with cattle membrane lipids. The cattle membrane contained a 4-fold greater concentration of n-3 PUFAs and a reduction in the level of n-6 PUFAs (29 % cattle vs. 43 % crocodile) with no significant difference in SFAs or MUFAs. Following this procedure the activity of the crocodile sodium pumps increased from 729 to 908 ATP min^{-1} ($P < 0.01$) after the first reconstitution and further increased to 1476 ATP min^{-1} ($P =$

0.01) after the second reconstitution. The compilation of these findings has led to the hypothesis that membranes act as pacemakers of metabolism, actuated through their influence on the major energy-consuming processes that constitute BMR (Hulbert & Else, 1999; Else & Hulbert, 2003).

There are a number of physiological and environmental factors associated with changes in the composition and function of biological membranes. They may be pathophysiological, such as obesity and diabetes or normal physiological events such as exercise and diet.

1.2.2 Membranes and Metabolic Disorders

Considerable evidence exists linking changes in membrane phospholipid classes and FA content to various pathological conditions, such as schizophrenia (Horrobin, 1999), depression (Bruinsma & Taren, 2000), immunological dysfunction (Cunningham-Rundles, 2003; Kew *et al.*, 2003) and cardiovascular disease (Dewailly *et al.*, 2001; Dewailly *et al.*, 2002) to name a few. Nonetheless, this discussion will focus upon the link between membrane phospholipid FA composition and insulin sensitivity, particularly in skeletal muscle, as this tissue is the major site of insulin stimulated glucose uptake (DeFronzo *et al.*, 1981) and the focus of this thesis.

There are a number of peer-reviewed research papers and review articles discussing the link between phospholipid FA content and metabolic disorders typified by the metabolic syndrome. This syndrome, also known as syndrome 'X' or insulin resistance syndrome is a cluster of disease states that include hypertension, atherogenic dislipideamia, low-grade chronic inflammation, a high propensity for thrombus formation, type 2 diabetes and abdominal obesity (Grundy *et al.*, 2004; Hulbert *et al.*, 2004). While there is still debate over the causative aspect of the metabolic syndrome, resistance to insulin and its effects on carbohydrate and lipid metabolism appears to be a key factor (Reaven, 1988; Ferrannini *et al.*, 1991; Hulbert *et al.*, 2004).

Investigations by Zeghari and co-workers (Zeghari *et al.*, 2000) found that adipocyte membrane sphingomyelin content is a predictor of fasting insulin levels and is strongly associated with insulin resistance in obese women. Similar results have also been observed for erythrocyte membranes along with increased levels of total PE species in subjects with impaired glucose tolerance (Candiloros *et al.*, 1996). In addition, the link between membrane phospholipid fatty acid composition and diabetes is not limited to type 2 and insulin resistance. Data published by Ruiz-Guitierrez and co-workers reveals a reduction in PUFAs, especially 22:6 n-3 and 20:4 n-6, in erythrocyte membranes taken from sufferers of type 1 (insulin-dependent) diabetes (Ruiz-Gutierrez *et al.*, 1993). They also found that 22:6 n-3 and total n-3 content had a significant positive association with sodium transport and other metabolic markers, suggesting reductions in these FAs may also be related to poor metabolic control in type 2 diabetes.

The association between skeletal muscle membrane FAs and insulin resistance, seen in type 2 (non-insulin dependent) diabetes is well established in both rodents and humans (Borkman *et al.*, 1993; Vessby *et al.*, 1994; Pan *et al.*, 1995; Helge *et al.*, 1998b; Storlien *et al.*, 1998). In summary, the higher the levels of saturated FAs the poorer the insulin action. Conversely, a greater proportion of long chain PUFAs, particularly n-3s is associated with a higher level of insulin sensitivity. A study on animal liver and skeletal muscle membranes found a diet rich in saturated FAs had a reduced degree of membrane unsaturation in both of these tissues, owing mostly to reductions in 22:6 n-3, and double the concentration of plasma insulin compared to animals fed a diet rich in unsaturates (Loizou *et al.*, 2001).

It is possible that n-3 PUFAs and insulin resistance are linked through the influence of these PUFAs on insulin receptors. A recent study on dietary fat-induced insulin-resistant rats suggests that n-3 PUFAs can restore the level of insulin receptor phosphorylation in response to insulin stimulation to normal levels (Taouis *et al.*, 2002). Other studies have also found that increased levels of membrane n-3 PUFAs are beneficial to insulin receptor

function in both skeletal muscle (Sohal *et al.*, 1992) and adipocytes (Clandinin *et al.*, 1993). Conversely, evidence exists to suggest that increased levels of skeletal muscle n-6 fatty acids is linked to poor insulin binding (Peliknova *et al.*, 1989) and reduced insulin receptor phosphorylation (Taouis *et al.*, 2002). Of interest is the finding that some insulin sensitising drugs such as bezafibrate are known to increase the level of membrane unsaturation (Matsui *et al.*, 1997) suggesting a secondary effect on the insulin receptor through manipulation of membrane lipid content. Clore *et al* (1998) have also reported correlations between insulin sensitivity and the FA composition of PC but not PE in vastus lateralis of healthy humans. This suggests that the combination of particular FAs and headgroups, *i.e.*, phospholipid molecular species, may be important determinants of insulin sensitivity.

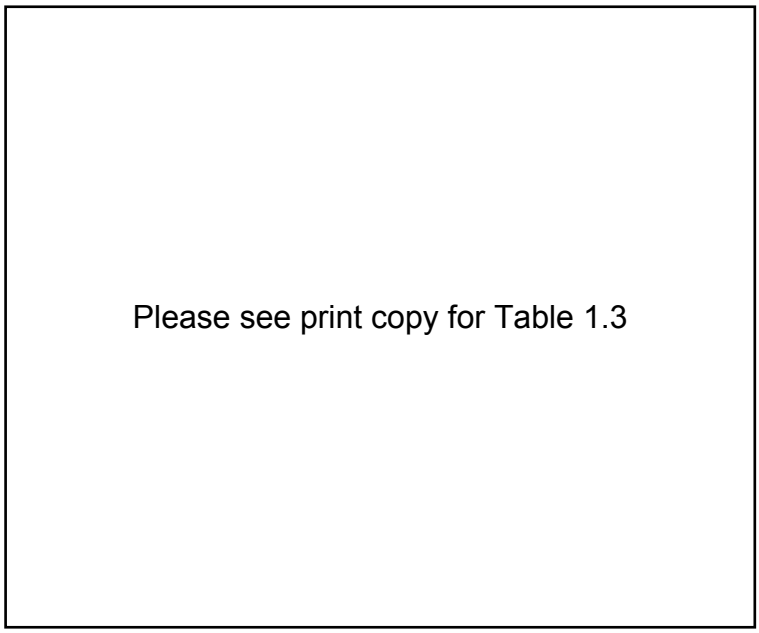
1.3 Skeletal Muscle Phospholipids

In 1964, Masoro and colleagues at the University of Washington separated phospholipid classes by column and thin layer chromatography and reported that PC accounted for more than 50 % and PE ~ 25 % of monkey skeletal muscle phospholipids (Masoro *et al.*, 1964). The first insights into membrane phospholipid FA composition of skeletal muscle came in the early 1970's when Bruce & Svennerholm (1971) analysed PC from human gastrocnemius muscle. Their data demonstrated a large proportion (~ 40 %) of the PC fatty acid content to be linoleic acid (18:2 n-6). A number of subsequent studies have shown this trend to be consistent for total human skeletal muscle phospholipid FA composition (see Table 1-2). Bruce also published data in 1974 describing the abundance of different phospholipid headgroup classes in human gastrocnemius muscle that supported the findings of Masoro (Bruce, 1974). The most abundant glycerophospholipid class was found to be PC (47.4 %) followed by PE (24.1 %), PI (8.9%) and PS (3.3%).

Table 1-3 shows the FA composition of human (red and white vastus lateralis) and rat (red and white vastus lateralis, soleus and extensor digitorum longus) skeletal muscle. The

values have been calculated from a compilation of data available from four human studies (Borkman *et al.*, 1993; Andersson *et al.*, 1998; Andersson *et al.*, 2000; Helge *et al.*, 2001) and four rat studies (Kriketos *et al.*, 1995; Ayre *et al.*, 1998; Helge *et al.*, 1999; Turner *et al.*, 2004). The most abundant FA observed in human skeletal muscle is linoleic acid (18:2 n-6) with large contributions by the saturated FAs palmitic acid (16:0) and stearic acid (18:0). The n-6 PUFA arachidonic acid (20:4 n-6) is also found in relatively high abundance. The largest differences in the rat skeletal muscle when compared to humans are the approximately four-fold increase in DHA (22:6, n-3) and two-fold decrease in linoleic acid (18:2, n-6). Even when rats are fed a diet completely deficient in n-3 FAs, the percentage of skeletal muscle 22:6 n-3 remains much higher than the human average shown in Table 1-2 (Turner *et al.*, 2004). As a consequence of these differences the ratio of n-6 to n-3 FAs is ~ 8 in humans compared to ~ 2 in rats. This is in spite of the fact that the total percentage of PUFAs is similar in both species. While there are a number of variables that should be considered in this compilation of data, *e.g.*, genetic, dietary, muscle and fibre type and FA isolation techniques, the difference between the two species is large and consistent.

Table 1-3: ***Phospholipid fatty acid composition of skeletal muscle***



Fatty acid composition of human (red and white vastus lateralis) and rat (red and white vastus lateralis, soleus and extensor digitorum longus) skeletal muscle. Values are a compilation of mean data from four human (Borkman et al., 1993; Andersson et al., 1998; Andersson et al., 2000; Helge et al., 2001) and four rat (Kriketos et al., 1995; Ayre et al., 1998; Helge et al., 1999; Turner et al., 2004) studies and are presented as mean ± S.E.M. FA, fatty acid

Studies have also revealed differences in phospholipid FA composition between muscle fibre types in the rat. Work by Kriketos *et al.* (1995) demonstrated differences in the FA profile between muscle fibre types, with the insulin sensitive type I and type IIa fibres containing a greater proportion of long-chain PUFAs, in particular 22:6 n-3, than type IIb fibres. The data also demonstrate a greater incorporation of stearic acid and a lower incorporation of palmitic acid into the membranes of type I and IIa fibres compared to type IIb fibres. A later study by Blackard *et al.* (1997) found a similar trend in regard to the stearic and palmitic acid concentrations but did not find the difference in 22:6 n-3. This inconsistency may be explained by the different percentage of fibre types in the muscles analysed by the two studies. The Kriketos study compared white quadriceps

(>86% type IIb fibres) to soleus (~87% type I fibres) and red quadriceps (> 62% type I and IIa fibres) while the Blackard study compared extensor digitorum longus (EDL, 59% type IIb and 38% type IIa fibres) to soleus. Owing to the relatively high levels of type IIa fibres, EDL has a high concentration of DHA compared to white quadriceps and therefore may not have shown the significant difference observed between EDL and soleus muscles. Two key environmental factors that have been found to impact upon the phospholipid FA profile of skeletal muscle are exercise and diet.

1.3.1 Exercise and Skeletal Muscle Phospholipid FA Composition

A recent study examining the response of skeletal muscle membranes to endurance training reported alterations in the concentration of phospholipid head group classes. Gorski *et al.* (1999) described increases in total PE and cardiolipin in red gastrocnemius, PI and SM in white gastrocnemius and PI in diaphragm muscle of rats after 7 wk of treadmill running.

There is also considerable data for both rodents and humans indicating that short-term exercise training (4 - 6 wk) has the ability to alter the phospholipid FA composition of skeletal muscle. In rats, the largest effects appear to be decreases in the content of both the major long-chain PUFAs, *i.e.* 22:6 n-3 and 20:4 n-6 and a concurrent increase in the major short-chain PUFA, *i.e.*18:2 n-6 (Kriketos *et al.*, 1995; Ayre *et al.*, 1998; Helge *et al.*, 1999). In contrast, increases in 22:6 n-3 and total long-chain PUFAs are commonly observed in human studies (Andersson *et al.*, 1998; Andersson *et al.*, 2000; Helge *et al.*, 2001; Helge & Dela, 2003).

Work on rats has shown a small exercise-induced decrease in the level of 22:6 n-3 in soleus muscle but no significant differences in the phospholipid FA composition of EDL (Kriketos *et al.*, 1995). Helge *et al.* (1999) also demonstrated an exercise-induced decrease in the level of 22:6 n-3 in red quadriceps muscle. In contrast to the Kriketos

study, however, Helge *et al.* (1999) found no reduction in the long-chain PUFA 22:6 n-3 in soleus or white quadriceps muscle. Conversely, both muscles had lower levels of 20:4 n-6 in the exercised compared to the sedentary animals. An increase in the level of 18:2 n-6 was also apparent in all muscles after 4 wk of exercise training (Helge *et al.*, 1999). A study of genetically obese (*fa/fa*) Zucker rats has also demonstrated a decrease in the content of 20:4 n-6 in insulin sensitive muscle fibres (type I and IIa) but not in insulin resistant fibres (type IIb) after exercise training (Ayre *et al.*, 1998).

An early human study, produced data comparing the FA and cholesterol content of skeletal muscle (vastus lateralis) in 10 untrained and 10 endurance-trained men (Thomas *et al.*, 1977). The results of this study showed a small yet statistically significant reduction in the content of palmitic acid (16:0) in the trained compared to the untrained group. Nevertheless, the FA content of the diet was not taken into account and consequently its influence cannot be assessed. Furthermore, the lipid fraction examined was not separated and so a considerable proportion of the FAs analysed may have come from triacylglycerols, free FAs and cholesterol esters. In addition, 22:6 n-3 was not analysed as gas chromatography (GC) was not performed for a sufficient period of time to allow it to elute from the column.

More recent work by Anderson *et al.* describes increases in 22:6 n-3, 18:0 and 18:1 n-9 and decreases in 16:0, 18:2 n-6 and 20:3 n-6 in vastus lateralis (Andersson *et al.*, 1998; Andersson *et al.*, 2000). These studies have attempted to overcome the problem of dietary fatty acids by placing subjects on controlled diets. Unlike laboratory animals, however, humans are free living and dietary compliance is often an issue. An elegant solution by Helge *et al.* (2001) was to use subjects as both control and experimental groups, effectively eliminating dietary variations. To achieve this, subjects performed one-leg knee extensions for a 4 wk training period using one leg exclusively while the other performed no training during this period. The ‘sedentary’ leg was then used as a control.

They reported significantly higher levels of 18:1 n-9, 18:1 n-7 and 22:6 n-3 in the quadriceps muscle of the trained compared to the untrained leg.

A recent study in healthy humans by Helge and Dela (2003) found that exercise training was associated with minor increases in phospholipid long-chain polyunsaturated fatty acid content in skeletal muscle that were correlated with leg glucose uptake during a hyperinsulinemic clamp ($r = 0.57$, $p < 0.04$, $n = 8$). Clore *et al* (1998) have also reported correlations between the FA composition of PC but not PE in vastus lateralis of healthy humans and insulin sensitivity, as determined by the plasma insulin concentration required to achieve half-maximal glucose disposal (ED_{50}) during a hyperinsulinemic euglycemic clamp. The ED_{50} was found to inversely correlate with the PC content of long chain PUFAs ($r = 0.45$, $P < 0.01$) and directly correlate with the ratio of 16:0 to 18:0 ($r = 0.45$, $P < 0.05$) in PC. These studies suggest that although changes observed in the FA content of skeletal muscle with exercise appear to be small they may be functionally significant. It also appears that the combination of head group and FAs, *i.e.* phospholipid molecular species may also be an important factor.

In summary, exercise training has the ability to alter the relative composition of both phospholipid classes and their FA esters in rat and human skeletal muscle. While some changes are quite consistent between studies, others are more varied. It is possible that the problem lies in the methodology, *i.e.* the small changes in total FA content may be near the limit of sensitivity for gas chromatography (GC). The common finding that 22:6 n-3 is reduced in rat skeletal muscle and increased in human skeletal muscle is interesting, especially considering that the 'pool' of rat skeletal muscle 22:6 n-3 is ~ 4 fold that of humans (see Table 1-2). Perhaps the mechanism for the reduction in rat skeletal muscle 22:6 n-3 is not observed, or even reversed in human skeletal muscle as a result of 22:6 n-3's relatively low abundance. There is also evidence to suggest, at least in humans, that even though exercise-induced changes in skeletal muscle phospholipids appear to be

small they may be important in the regulation of physiological functions such as insulin action.

1.3.2 Diet and Skeletal Muscle Phospholipid FA Composition

In comparison to the small changes in skeletal muscle phospholipids observed with exercise training, diet induced changes are larger and more rapid. A study on rats by Pan and Storlien (1993) comparing three diets, a high saturated fat diet (tallow), a high n-9 diet (olive oil) and a high n-6 diet (safflower oil), found that the fat content of the diet was reflected in the ratio of FA classes (n-9, n-6, n-3) in red quadriceps muscle after 30 days. Unfortunately, the FA profile of the diets was not analysed and therefore a direct comparison of the FA profiles of the diet and skeletal muscle cannot be performed.

In 1996, Ayre and Hulbert published data demonstrating that diet has a greater effect on skeletal muscle PUFA than SFA content (Ayre & Hulbert, 1996). In feeding rats a diet either deficient in essential FAs (DEFA), high in essential n-6 FAs or high in essential n-3 FAs for 9 weeks, they found little variation in saturated FA content despite large differences in the saturated FA content of the diets. In contrast, the content of n-9 PUFAs where significantly higher in both EDL and soleus muscle of the rats fed the DEFA diet than those fed the n-6 or n-3 diet, while rats fed n-6 or n-3 diets had increased levels of n-6 and n-3 PUFAs respectively. After feeding the rats a non-purified 'washout' diet with no deficiencies in FA content, there was a shift towards the skeletal muscle FA profile of the rats fed the high n-3 diet. The data also demonstrate that this shift is more rapid in soleus (2 wk) than EDL muscle (6 wk). This study demonstrates that there is a hierarchy of preference for PUFA classes in rat skeletal muscle. Predictably, when the diet was deficient in essential FA, the level of n-9 PUFAs is increased. When n-6 fatty acids are available, they are incorporated into the membrane in preference to n-9 PUFAs and n-3s are incorporated in preference to both n-9 and n-6 PUFAs if they are present in the diet. The difference in apparent plasticity between the two muscle types may be due to a

difference in their requirement for n-3 PUFAs. A higher level of skeletal muscle n-3 content is linked to increases in insulin sensitivity (see section 1.2.2). As soleus is high in insulin-sensitive type I and IIa fibres it may have a higher requirement for n-3s than does EDL. It would therefore, be interesting to test if soleus muscle is less "plastic" than EDL when the supply of n-3 PUFAs is removed after a period of feeding an n-3 rich diet.

Work in humans has also demonstrated the close relationship between skeletal muscle and dietary FA composition. In a study conducted by Andersson *et al.* (2002), subjects were fed a diet high in SFAs or MUFAs. The diet groups were divided into a further two groups, one taking n-3 (fish oil) capsules and the other a placebo capsule. Predictably, the SFA group had higher levels of saturated phospholipid FAs than did the MUFA group with the inverse levels observed for oleic acid (18:1, n-9). There were also significantly higher levels of long chain n-3s (20:5, 22:5, and 22:6) and lower levels of long chain n-6s (20:3, 20:4) in the skeletal muscle phospholipids of the fish oil capsule supplemented groups in both diets. Although the FA content of the diets was not analysed and a direct comparison of n-6 and n-3 content is not possible, the data again reinforce the preference for incorporation of n-3 FAs into skeletal muscle phospholipids over n-6 FAs when both are available in the diet.

In summary, diet has a large influence on the phospholipid FA profile of skeletal muscle. Not surprisingly, this influence is most dominant in relation to the content of n-6 and n-3 PUFAs and the resultant n-6 to n-3 ratio, as the diet is the sole source of these lipids (see section 1.1.1). This appears to be a recurring theme and has also been demonstrated for cardiac sarcolemma, liver plasmalemma, cerebral synaptosomes and cerebral myelin (Hulbert *et al.*, 2004).

1.4 Conclusions

In conclusion, biological membranes are dynamic structures that participate both directly and indirectly in a number of cellular functions. There is considerable evidence demonstrating the association between various metabolic disorders and skeletal muscle phospholipid content. There is also a large pool of evidence confirming an effect of diet and exercise on the phospholipid FA content of skeletal muscle membranes. Furthermore, these changes may have ameliorating effects upon the aforementioned metabolic disorders. Nevertheless, an understanding of alterations in whole phospholipid molecular species induced by exercise and diet is very limited. This lack of knowledge is a function of the methods previously employed to study membrane lipids in this field. To date our understanding of membrane lipid composition has been limited to phospholipid class or fatty acid analysis, primarily by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and GC. Each of these techniques requires extensive sample preparation and relatively large amounts of starting material. The most commonly applied method to determine the FA composition of phospholipids is GC. This technique requires the degradation of whole phospholipids to FAs and their derivatisation to methyl esters. GC provides excellent qualitative and quantitative information on the phospholipid FA content of membranes but no information on molecular species. HPLC and TLC may also be used for the identification of phospholipid head groups yet lack FA information. A combination of these methods may be used to identify the range of FAs attached to particular head groups, however, exact molecular speciation still cannot be ascertained. A method developed in 1991 is able to overcome this problem through multiple TLC, derivatisation and HPLC steps (Takamura & Kito, 1991). Nevertheless, this method has not been widely adopted as the high level of sample handling required is labour intensive and amplifies the probability of loss and contamination. In fact, De Long and co-workers have demonstrated an overestimation of long chain PUFAs when phospholipids are separated by either TLC or HPLC (DeLong *et al.*, 2001). Recent advances in mass spectrometry will revolutionise this field. This technique allows the analysis of

phospholipid molecular species profile, providing a greater level of information than the total phospholipid FA profile provided by GC.

1.5 Thesis Outline

The general aim of this thesis is to expand our current understanding of skeletal muscle membrane lipid composition and how it is influenced by exercise training. This will be achieved using the relatively new technique of electrospray-ionisation mass spectrometry (ESI-MS). Accordingly, Chapter 2 will present a review of the current 'state-of-play' in the mass spectrometric analysis of phospholipids. In order to arm the reader with the knowledge required to obtain a full understanding of these studies the initial sections of Chapter 2 will provide a brief description of mass spectrometry with particular emphasis on the techniques used in this thesis.

The aim of the work described in Chapter 3 was to develop a standard mass spectrometric method to be used in the analysis of phospholipids in later chapters. This work involved (i) optimisation of instrument parameters, (ii) ensuring an accurate identification of phospholipid molecular species, (iii) testing the reproducibility of the technique and, (iv) the application of a normalisation procedure. In addition, a validation of the method, *i.e.*, a comparison with the established method of GC will be described.

In Chapter 4, the developed MS method is used to provide the first set of data describing the effect of exercise training on rat skeletal muscle phospholipid molecular species. This chapter will also provide a novel comparison between the phospholipid molecular species profile of both glycolytic and oxidative muscle fibres.

Chapter 5 will describe the effect of exercise training on rat skeletal muscle phospholipid molecular species when they are fed a diet high in saturated fat. This diet alters the availability of essential FAs and may therefore mediate the exercise effect described in the previous chapter.

The final chapter (Chapter 6) will provide a discussion on the strengths and limitations of the MS techniques. An overview of the key findings and their possible implications for skeletal muscle membrane function will also be provided.

Chapter 2

Mass Spectrometry and the Analysis of Phospholipids

2.1 Mass Spectrometry

Mass spectrometry (MS) had its beginnings in Nobel Laureate J. J. Thomson's vacuum tube at the beginning of the last century (Thompson, 1911). Thomson, a physicist, analysed a sample of marsh gas and identified the presence of methane and acetylene through the observation of ions with a mass-to-charge ratio (*m/z*) of 16 and 26, respectively. Mass spectrometry remained a technique used solely by physicists for almost three decades. During this period, mass spectrometry was predominantly used in the analysis of isotopes, because of its ability to measure atomic masses to a precision of one part-per-million or better. Now, almost a century after its conception MS has developed into a diverse field with applications ranging from geochronology to biochemical research.

The modern mass spectrometer may incorporate a broad range of technologies, however, several steps are common, namely; (i) vaporisation, (ii) ionisation, (iii) mass analysis, (iv) ion detection, and (v) data collection and display. A schematic diagram of a mass spectrometer highlighting these features is shown in Figure 2-1.

This discussion will describe some of the different methods of ionisation and mass analysis with particular emphasis on the techniques used in this study, specifically electrospray ionisation (ESI) and quadrupole and time-of-flight (ToF) mass analysers. A brief discussion of fast atom bombardment (FAB) and matrix-assisted laser desorption/ionisation (MALDI) will also be presented. FAB was historically significant in the development of phospholipid mass spectrometry while MALDI is beginning to find some use in the analysis of these biomolecules.

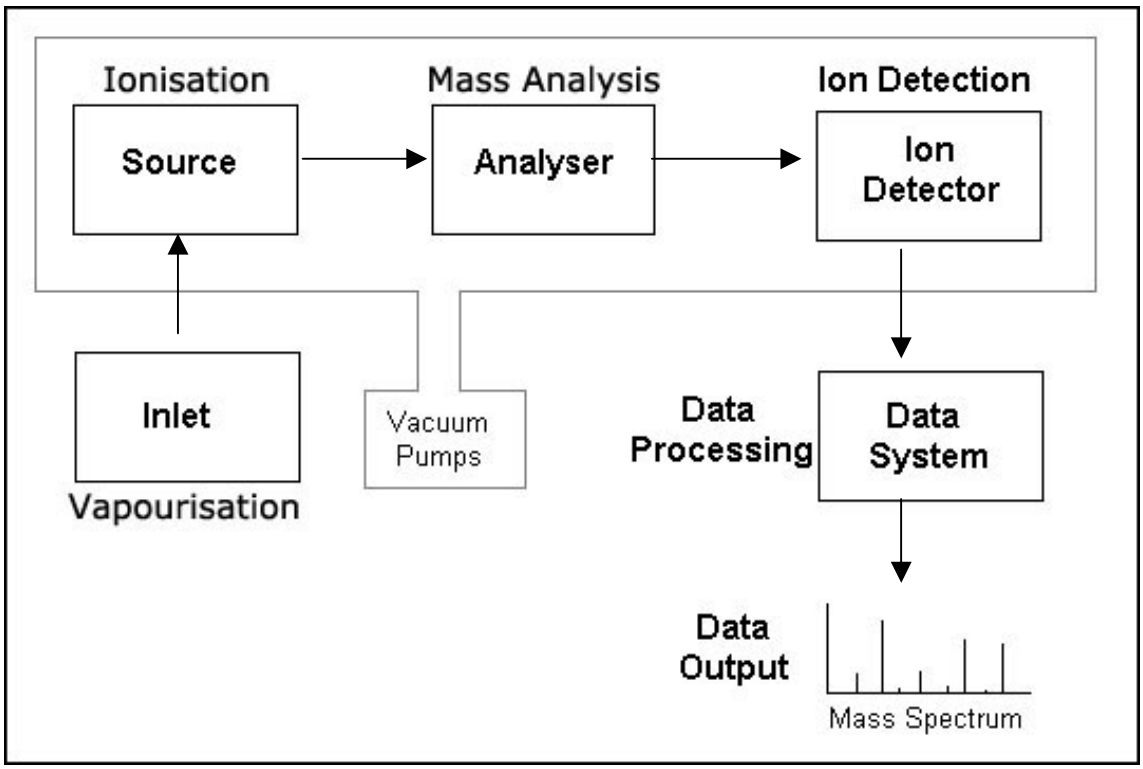


Figure 2-1: ***Basic components of a mass spectrometer.***

2.1.1 Ionisation Techniques

The method of ionisation used in the analysis of a biological sample is determined by its physical properties. Traditional techniques, such as electron ionisation (EI) and chemical ionisation (CI) require the introduction of the analyte in the vapour phase. Therefore, the analysis of compounds by these ionisation techniques requires volatilisation as an initial step prior to ionisation. For non-volatile samples, however, achieving sufficient vapour pressure of the sample for efficient ionisation presents a significant obstacle for analysis. Historically, volatilisation was achieved through thermal desorption or chemical derivatisation. This increases the possibility of contamination and error through additional sample handling and may produce decomposition of thermally labile target compounds

leading to a loss of important structural information. Over recent years, ESI and MALDI have been developed that combine volatilisation and ionisation into a single step and provide simple methodologies for MS analysis of high molecular weight, thermally labile or non-volatile compounds.

2.1.1.a Electrospray Ionisation

The first reported use of ESI in mass spectrometry appeared in the mid eighties (Yamashita & Fenn, 1984; Aleksandrov *et al.*, 1985) but it was not until several years later when its ability to improve the mass spectral analysis of large biomolecules was demonstrated (Fenn *et al.*, 1989). Since then ESI has proven so valuable in expanding the capabilities of MS in the analysis of biomolecules that John Fenn was awarded the Nobel Prize for chemistry in 2002 for his role in its development.

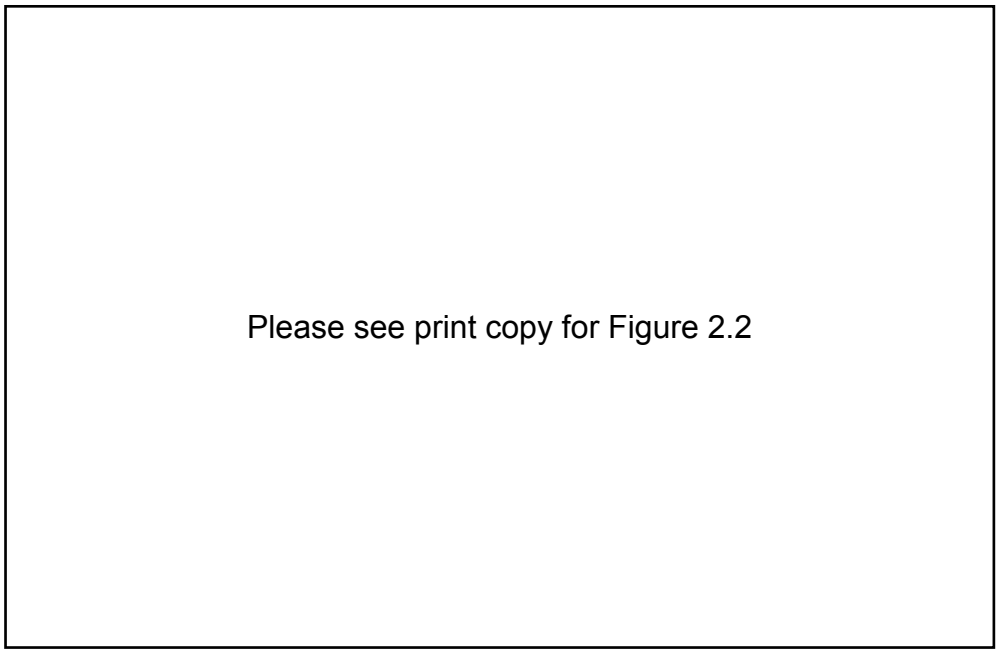


Figure 2-2: ***Drawing of the electrospray process operating in positive ion mode.***
See text for explanation. Adapted from Cech & Enke (2001).

The ESI process is outlined schematically in Figure 2-2. The first step in ESI involves the injection of the analyte in solution through a capillary, to the tip of which, a strong electric field is applied. This electric field causes the accumulation of ions at the surface of the liquid (Murphy, 1993; Smith *et al.*, 1994; Kebarle, 2000). The pull of the electric field and the repulsive forces of the surface ions stretch the liquid into what is known as the Taylor cone (Cole, 2000). Once the liquid has stretched sufficiently to overcome the surface tension, the elongated tip of the cone breaks up into charged droplets (Murphy, 1993; Kebarle, 2000) each of which has a surface enriched with unneutralised ions and a core containing uncharged molecules and neutral salts (Enke, 1997). The droplets then reduce in size by the evaporation of solvent as they pass through the source. Evaporation is facilitated by the heating of the ion source and the application of a warm counter-current drying gas (commonly nitrogen). Once the droplets have become small enough that the Rayleigh limit has been exceeded, *i.e.*, the repulsive Columbic forces exceed the surface tension of the droplet, fission occurs, producing smaller progeny droplets (Kebarle & Ho, 1997; Kebarle, 2000; Vestal, 2001).

While there is a general consensus on the process of charged droplet formation and evolution, the second step, *i.e.*, the formation of gas phase ions from the charged droplets, is still a point of conjecture. There have been two theories proposed to explain this mechanism, namely the ion evaporation model (IEM) and the charge residue model (CRM) (Smith *et al.*, 1994; Kebarle & Ho, 1997; Kebarle, 2000; Vestal, 2001). According to the IEM, when solvent evaporation and Columbic fission have reduced the droplet size sufficiently, there is direct emission of ions into the gas phase. It is argued that this occurs as the charge on the droplet required for this emission is lower than that required for further fission (Murphy, 1993; Smith *et al.*, 1994; Kebarle & Ho, 1997; Kebarle, 2000; Vestal, 2001). In contrast the CRM suggests that the evaporation/fission cycles continue until a single ion is left and the remaining solvent is evaporated, leaving the gas phase ion (Smith *et al.*, 1994; Kebarle & Ho, 1997; Kebarle, 2000; Vestal, 2001).

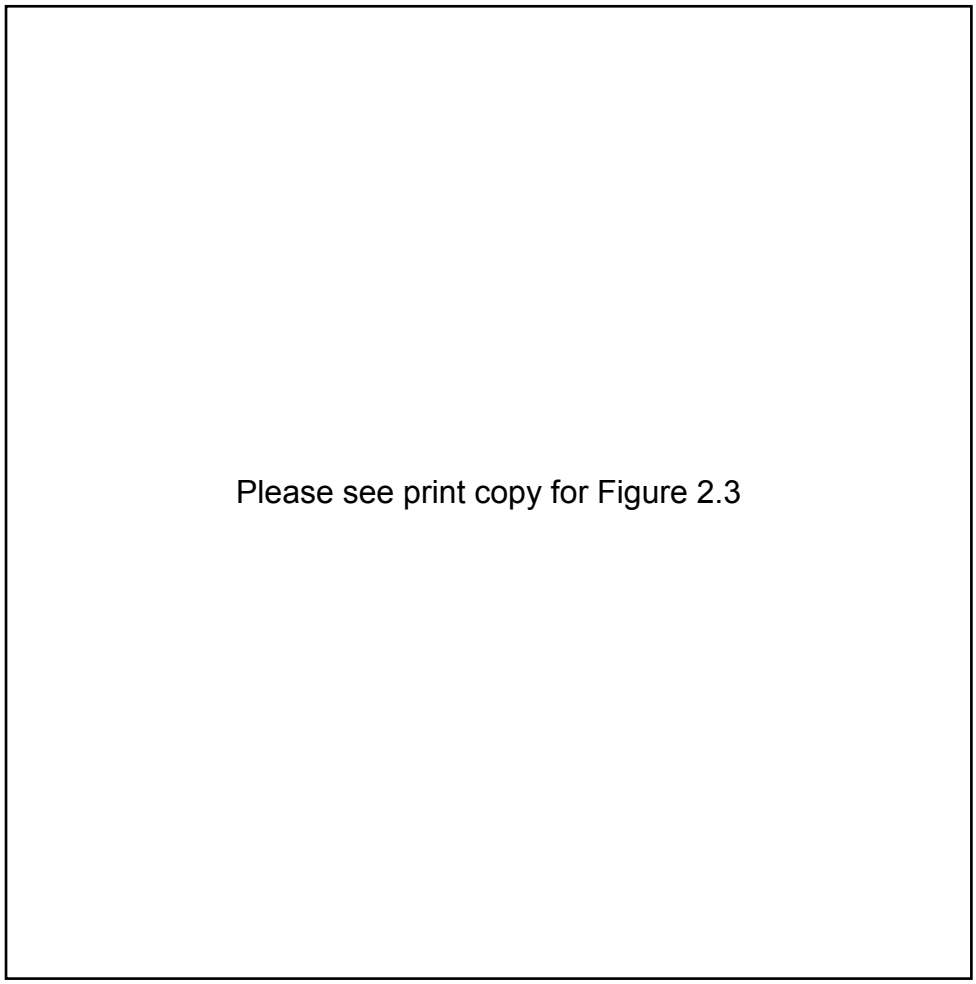


Figure 2-3: ***Schematic representation of the ion evaporation model (IEM) and the charge residue model (CRM) of ion formation in ESI.***
Adapted from Bottrill (2000).

Recent evidence suggests that both models may operate to a greater or lesser exetent depending on the circumstances. The IEM appears to be the more valid mechanism for the production of small ions while the CRM mechanism better explains the production of larger ions such as globular proteins (Cole, 2000; Kebarle, 2000).

Charge separation in the electrospray source is achieved by the attraction to the capillary of charges that have opposite polarity and the repulsion of like charges towards the

surface of the Taylor cone (Van Berkel, 1997). The charge imparted to the droplets, termed 'excess charge' (Enke, 1997) is equal to the amount of charge separation. The differentiation between the excess charge and neutralised charges is important as the neutralised charges require desolvation energies in excess of the Columbic forces between the counter ions to form gas-phase ions. Therefore, the maximum rate of formation of gas-phase ions is equal to the rate of charge separation (Enke, 1997; Cech & Enke, 2001).

During the ESI process ionisation may occur through one or more of the following mechanisms; (i) charge separation; (ii) adduct formation; (iii) gas phase reactions; and (iv) electrochemical oxidation and reduction (Cech & Enke, 2001).

Pre-existing analyte ions in solution may become part of the excess charge after charge separation in the Taylor cone (as shown in Equation 1). The gas-phase ions are produced when the ions carrying the excess charge have been completely desolvated. Ions formed from inorganic species such as sodium, chloride and nitrate; organic and biological species with acidic or basic functional groups; and species that contain ammonium, phosphonium or oxonium groups readily form ions through charge separation (Cech & Enke, 2001). For example, proteins form positively charged ions through protonation of basic amino acid residues, whereas fatty acids (FAs) tend to form negatively charged ions by the deprotonation of carboxylic acidic moieties.

$$X^-_{(aq)} \xrightarrow{ESI} X^-_{(g)} \qquad \text{Equation 1}$$

Adduct formation is essentially a unique form of ionisation by charge separation. By forming adducts with various ions in solution (before charge separation) polar analytes without basic or acidic groups can be induced to form gas-phase ions (Cech & Enke, 2001). This can be used to create either negatively charged ions, using adducts such as chloride ions (as displayed in Equation 2) (Cole & Zhu, 1999), or positively charged ions, using adducts such as sodium, lithium or potassium ions (Saf *et al.*, 1994; Hsu *et al.*,

1998; Domingues *et al.*, 2001). Even though the addition of a salt can aid in the ionisation process, high concentrations lead to the formation of clusters (*e.g.* equation 3) with the analyte and solvent, increasing the background and complexity of the spectrum and in some cases suppressing ionisation of the analyte.

$$X + Cl^-_{(aq)} \xrightarrow{ESI} XCl^-_{(g)}$$ Equation 2

$$nX + mCl^-_{(aq)} \xrightarrow{ESI} X_nCl_m{}^{m-}_{(g)}$$ Equation 3

The ions produced by ESI may also be affected by gas-phase interactions as a large number of both solvent and analyte ions are produced at atmospheric pressure (Stephenson & McLuckey, 1996; Amad *et al.*, 2000). Charging of analytes in the gas phase during ESI is generally driven by gas-phase proton transfer (Amad *et al.*, 2000). As gas-phase proton affinity and solution-phase basicity are not necessarily related, a species that is highly basic in solution may not have a high proton affinity in the gas phase (Cech & Enke, 2001). Such species yield their protons to analytes or solvents with a higher proton affinity upon entering the gas phase (Equation 4). In so doing, analytes that enter the gas phase as neutrals may become charged through gas-phase interactions.

$$X + NH_4^+ \longrightarrow XH^+ + NH_3$$ Equation 4

Electrochemical reactions (oxidation and reduction) are necessary for the transfer of charge from the metal capillary to the solution, as discussed above. They may also be used, in some cases, to convert an uncharged analyte into an ionic form that is compatible with ESI. For example, the derivatisation of alcohols to highly-oxidisable alcohol ferrocenecarbamate esters for positive ion ESI analysis (Van Berkel *et al.*, 1998). Electrochemical reactions (*e.g.*, Equation 5) may, however, be detrimental, as they can compete with the analyte for the excess charge and increase the spectral background (Cech & Enke, 2001).

$$X + e^-_{(aq)} \xrightarrow{\text{reduction}} X^-_{(aq)} \xrightarrow{\text{ESI}} X^-_{(g)}$$ Equation 5

The efficiency of ion formation of a particular analyte is affected by its ability to be ionised by the pathways discussed above, additionally two other factors, namely surface activity (Cech & Enke, 2001) and the presence of electrolytes (Kebarle, 2000) also play an important role.

Surface-active analytes, *i.e.*, nonpolar ions with a high affinity for the surface of ESI droplets, generally have a higher ESI response (Cech & Enke, 2001). As ESI droplets fission the progeny droplets remove a greater fraction of the parent droplet's charge than its mass (Cech & Enke, 2001). It is believed that the progeny droplets are enriched with surface-active analytes as it is from the surface layer of the parent droplet that they are formed (Tang & Smith, 2001). Hence, the progeny droplets have high levels of both excess charge and surface-active analytes. This means that analytes with a low affinity for the droplets surface do not form part of the progeny droplets and are therefore less likely to be charged during the ESI process.

The presence and concentration of other electrolytes in the solution also play an important role in the efficiency of ion formation of a particular analyte. Kerbale has proposed a simple equation for the interaction of three components shown in equation 6 (Kebarle, 2000). Two analytes, AX that dissociates into A^+ and X^-, and BX that dissociates into B^+ and X^-, and an *electrolyte* EX that dissociates into E^+ and X^-.

$$I_A^+ = pf \frac{k_A C_A}{k_A C_A + k_B C_B + k_E C_E} I$$ Equation 6

Where I_A is the ion current detected by the mass spectrometer at the *m/z* ratio of A^+ and C_A, C_B, C_E are the initial molar concentrations in the solution. I is the total current leaving the capillary (determined from the current meter as shown in Figure 2-2). The proportionality constants p and f correspond to the sampling efficiency of the mass

spectrometer to detect an ion relative to the amount of that ion produced (p) and the fraction of charge on the droplets leaving the capillary that is converted to gas-phase ions (f). The coefficients k_A, k_B and k_E express the relative 'efficiency' with which each species is converted to gas phase ions. These coefficients will vary depending on the mechanism of ionisation, *i.e.*, IEM or CRM. From equation 6 it can be seen that as the concentration and/or ion efficiency of the competing ion (B^+) or electrolyte (E^+) increase the ion current of A^+ is reduced.

The use of the ESI technique in MS has a number of advantages. First, the ability to introduce sample in solution is of great value in the analysis of biomolecules and allows direct coupling of MS to liquid chromatography (LC/MS). Second, the amount of energy imparted to the ions is the lowest of all ionisation techniques ensuring large analytes, up to several MDa, remain intact during ionisation (Tito *et al.*, 2000). Finally, ESI can effectively extend the mass range of the analyser by increasing the number of charges on large molecules thus reducing the *m/z* ratio to within the detection range of the analyser.

2.1.1.b Matrix Assisted Laser Desorption / Ionisation

Attempts to use laser ionisation for MS analysis first began over three decades ago (Vastola *et al.*, 1970). It was not, however, until a breakthrough in the late eighties that the merits of this technique were realised. In their work published in 1988, Karas and Hillenkamp (Karas & Hillenkamp, 1988) demonstrated that singly charged ions of large biomolecules could be produced by embedding them in a matrix able to absorb the radiation from a UV laser. This followed earlier work by (Tanaka *et al.*, 1988) on the use of inorganic matices with laser desorption. Along with ESI, MALDI is now one of the most important methods for the ionisation of non-volatile, high molecular weight compounds (Zenobi & Knochenmuss, 1998).

Sample preparation for MALDI involves the mixing of the sample with a large molar excess of solid matrix (Hillenkamp, 1994). A few μL of the matrix/sample solution is

placed on a sample target or plate and allowed to dry. The sample is then subjected to laser pulses that result in the excitation of the solid and in turn desorption of ions from the surface layers of the solid solution (Karas *et al.*, 2000; Vestal, 2001) as shown in Figure 2-4.

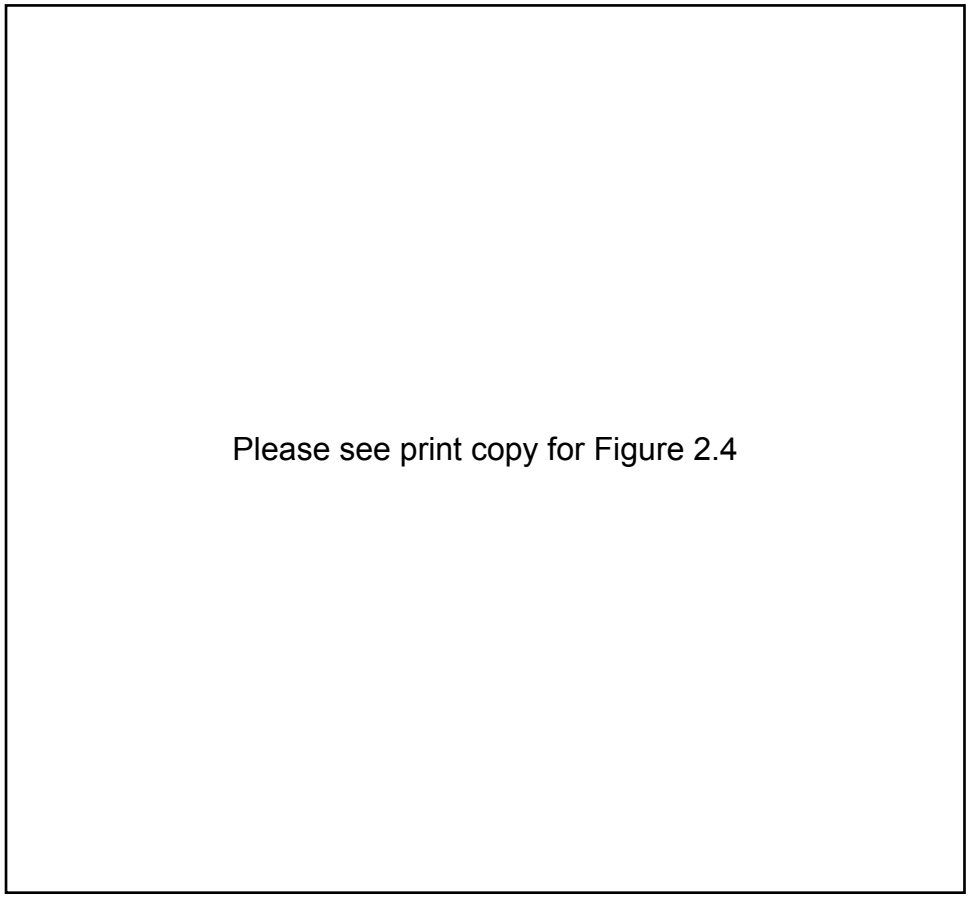


Figure 2-4: ***Diagram of the matrix assisted laser desorption / ionisation technique.***
Taken from Gates (2000).

There is at present, no general agreement on the exact mechanism of ionisation in MALDI. It appears that the ionisation process is affected by many factors including the sample, the matrix and the wavelength and power of the laser (Karas *et al.*, 2000; Vestal,

2001). A review on ionisation in MALDI (Karas *et al.*, 2000) suggests that large highly-excited matrix/analyte clusters with a deficit or excess of cations or anions are expelled upon laser irradiation. These initially generated clusters quickly evaporate by loss of neutral matrix molecules or neutral reaction products to produce the final gas phase ions as displayed in Figure 2-5.

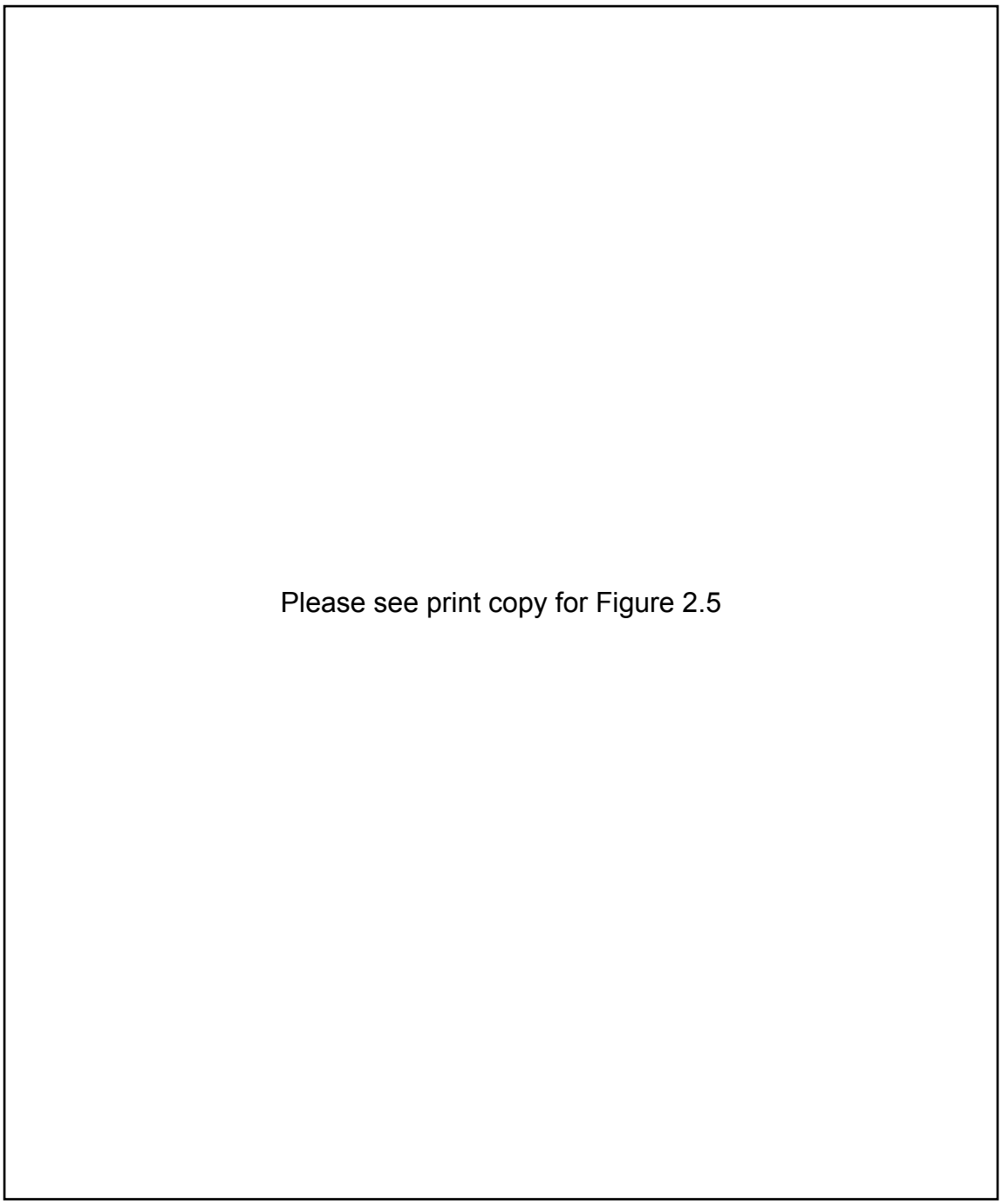


Figure 2-5: ***Some simple matrix/analyte clusters and their ionic and neutral products produced by MALDI when operating in positive (A) and negative ion mode (B).***
M, neutral analyte; T, matrix; Cat, cations; Me, doubly charged metal cations; B, anions. Adapted from Karas et al. (2000)

A major disadvantage MALDI had to overcome was its poor resolution and mass accuracy, especially with larger molecules. The use of a reflectron time of flight (ToF)[3] mass analyser helped to improve the resolution and accuracy for smaller molecules, such as peptides, however, there was little improvement for larger molecules, such as proteins and oligonucleotides (Vestal, 2001). The reason for this poor accuracy and resolution is the large variation in initial velocity of desorbed ions that is nearly independent of mass (Zhou *et al.*, 1992). The development of 'delayed extraction', whereby ions are produced in a field-free region and the accelerating field is turned on by application of a fast pulse after a time delay gives the initially slow ions enough additional energy to catch the initially fast ions at the detector (Vestal, 2001). The delayed extraction technique has improved both the resolving power and mass accuracy of MALDI, however, it only works effectively with analytes that are less than 10 000 Da. A major source of inaccuracy for very large species is the formation of adducts with water, metal ions and matrix molecules that cannot be rectified by improvements in the instrument itself.

2.1.1.c Fast Atom Bombardment

A major step in the application of mass spectrometry to the routine analysis of biological samples was the development of fast atom bombardment (FAB) ionisation (Barber *et al.*, 1981). FAB was developed as a progression of the well-established technique known as secondary ion mass spectrometry (SIMS). SIMS involves the mounting of an analyte on a metal surface and its bombardment with a beam of mass-selected ions generating secondary ions that can be analysed and used in the characterisation of the analyte (Watson, 1994). There are two major differences between SIMS and FAB. The first is that FAB uses neutralised atoms travelling at high velocity ('fast atoms') to bombard the analyte and the second is the use of a liquid matrix in which the analyte is embedded (Watson, 1994; Vestal, 2001). The bombardment of the analyte/matrix liquid solution

[3] ToF mass analysers will be discussed in detail in section 2.1.2.b

with ions rather than neutralised atoms is also used. This technique is known as liquid SIMS (LSIMS).

The desorption/ionisation mechanisms involved in FAB are not completely understood. A number of theories, however, have been proposed that all involve the formation of a thermally excited zone around the area of particle impact from which secondary ions are transferred to the gas phase (Kosevich *et al.*, 2003). A recent model proposed by Kosevich *et al.*, (2003) has attempted to explain why the impact of the high-energy particle with the matrix/analyte solution does not cause fragmentation of fragile biomolecules and weakly bound clusters. This 'bubble chamber model' is based upon the observation that all FAB/SIMS experiments, owing to the low-pressure conditions, are conducted with the liquid in a superheated state. The interaction of the energetic particles with the superheated liquid initiates a local boiling event creating bubble formation in the bulk liquid below its surface. This boiling event occurs at the current temperature of the bulk liquid and is, therefore, non-destructive for biomolecules.

The major advantage of the FAB ionisation technique has over its predecessors is its 'soft' nature, *i.e.* its ability to produce intact biomolecular ions. FAB was also readily compatable to MS instruments already in use when it was developed, leading to a rapid implementation. A disadvantage of FAB is the significant level of background created by the ionisation of the matrix and its fragments that can complicate interpretation of spectra (Murphy, 1993; Vestal, 2001). Other soft ionisation techniques such as ESI and MALDI have since been developed with a number of advantages over FAB that have essentially superseded FAB technology.

2.1.2 Mass Analysers

The ability to separate ions according to their mass-to-charge ratio by the application of magnetic and electric fields was first described at the end of the 19th century (Wien,

1898). At present, there is a wide variety of fundamentally diverse mass analysers used in the field of mass spectrometry. Some of the more common analysers include magnetic sector, electric field sector, quadrupole, time-of-flight, ion trap and ion cyclotron analysers. Each mass analyser has its advantages and disadvantages that must be considered when selecting a mass analyser for a specific application. Important characteristics to be considered include resolving power, mass accuracy and range, linear dynamic range, abundance sensitivity, precision, efficiency, speed and compatibility with the ioniser (McLuckey & Wells, 2001).

2.1.2.a Quadrupole Mass Analysers

Another Nobel Laureate, Wolfgang Paul created the first quadrupole mass analyser, also known as a quadrupole mass filter, in the 1950's (Dawson, 1976; Murphy, 1993). It has since become the most widely used form of mass analyser in mass spectrometry (Dawson, 1976; Murphy, 1993; McLuckey & Wells, 2001).

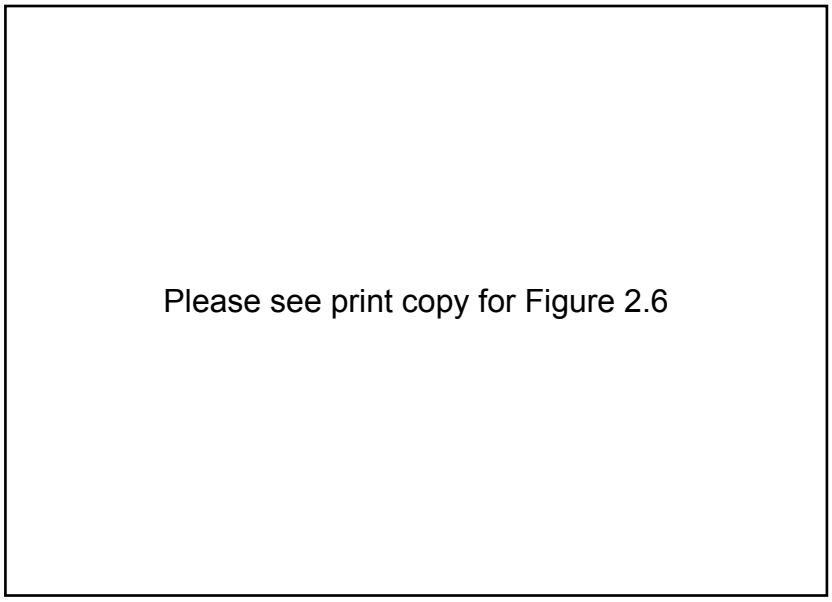


Figure 2-6: ***A schematic diagram of a quadrupole mass analyser.***
Taken from Gates (2000)

Mass separation in quadrupole mass analysers is achieved by inducing oscillations in ions of different mass by the effect of a hyperbolic electric field (Murphy, 1993; Yates, 1998). The three dimensional electric field is created by simultaneously applying an AC potential at radio frequencies (RF) and a DC potential to four parallel metal rods. The same potentials are applied to opposite rods while adjacent rods have opposite DC polarity and AC potentials phase shifted by 180° (Dawson, 1976; Murphy, 1993; Yates, 1998). Ions traverse the rods with a complex trajectory, characteristic of their *m/z*. At a given set of potentials, ions of a particular *m/z* will have a trajectory stable enough to pass along the quadrupoles entire length as displayed in Figure 2-6. By varying the magnitude of these potentials while maintaining their ratio (generally 0.167, DC/RF), ions of different *m/z* will be transmitted according to the following equation:

$$m/z = 0.136\frac{V_0}{r_o^2 f^2} \qquad \text{Equation 7}$$

Where V_0 is the RF voltage when DC/RF voltage = 0.167, r_o is half the distance between opposite rods, and f is the RF voltage frequency.

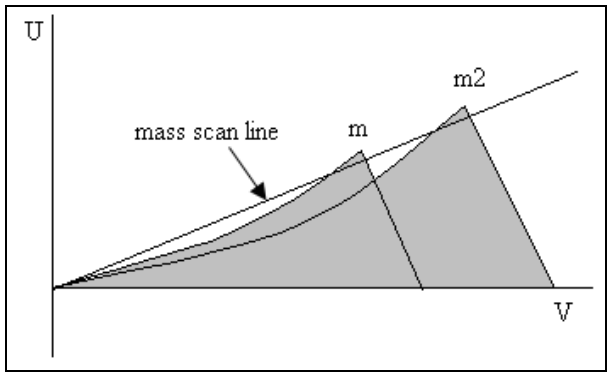


Figure 2-7: ***Stability diagram for ions in a hyperbolic quadrupole field as a function of DC (U) and RF (V) voltage.***
The shaded area represents the region of stable ions, i.e., those with trajectories stable enough to traverse the quadrupole. Increasing the magnitude of U linearly as a function of V will increase the m/z value of the stable ions. (e.g., m to m2).

Quadruploes may also be operated as 'beam guides' by applying the RF voltage only. From the ion stability diagram (Figure 2-7) it can be seen that all of the ions will have a stable trajectory when the DC voltage is held at zero. This technique may be used in instruments with multiple mass analysers to focus ions for subsequent mass analysis. In many instruments an RF only quadrupole is used as a collision cell in tandem MS experiments.[4]

Quadrupole mass analysers generally have an upper *m/z* limit of approximately 4000 (Murphy, 1993; McLuckey & Wells, 2001). It has been shown, however, that the *m/z* range can be extended, at the expense of resolution, by reducing the operating frequency (Light-Wahl *et al.*, 1993; Light-Wahl *et al.*, 1994).

A major advantage of quadrupole mass analysers are their ability to withstand high pressures, up to 10^{-4} torr, making them ideal for use in conjunction with gas chromatography (GC) and more importantly ESI (Murphy, 1993).

2.1.2.b Time-of-flight Mass Analysers

The first proposed time-of-flight mass spectrometer was presented in the mid 1940's (Stephens, 1946). It was not until almost a decade later, however, that the first commercial instrument was produced (Katzenstein & Friedland, 1955).

In a ToF mass analyser, ions are accelerated in the source by an electric potential to a fixed kinetic energy, with their velocity inversely proportional to square root of their *m/z* ($v \propto (m/z)^{-1/2}$). The ions then pass through a field-free region resulting in a spatial separation of ions according to their *m/z*, *i.e.*, ions with a higher *m/z* will have a lower velocity and longer flight time (Hillenkamp *et al.*, 1991; Yates, 1998). A detector at the end of the field-free region then records a signal as it is struck by each ion. The difference

[4] Tandem MS will be discussed in detail in section 2.1.3

between the common start time of all ions and the detection time of an individual ion can then be used to calculate its *m/z* (Hillenkamp *et al.*, 1991).

As discussed in section 2.1.1 early problems with variability in initial velocity distribution of ions with the same *m/z* ratio in ToF instruments resulted in poor mass resolution. These issues have been largely addressed by delayed extraction for MALDI sources (as discussed earlier) and the introduction of an ion mirror or 'reflectron' (Mamyrin *et al.*, 1973). A diagram of a reflectron ToF is shown in Figure 2-8.

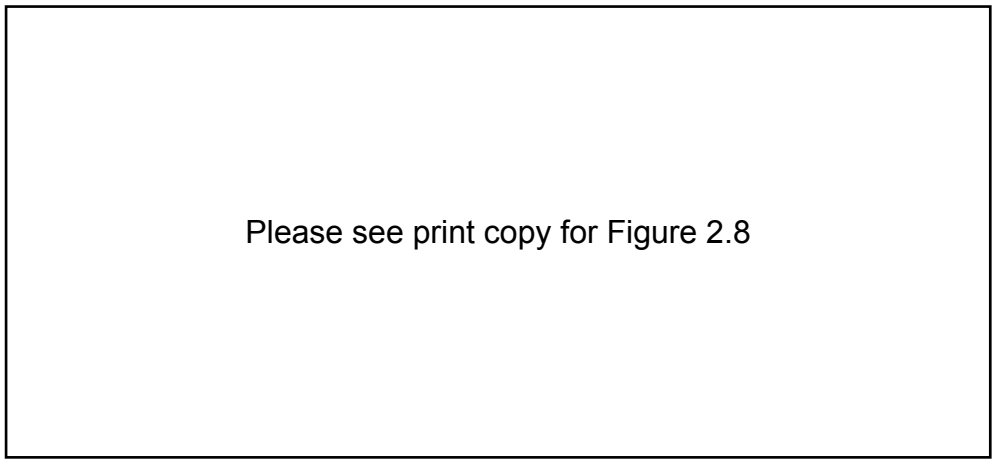


Figure 2-8: ***Schematic diagram of a linear ToF (A) and a reflectron ToF (B) mass spectrometer with a MALDI ion source.***
Taken from Hillenkamp et al. (1991)

Ions pass along the flight tube until they reach the reflectron. The reflectron then redirects the ions back along the same flight tube at a slightly different angle so as not to interfere with ions entering it. Ions of a higher kinetic energy arrive at the reflectron earlier and penetrate deeper than ions of the same *m/z* but with lower kinetic energy. Therefore, ions with the same *m/z* yet different energies will meet at the reflectron (Yates, 1998). The addition of a reflectron also increases the length of the flight path, further enhancing resolution by allowing more time for separation of ions of differing *m/z* ratio, without unduly increasing the size of the instrument (Yates, 1998).

While ToF analysers are ideally suited to pulsed ion sources such as MALDI, a limiting factor in their range of application was their incompatibility with continuous ion sources such as ESI. This is because all ions must enter the field free flight tube with a defined time. The development of orthogonal acceleration (oa) ToF overcame this problem (Guilhaus *et al.*, 1997; Guilhaus *et al.*, 2000; McLuckey & Wells, 2001). Orthogonal acceleration is achieved by the application of a fast pulse electrostatic field (10-100 ns) exerting a force on the ions that is orthogonal to the ion beam axis. This acceleration creates a vectorially independent component of velocity that is not effected by the kinetic energy spread of ions along the beam axis (Guilhaus *et al.*, 1997; Guilhaus *et al.*, 2000).

ToF analysers have some major advantages over their scanning counterparts (*e.g.* quadrupole and sector instruments); (i) the mass range of the ToF is much larger (although sensitivity is decreased when analysing very large *m/z* ranges as the repetition rate of the extraction pulse has to be reduced to allow for large ions; Chernushevich *et al.*, 2001); (ii) the acquisition time of the ToF is more rapid; and (iii) the ToF has a greater efficiency, that is, a greater percentage of ions are transmitted to the detector (McLuckey & Wells, 2001). The last two advantages are achieved owing to one characteristic; ToF analysers acquire a complete mass spectrum with each pulse.

2.1.3 Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) has become an extremely important tool in the structural characterisation of biomolecules. Ions that resulted from fragmentation of the analyte ion were often observed in early MS instruments owing to the relatively high background pressures in the analysers as a result of the crude vacuum pumps of the day. Fragment ions were, however, considered a nuisance to be ignored (Benyon, 1960). It was not until the mid 1970's, when multi-sector high-resolution MS was developed that MS/MS was explored for analytical and structural characterisations (Mc Lafferty *et al.*, 1973a; Mc Lafferty *et al.*, 1973b).

MS/MS is achieved by two stages of mass analysis. In the first mass analyser, a 'precursor' or 'parent' ion is selected on the basis of its *m/z* and allowed to pass into a collision cell. The ion is then activated by collisions with an inert gas such as argon. The amount of internal energy transferred to the analyte ion depends on its incident kinetic energy and the mass of the collision gas. Low kinetic energy, usually refers to the 1 – 100 eV range, as used in quadrupole or ion trap instruments and high kinetic energy ≥ 1000 eV, used in magnetic sector instruments (De Hoffmann & Stroobant, 2002). If sufficient energy is transferred to a precursor ion it fragments, a process known as collision-induced dissociation (CID) or collision-activated dissociation (CAD) (Shukla & Futrell, 2000; Yates, 2000). The second mass analyser then records the 'product' or 'daughter' ions. A schematic diagram of this process is depicted in Figure 2- 9.

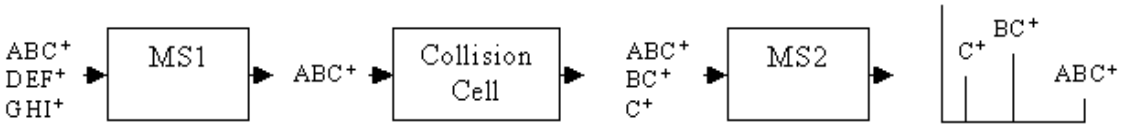


Figure 2-9: ***Schematic diagram of the steps involved in tandem mass spectrometry.***
Where MS1 is the first mass analyser and MS2 is the second mass analyser.

The amount of energy internalised by the precursor ion after collision is given by the following equation:

$$E_{cm} = E_{lab}\frac{M_t}{M_t + M_i} \qquad \text{Equation 8}$$

Where, E_{cm} is the maximum energy fraction converted to into internal energy, E_{lab} is the precursor ion kinetic energy that is determined in the mass spectrometer by the accelerating voltage, M_t is the collision (target) gas mass and M_i is the ion mass.

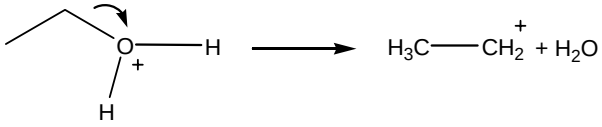
It is important to note that CID-induced fragmentation is not a random destruction of the target ion but a structurally specific process usually driven by charge-mediated rearrangements (DeHoffmann & Stroobant, 2002). Fragmentation reactions in both positive and negative ions may be divided into those experienced by odd-electron (radical) ions or those experienced by even-electron ions. As the focus of this thesis is on the characterisation of even-electron molecular ions, the fragmentation of radical ions will not be discussed.

Fragmentation reactions of even-electron cations often obey the parity rule (fragmentation of an even-electron ion yields an even-electron ion plus a neutral fragment) (De Hoffmann & Stroobant, 2002). For example the neutral loss of water from a protonated alcohol:

$$R\text{-}OH_2^+ \rightarrow R^+ + H_2O$$ Equation 9

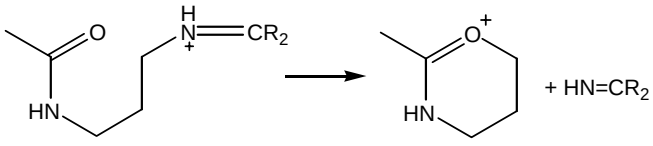
These reactions have been divided into four main classifications (De Hoffmann & Stroobant, 2002):

1. Bond cleavage with charge migration:



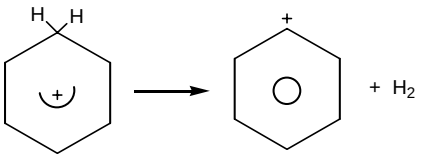
Equation 10

2. Bond cleavage with cyclisation and charge migration



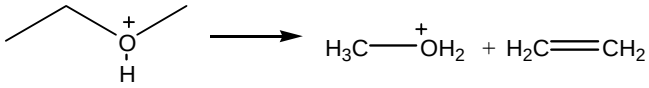
Equation 11

3. Cleavage of two bonds with charge retention in a cyclic ion



Equation 12

4. Cleavage of two bonds with rearrangement and charge retention

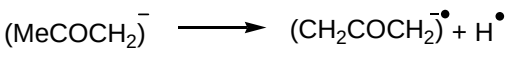


Equation 13

Some even electron cations do not obey the parity rule and fragment to produce a radical ion. These fragmentations are much less frequent and require more complex rearrangements (De Hoffmann & Stroobant, 2002).

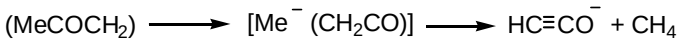
The fragmentation of even-electron anions has also been divided into four general classifications (Bowie, 1990; Bowie, 1994).

1. Homolytic cleavage of a radical forming a stable radical anion



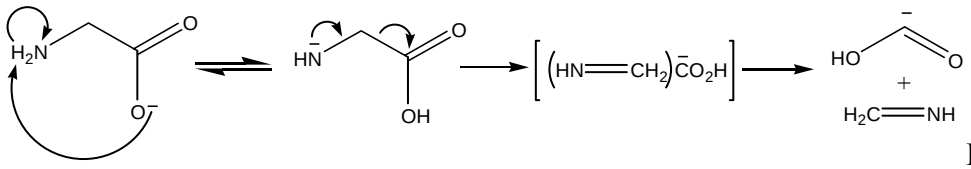
Equation 14

2. Fragmentation through an intermediate ion complex that is subsequently fragmented resulting in the elimination of neutrals



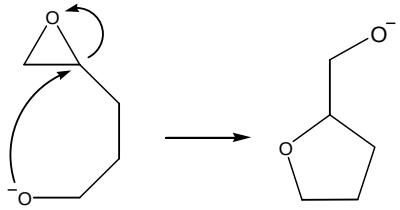
Equation 15

3. Fragmentation preceded by proton transfer to the initial deprotonation site followed by an intermediary and subsequent elimination of neutrals, often through an intermediate as in point 2 above



Equation 16

4. Fragmentations preceded by rearrangement of the initial anion



Equation 17

MS/MS can be divided into two classes, tandem in space and tandem in time (Shukla & Futrell, 2000; McLuckey & Wells, 2001; DeHoffmann & Stroobant, 2002). Tandem in space instruments follow the sequence of events listed above as the ions pass through two or more coupled mass analysers (as in Figure 2-9). Tandem in time instruments also follow the same sequence of events, however, they all take place in the one mass analyser *e.g.* Fourier transform and ion trap instruments and are separated chronologically (Shukla & Futrell, 2000; Hoffmann & Stroobant, 2002). In this study, tandem in space instruments have been used exclusively and therefore, further discussion will be focussed upon these instruments.

The combinations of mass analysers used in MS/MS are numerous. For example, multiple magnetic and electric sector, quadrupole and ToF instruments have been constructed, as well as, hybrid combinations thereof (Shukla & Futrell, 2000). One hybrid combination that has gained popularity in recent years is the quadrupole-oaToF (Q-ToF) (Figure 2-10). This instrument has ultra high sensitivity and sufficiently high resolution for MS/MS characterisation of large biomolecules (Shukla & Futrell, 2000). Current Q-ToFs (Figure 2-10) consist of an RF only quadrupole/hexapole ion guide, a quadrupole mass filter, an RF only quadrupole/hexapole collision cell and a reflectron oaToF (Shevchenko *et al.*,

1997; Chernushevich *et al.*, 2001; Ishigai *et al.*, 2001; Guan *et al.*, 2002; Marchese *et al.*, 2003; Miao *et al.*, 2003; Wan & Desiderio, 2003). The quadrupole mass filter may be used to select the ion of interest for an MS/MS experiment or operated in RF only mode allowing all ions to pass through to the ToF for single stage MS. In either MS or MS/MS operation the ToF analyser acquires all spectral data, providing high speed, resolution and efficiency (Chernushevich *et al.*, 2001). A schematic diagram of the Micromass Q-ToF 2®, an instrument used in this project can be seen in Figure 2-10.

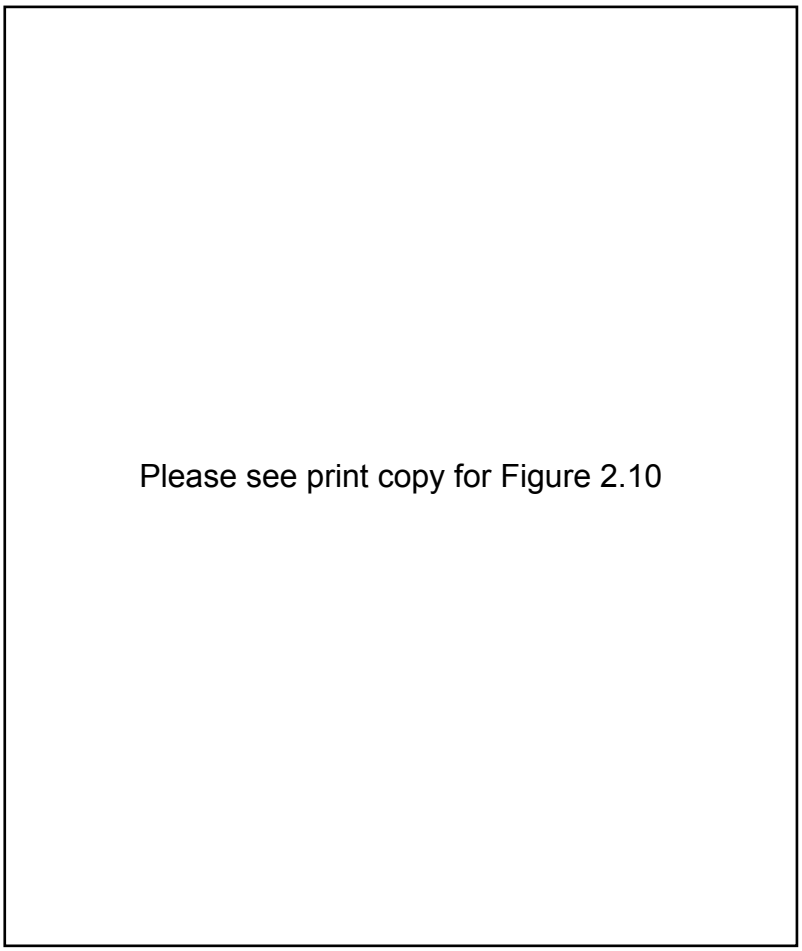


Figure 2-10: ***A schematic diagram of the Q-ToF 2®.***
Taken from the Micromass Q-ToF 2 users guide.

2.2 Mass Spectrometric Analysis of Lipids

Lipids are defined as any substance derived from living tissue that can be extracted or solubilized in organic solvents (Murphy *et al.*, 2001). Thus, lipids by definition encompass a broad spectrum of important biomolecules ranging from simple fatty acids (FAs) through to large peptidolipins. Other lipids include eicosanoids, phospholipids, steroids, triacylglycerols (TAGs) and sphingolipids to name a few.

This area of MS is extensive and extends beyond the scope of this discussion. There are two reviews (Murphy, 1993; Murphy *et al.*, 2001) that provide an excellent account of the MS analysis of lipids. The aim of this section is to provide a brief overview of the mass spectrometric analysis of selected lipid classes that are of importance to this study.

2.2.1 Mass Spectrometric Analysis of Fatty Acids

The lack of lipid volatility posed a problem for the analysis of FAs by early ionisation techniques such as EI and CI. This was overcome through the synthesis of volatile derivatives of FAs such as methyl esters (Murphy, 1993). Mass spectrometrists soon discovered they were able to establish the structure of even complex FAs with very little sample. In fact, MS study of FAs in the late 1950's provided the basis for our understanding of electron ionisation (EI) mass spectrometry (Murphy, 1993).

These early experiments using EI and CI generally involved a combination of derivatisation, GC and at times degradation to known molecules (Murphy *et al.*, 2001). With the development of new ionisation techniques such as FAB, ESI and MALDI, MS/MS methods have been developed to characterise FAs without such requirements.

A study by Kerwin *et al.* (1996) using negative ion ESI-MS and MS/MS was able to characterise various SFAs, MUFAs and PUFAs with samples as small as 1 pg. They used CID to produce typical product ion spectra that allowed them to localise the position of

double bonds in the unsaturates (Figure 2-11). While the fragmentation spectra of all FAs were dominated by an ion corresponding to the neutral loss of water, the MUFAs and PUFAs also displayed minor peaks produced by cleavage at the carboxyl and/or methyl side of the double bonds. MALDI-ToF MS has also recently been used to identify and quantify the FA composition of vegetable oils (Ayorinde *et al.*, 2000).

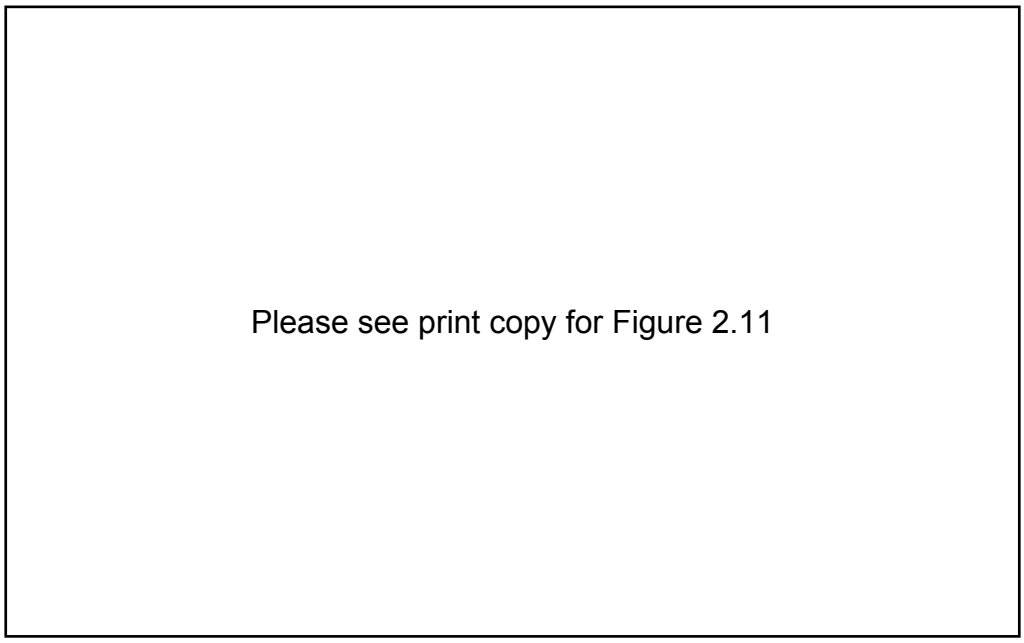


Figure 2-11: ***Negative ion CID spectra from m/z 60 to 265 of γ- and α-linolenic acids.***
Taken from Kerwin et al. (1996)

2.2.2 Mass Spectrometric Analysis of Triacylglycerols

Triacylglycerols are highly concentrated stores of energy, providing approximately 37.7 kJ g^{-1} (Stryer, 1995). They consist of a glycerol backbone with a fatty acid attached to each of its three carbon atoms as displayed in Figure 2-12. With up to 20 different fatty acids present in a given cell (Murphy *et al.*, 2001), TAGs can be found with a diverse range of FA permutations.

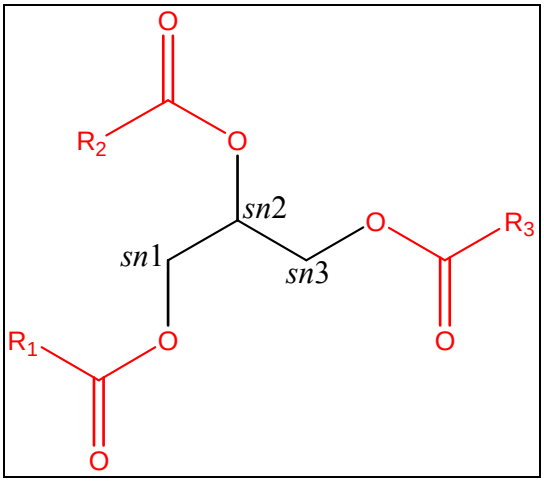


Figure 2-12: ***General structure of a triacylglycerol.***
The glycerol backbone is highlighted in black and the three fatty acid chains in red.

Initial MS experiments suffered from problems with TAGs adsorbing to surfaces within the source leading to the inability to produce any molecular ions. It was soon discovered, however, that EI-MS could be used for successful qualitative and quantitative analysis of TAGs (Murphy, 1993). Many other ionisation techniques (CI, APCI, FAB, thermospray and ESI) have been used in the study of TAGs, often in combination with either GC or LC (Murphy *et al.*, 2001). Positive ion MALDI has also been used for the analysis of TAG $[M+Na]^+$ ions in various naturally occurring oils (Ayorinde *et al.*, 1999; Ayorinde *et al.*, 2000; Hlongwane *et al.*, 2001).

Recent work by Han and Gross has focussed on the direct characterisation and quantification of TAG $[M+Li]^+$ ions using ESI-MS and ESI-MS/MS (Han & Gross, 2001). By spiking the biological sample with synthetic TAGs, they are able to correct for variations in ionisation efficiencies and quantify the various molecular species. In addition, CID of these molecular species produces peaks corresponding to the neutral loss of each of the FAs allowing structural characterisation. They have also used this

technique to study changes in TAG molecular speciation in rat myocardium associated with diabetes (Han *et al.*, 2000).

2.3 Mass Spectrometric Analysis of Phospholipids

Direct analysis of phospholipids by MS began in the early seventies using a variety of ionisation techniques often requiring degradation and/or derivatisation (Murphy, 1993). The ability to analyse intact phospholipids was first reported in 1974 using field desorption ionisation (Wood & Lau, 1974). In this method, phospholipids are coated on a tungsten or rhenium filament that is covered with carbon needles, by the evaporation of a salt containing solution. The filament is heated until the sample melts and the ions migrate and accumulate at the tip of the needles. The ions then desorb carrying the sample molecules with them. The use of field desorption for the wide-spread analysis of phospholipids was hindered by a number of difficulties, such as sensitivity to sodium ions and the incorporation of artefact ions during heating of the emitter filament (Murphy, 1993).

2.3.1 FAB-MS

The development of FAB-MS in 1981 (Barber *et al.*, 1981) opened the door for the extensive use of mass spectrometry in the identification and structural analysis of phospholipids. Phospholipids are inherently compatible to this form of ionisation, as they tend to form layers on the surface of the matrix mixture, resulting in the emission of abundant secondary ions during the FAB ionisation process, even with small amounts of sample (subnanomole) (Murphy, 1993). In addition, the phospholipids are not heated, as there is no requirement of volatility. This removes the problem of pyrolysis reactions and the major ions observed are usually molecular ions (Murphy, 1993). Additionally, results obtained by FAB were found to be readily reproducible across different laboratories (Clay *et al.*, 1983).

The combination of FAB and MS/MS further enhanced the quality of structural information obtained. It was discovered that negative ion CID of phospholipid molecular species produced abundant product ions associated with the polar head group and the two FAs esterified to the glycerol backbone. This allowed the simple and definitive characterisation of phospholipids that was previously difficult to obtain. This technique dominated the MS analysis of phospholipids for a decade and provided an enormous body of information on the ion chemistry of phospholipids.

One disadvantage of FAB is the significant level of background created by the ionisation of the matrix and its fragments that can complicate interpretation of spectra (Murphy, 1993; Vestal, 2001). An example of these peaks (labelled G) produced when glycerol was used as the matrix for positive ion FAB-MS is shown in Figure 2-13. While this does not create a large problem when an individual molecular species at a relatively high concentration is analysed, it does increase the difficulty of interpreting spectra from a mixture where some species are less abundant. Furthermore, the FAB ionisation mechanism results in considerable fragmentation (Figure 2-13) precluding it from quantitative analysis of complex lipids (Murphy *et al.*, 2001).

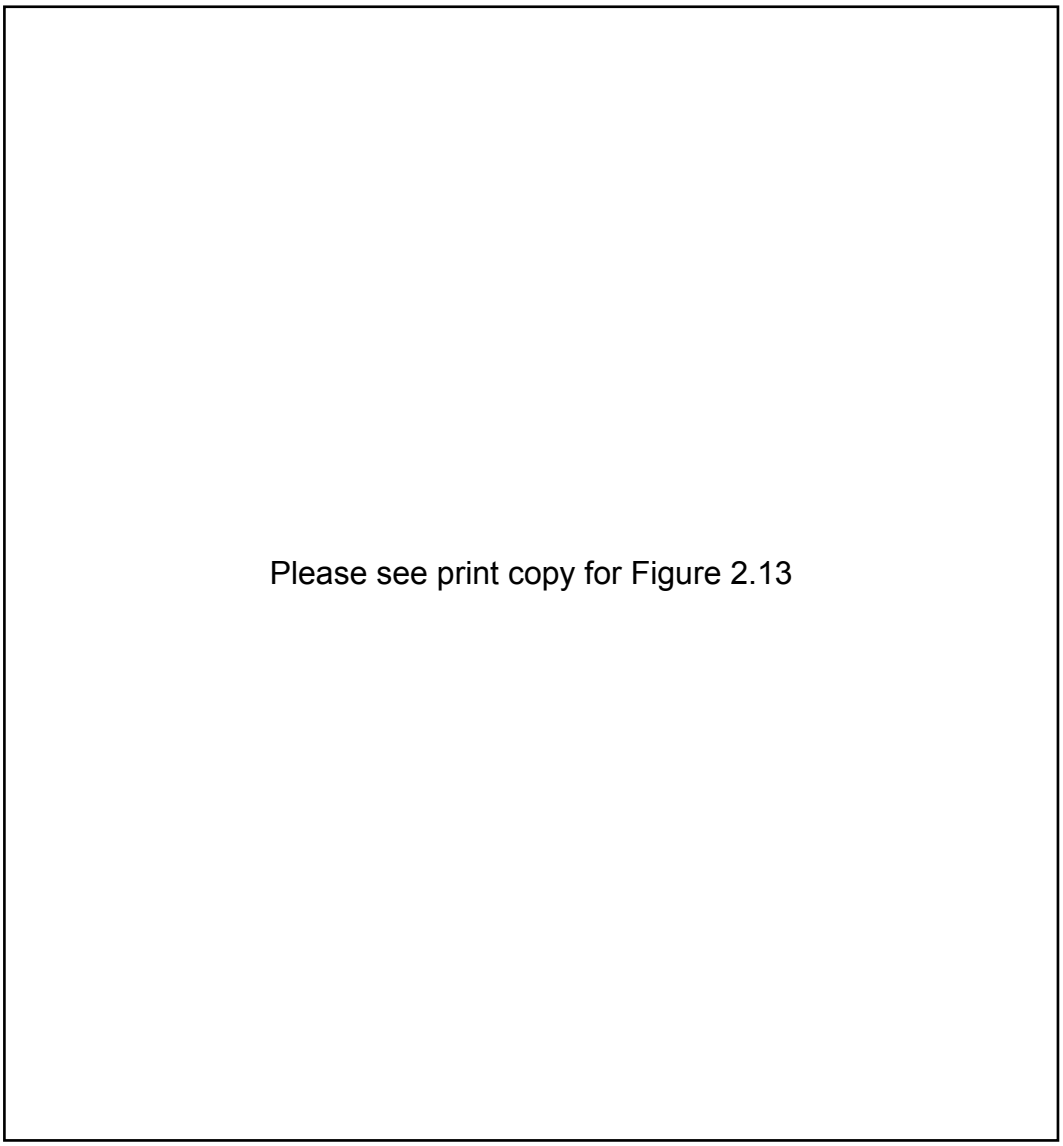


Figure 2-13: ***Positive ion FAB-MS spectra of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (A) and 1-O-hexadecyl-2-oleoyl-sn-glycero-3-phosphocholine (B).***
Ions arising from the glycerol matrix are labelled G. Taken from Murphy (1993).

2.3.2 ESI-MS

The application of ESI-MS to the analysis of phospholipids in the mid 1990s (Han & Gross, 1994; Kerwin *et al.*, 1994; Kim *et al.*, 1994) was able to overcome the problems encountered by FAB. ESI-MS and MS/MS investigation of phospholipids benefited greatly from the knowledge base acquired from FAB-MS allowing it to rapidly expand to the point where it is currently being applied to answer complex biological questions (Nowatzke *et al.*, 1998; Schneiter *et al.*, 1999; Hsu *et al.*, 2000; Ramanadham *et al.*, 2000; Williams *et al.*, 2000; Blom *et al.*, 2001).

An early publication on the use of ESI-MS for the examination of phospholipids reported an increase of 2-3 orders of magnitude in sensitivity over FAB-MS, enabling the identification of more than 50 phospholipid species in human erythrocyte plasma membranes from less than 1 μl of blood (Han & Gross, 1994). A positive ion MS spectrum from this study obtained from 13.5 pmol of total PC can be seen in figure 2-14.

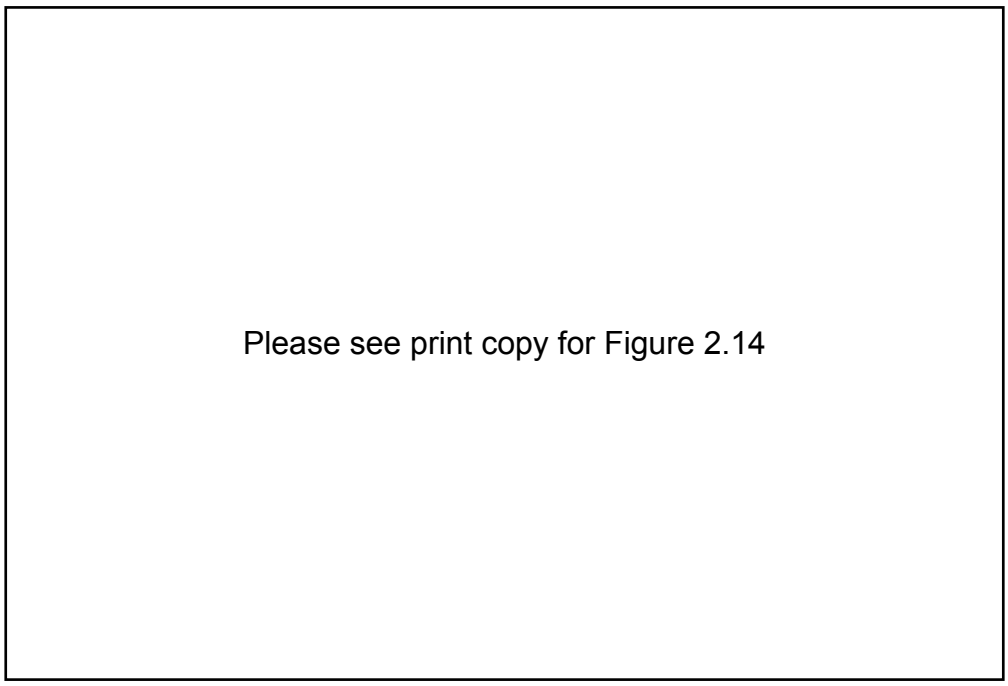


Figure 2-14: ***Positive-ion ESI-MS spectrum of human erythrocyte plasma membrane phospholipid extract containing 13.5 pmol of total phosphatidyl choline (PC) species.***
This spectrum shows a number of sodiated PC (m/z 757 = 32:0, 781 = 34:2, 783 = 34:1, 805 = 36:4, 809 = 36:2, 833 = 38:4), plasmenyl PC (m/z 767 = 34:1, 795 = 36:1, 823 = 38:1) and sphingomyelin (SM) (m/z 726 = 16:0, 754 = 18:0, 782 = 20:0) molecular species. Adapted from Han & Gross (1994)

2.3.2.a Neutral Phospholipid Species

Positive-ion ESI-MS spectra are dominated by phosphatidylcholine species as shown in Figure 2-15. PC species contain a quaternary nitrogen atom whose positive charge is neutralised by the negative charge of the phosphate group. They tend to form abundant $[M+H]^+$ ions as the phosphate anion can be protonated during the electrospray process. Sphingomyelin species also tend to form $[M+H]^+$ ions as they contain the same ionic groups as PC species. In this thesis the focus is on glycerophospholipids and therefore discussion of SM species will be limited. The first ESI-MS studies of phospholipids

illustrated that both $[M+H]^+$ and [M+alkali metal]$^+$ (see Figure 2-14) ions were observed during positive-ion ESI-MS analysis of these species, depending on the presence of the metals in the buffer (Han & Gross, 1994; Kerwin *et al.*, 1994).

Negative-ion ESI-MS will also produce PC $[M+OAc]^-$ ions when acetate is added to the buffer (Kerwin *et al.*, 1994; Harrison & Murphy, 1995) and $[M+Cl]^-$ ions when a chlorinated solvent is used (Harrison & Murphy, 1995). Additionally PC $[M-15]^-$ ions can be produced by the demethylation of the choline moiety by the above-mentioned anionic adducts when cone voltage is increased (Harrison & Murphy, 1995). Many of these ions, however, have the same *m/z* as other PE and PS $[M-H]^-$ ions and further complicate an already crowded negative ion spectrum.

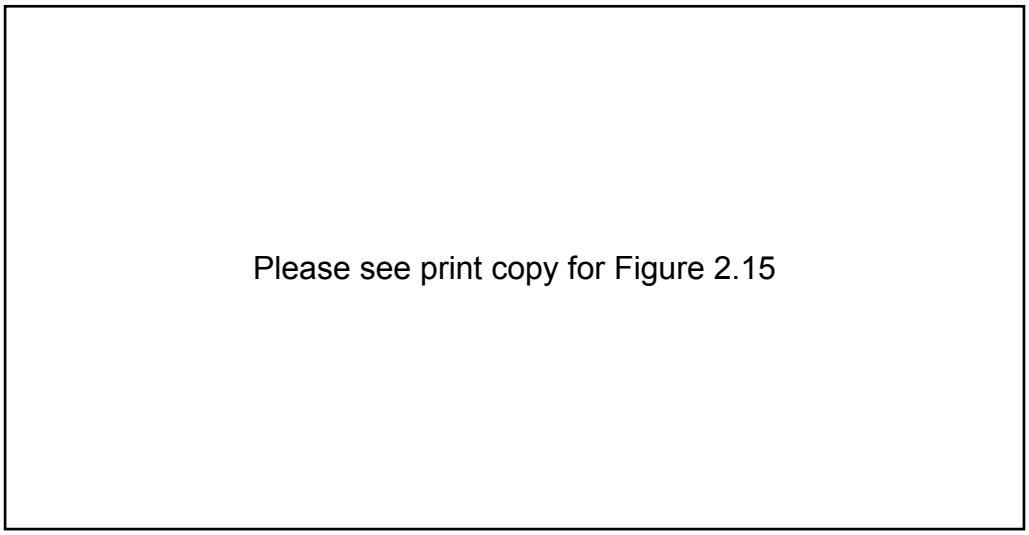


Figure 2-15: ***Positive ion ESI-MS spectrum of a total lipid extract from rat bile dominated by PC species.***
Adapted from Lehmann et al. (1997)

MS/MS of Protonated Ions

CID of protonated PC molecular ions produces a single predominate ion at *m/z* 184 corresponding to the charged phosphocholine head group (Kerwin *et al.*, 1994; Hsu &

Turk, 2003) and therefore provides little structural information aside from the presence of a choline moiety. An example spectrum of the CID of $[M+H]^+$ PC(16:0,18:1) ions obtained by Hsu and Turk (2003) displaying the single dominant product ion at *m/z* 184 is shown in Figure 2-16. Two possible pathways for the formation of this ion involving the *sn*-2 (pathway a) or *sn*-1 (pathway b) α-hydrogen are shown in Scheme 1. MS/MS of protonated SM species has also been shown to produce a phosphocholine peak at *m/z* 184 (Brugger *et al.*, 1997; Hsu & Turk, 2000e).

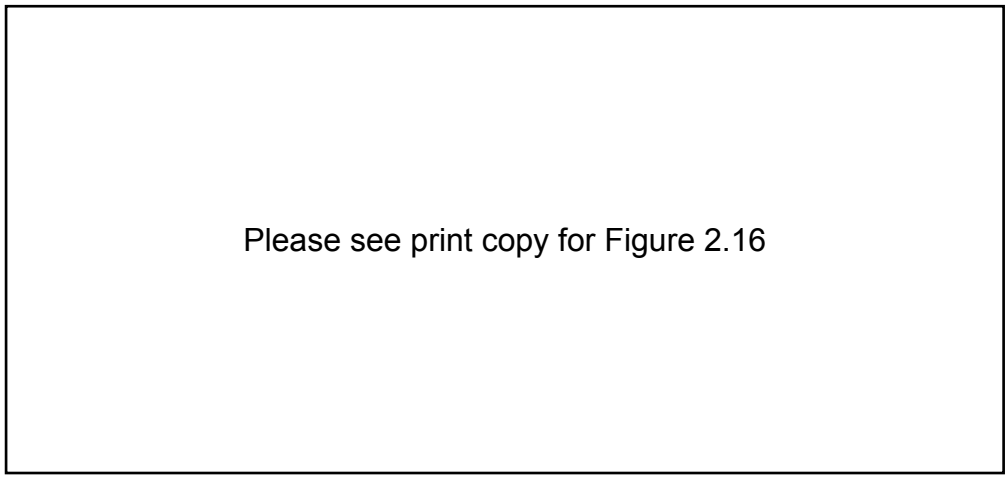
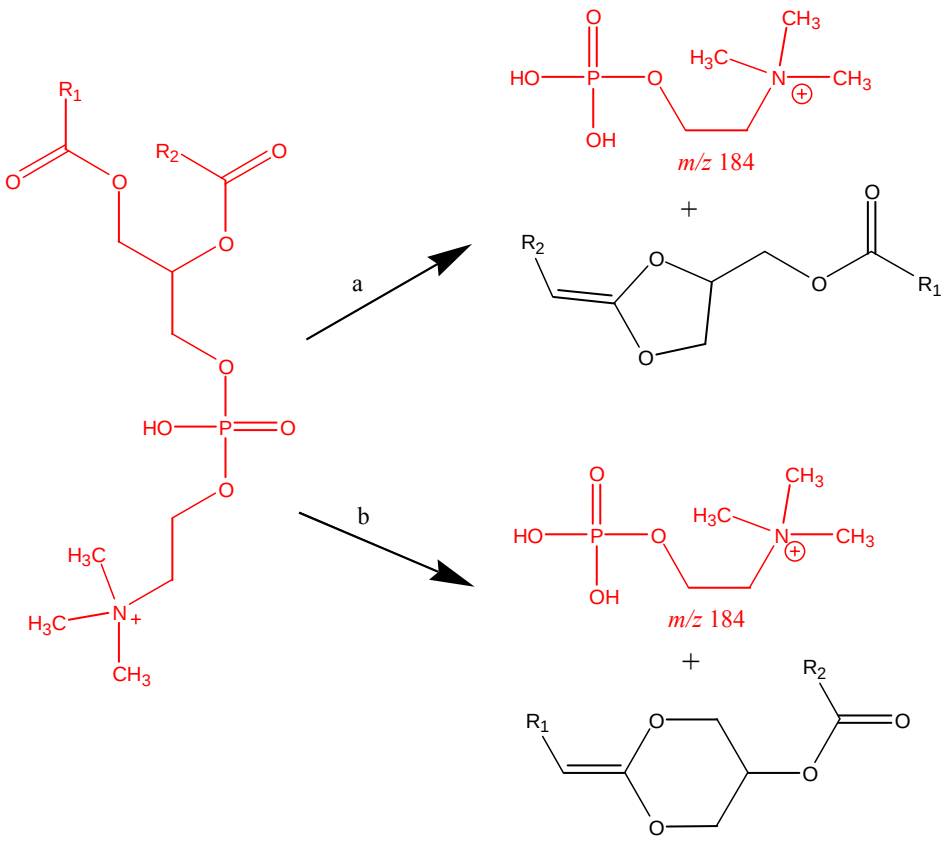


Figure 2-16: ***MS/MS spectrum obtained from the CID of PC(16:0,18:1) $[M+H]^+$ ions.***
The ion at m/z 184 is the characteristic phosphocholine cation. Adapted from Hsu & Turk (2003).



Scheme 1: ***Two proposed fragmentation pathways that produce phosphocholine cations.***

MS/MS of Lithiated Ions

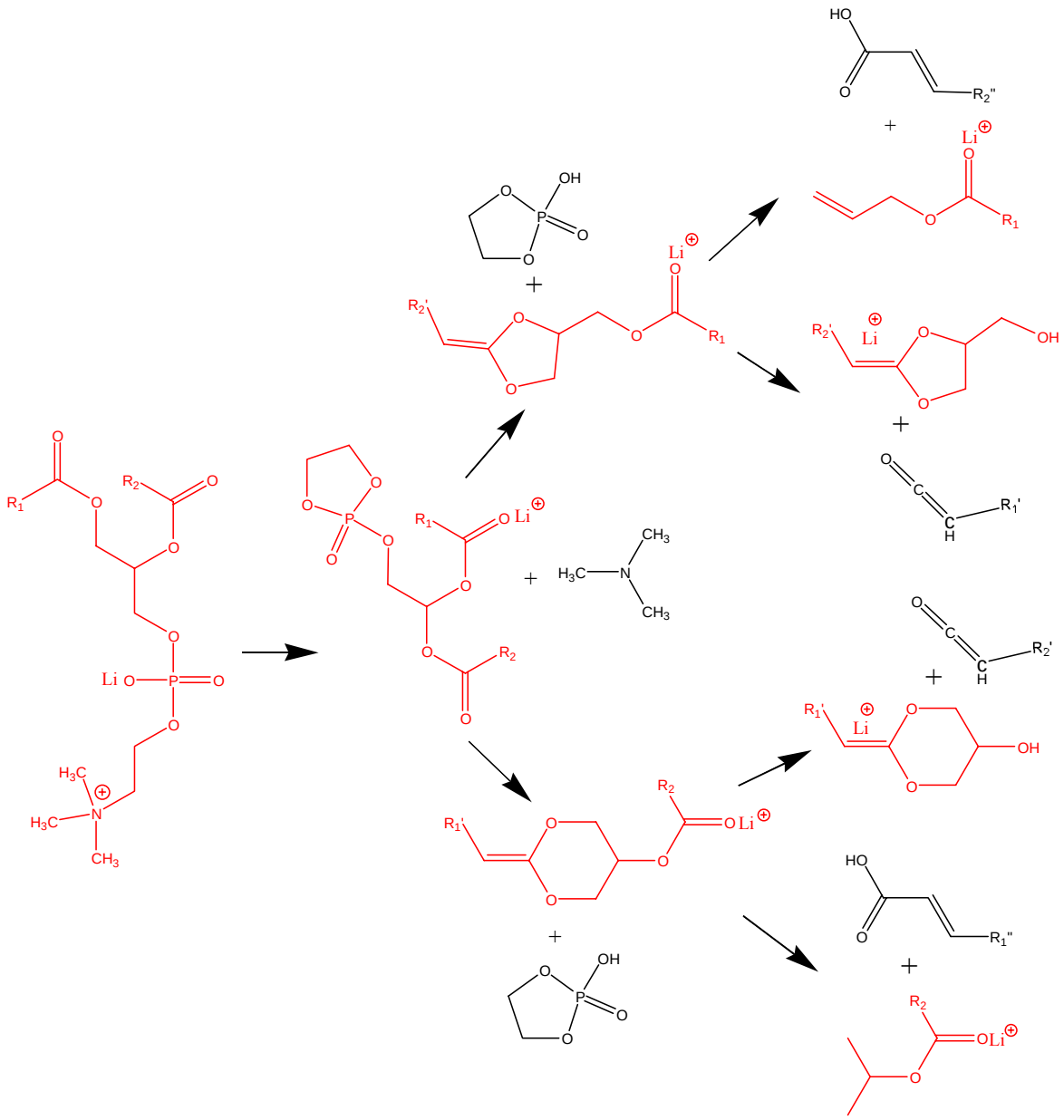
In contrast to the MS/MS analysis of $[M+H]^+$ ions, the CID of [M+alkali metal]$^+$ ions produces product ions corresponding to the neutral loss of both the *sn*-1 and *sn*-2 FA constituents providing a more informative spectrum. While sodiated molecular ions are sometimes seen in biological samples as naturally occurring adducts (Williams *et al.*, 2000) $[M+Li]^+$ ions are more commonly used in the structural characterisation of phosphatidylcholines. The use of lithium provides more abundant informative product ions than those of the $[M+Na]^+$ product ions (Ramanadham *et al.*, 1998; Hsu *et al.*, 2000; Ramanadham *et al.*, 2000). The major ions produced from CID of lithiated PC species are

from the neutral loss of trimethylamine ($[M+Li-59]^+$) followed by the further loss of either O,O'-dimethylenephosphoric acid ($[M+Li-183]^+$) or lithiated O,O'-dimethylenephosphoric acid ($[M+Li-189]^+$) (Hsu *et al.*, 1998; Hsu & Turk, 2003). In addition, peaks are also produced from the neutral loss of the FAs ($[M+Li-R_xCOOH]^+$) or the lithiated FAs ($[M+Li-R_xCOOLi]^+$) and by the same losses from the $[M+Li-59]^+$ ions ($[M+Li-59-R_xCOOH]^+$ and $[M+Li-59-R_xCOOLi]^+$) (Hsu *et al.*, 1998; Hsu & Turk, 2003).[5] An example of an MS/MS spectrum obtained from lithiated PC(16:0,20:4) ions displaying these characteristic product ions is shown in Figure 2-17 and some proposed pathways for their formation are displayed in Schemes 2 and 3. In summary, the use of lithium adducts greatly improves the structural information obtained from MS/MS analysis of PC species.

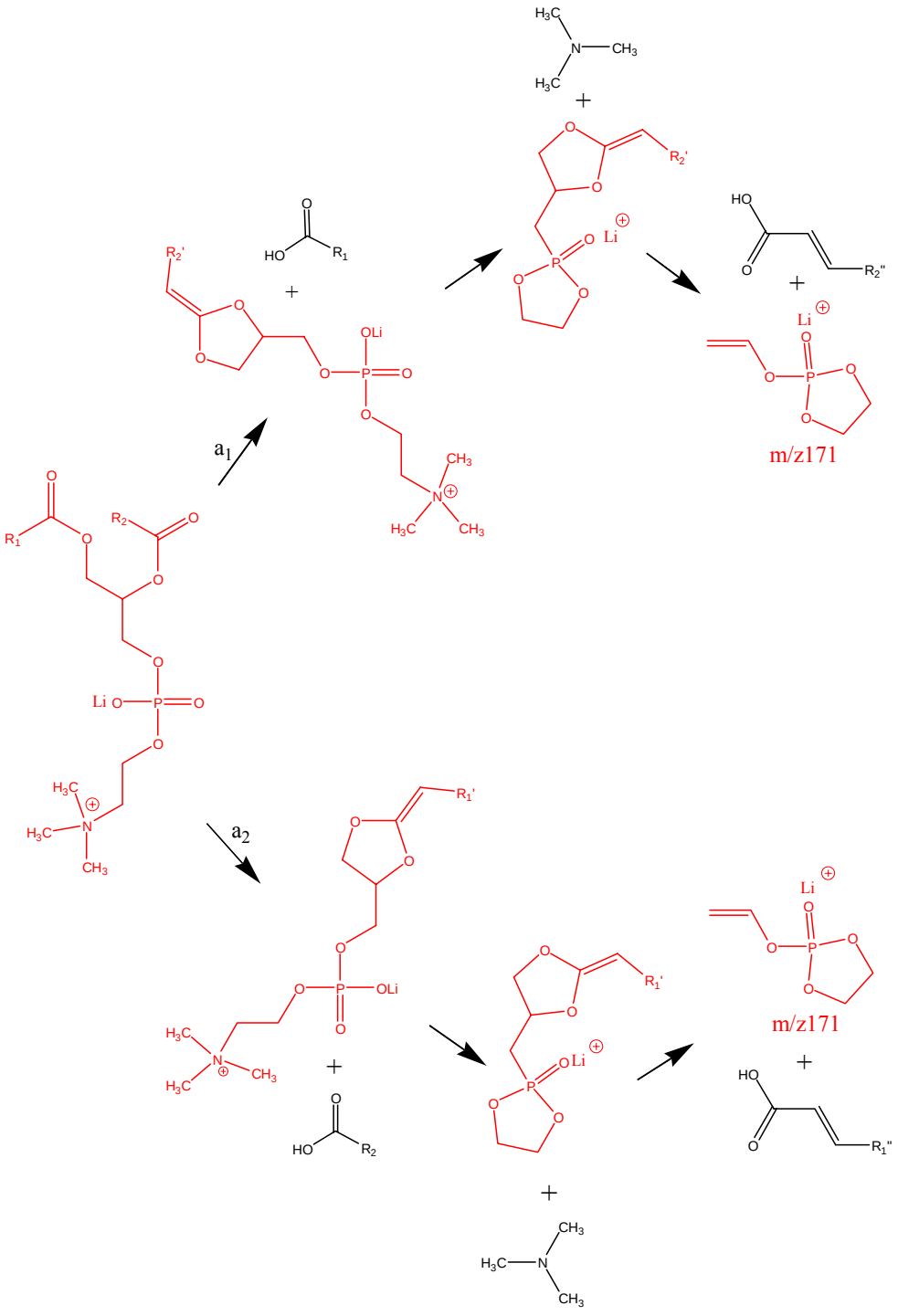
Please see print copy for Figure 2.17

Figure 2-17: ***MS/MS spectrum obtained from the CID of PC(16:0,18:1) $[M+Li]^+$ ions.***
The ions at m/z 510 and 484 represent the neutral loss of the two free fatty acids and at m/z 504 and 478 the two-lithiated fatty acids from the lithiated molecular ion. The ions at m/z 451 and 425 represent the neutral loss of the two free fatty acids from the $[M+Li-59]^+$ ion. Adapted from Hsu et al. (1998).

[5] Where x = 1 or 2



Scheme 2: ***Proposed fragmentation pathways of lithiated PC phospholipids with an initial loss of trimethylamine***



Scheme 3*: Proposed fragmentation pathways of lithiated PC phospholipids with an initial neutral loss of a fatty acid*

2.3.2.b Acidic Phospholipid Species

Negative ion ESI-MS produces abundant $[M-H]^-$ ions of PA, PG, PS and PI species (Kerwin *et al.*, 1994; Ivanova *et al.*, 2001). Abundant PE $[M-H]^-$ ions are also observed if the pH of the sample solution is increased (Han *et al.*, 2000). An example of a negative ion ESI-MS spectrum can be seen in Figure 2-18. PE and PS $[M+H]^+$ and [M+alkali metal]$^+$ ions may also be observed in positive-ion ESI-MS spectra, however, they are of relatively low abundance (Kerwin *et al.*, 1994; Brugger *et al.*, 1997).

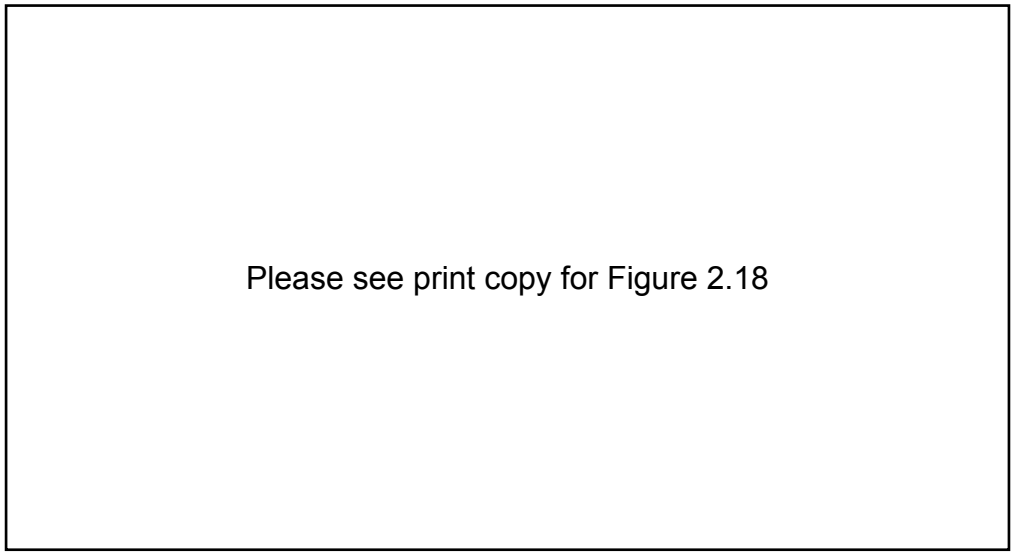


Figure 2-18: ***Negative ion ESI-MS spectrum of a total lipid extract from Chinese hamster ovary cells with various PE, PS and PI species labelled.*** *Both PA and PG species also produce $[M-H]^-$ ions, however, none are labelled on this spectrum. Adapted from Brugger et al. (1997)*

MS/MS of Deprotonated Ions

The MS/MS analysis of deprotonated phospholipid molecular ions produces a spectrum containing more structurally useful product ions than can be obtained from protonated and even lithiated species. Initial ESI-MS studies on the CID of the $[M-H]^-$ ions

demonstrated that abundant product ions are produced corresponding to the carboxylate ions of the acyl FA groups esterified to the glycerol backbone (Kerwin *et al.*, 1994). In addition, negative ion MS/MS also produces product ions characteristic of specific head groups (Brugger *et al.*, 1997).

The CID of PA $[M-H]^-$ ions produces product ions through three separate pathways. They are; i) the neutral loss of the two free FAs ($[M-H-R_xCOOH]^-$), ii) the neutral loss of the FAs as ketenes ($[M-H-R'_xCH=C=O]^-$) and, iii) the formation of the FA carboxylate anions ($[R_xCO_2]^-$). An example MS/MS spectrum obtained from the CID of deprotonated PA(16:0,18:1) ions can be seen in Figure 2-19 and the pathways for the formation of these product ions in Schemes 4 and 5. The three aforementioned pathways appear to be constant in all acidic phospholipid species with the addition of specific ions related to the species headgroup as discussed later. Data obtained by Hsu & Turk (2000c) indicates that the neutral loss of the free FA from both the *sn*-1 and *sn*-2 positions is more prevalent than the loss of the corresponding ketenes. Their results also suggest that the production of the *sn*-1 carboxylate anion is more favourable than that of the *sn*-2. This agrees with the observations of Hvattum *et al.* (1998) that PA species have a preferential formation of the *sn*-1 FA carboxylate anion and that the preference for this fragmentation pathway is augmented by increasing collision energy. In a low-energy source fragmentation experiment, Hsu & Turk (2000c) observed a large increase in the abundance of $[M-H-R_2COOH]^-$ ions with respect to other fragments. This suggests that a significant number of the *sn*-1 carboxylate anions may be produced by secondary fragmentation of $[M-H-R_2COOH]^-$ ions (Scheme 5, pathway c'_{2b}). This may occur at laboratory frame collision energies as low as 15 eV as the *sn*-1 carboxylate anion is known to be more abundant even at this low energy (Hvattum *et al.*, 1998)

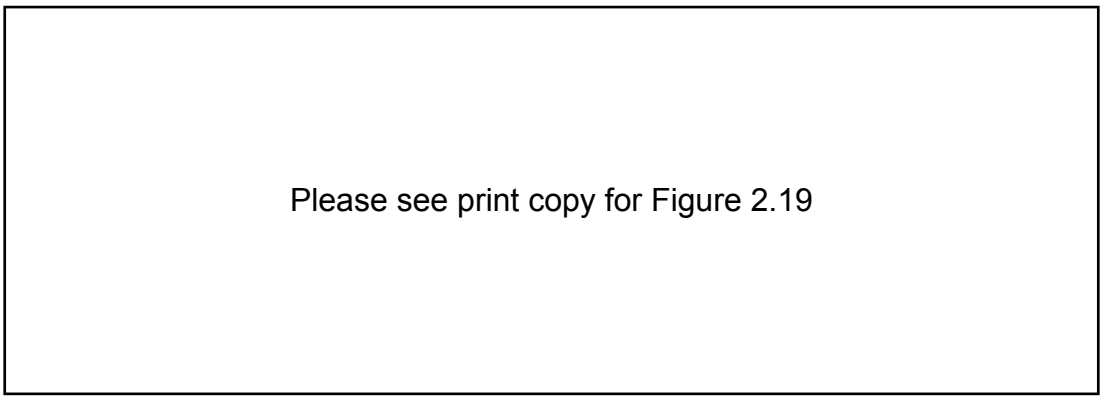
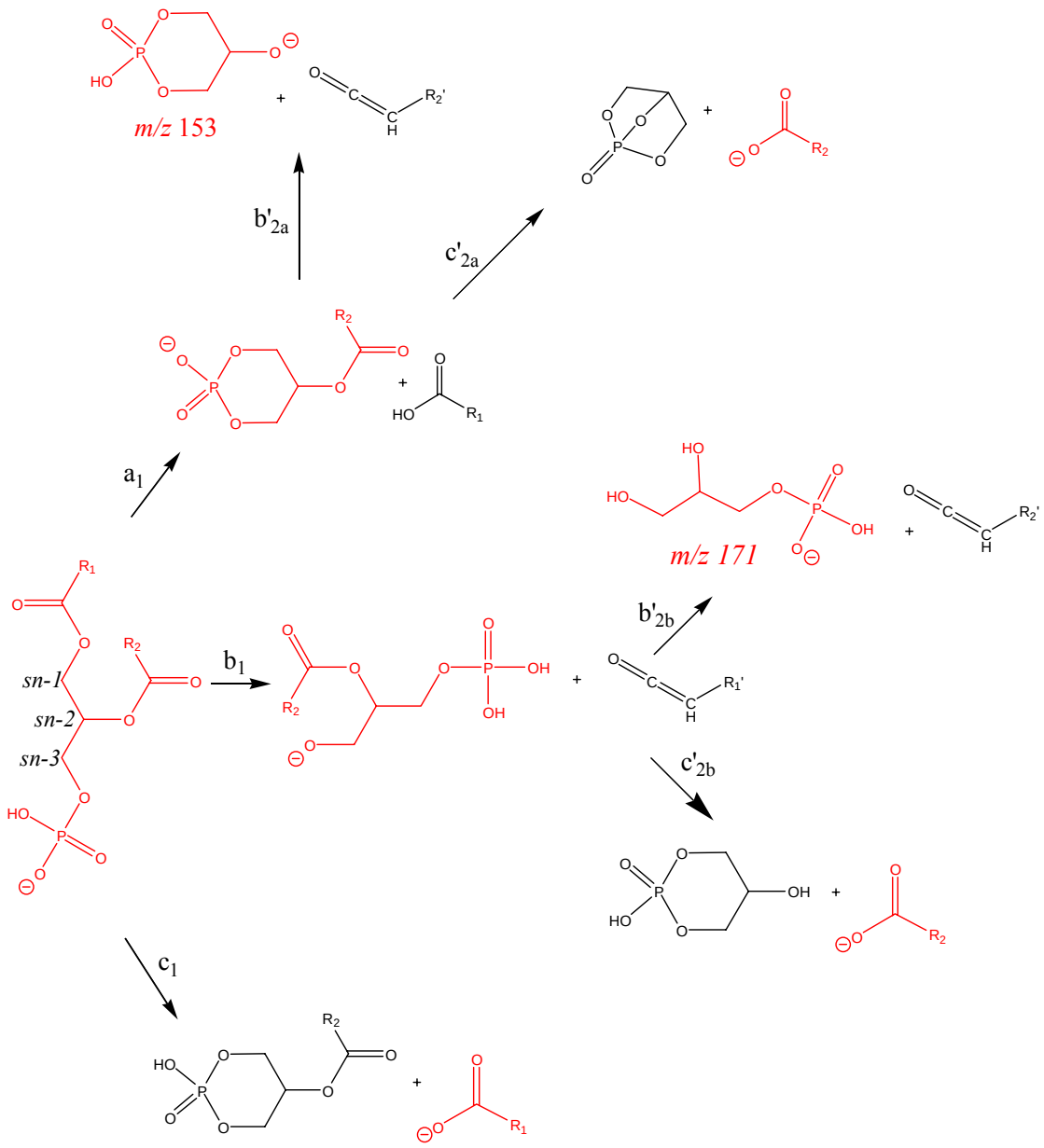
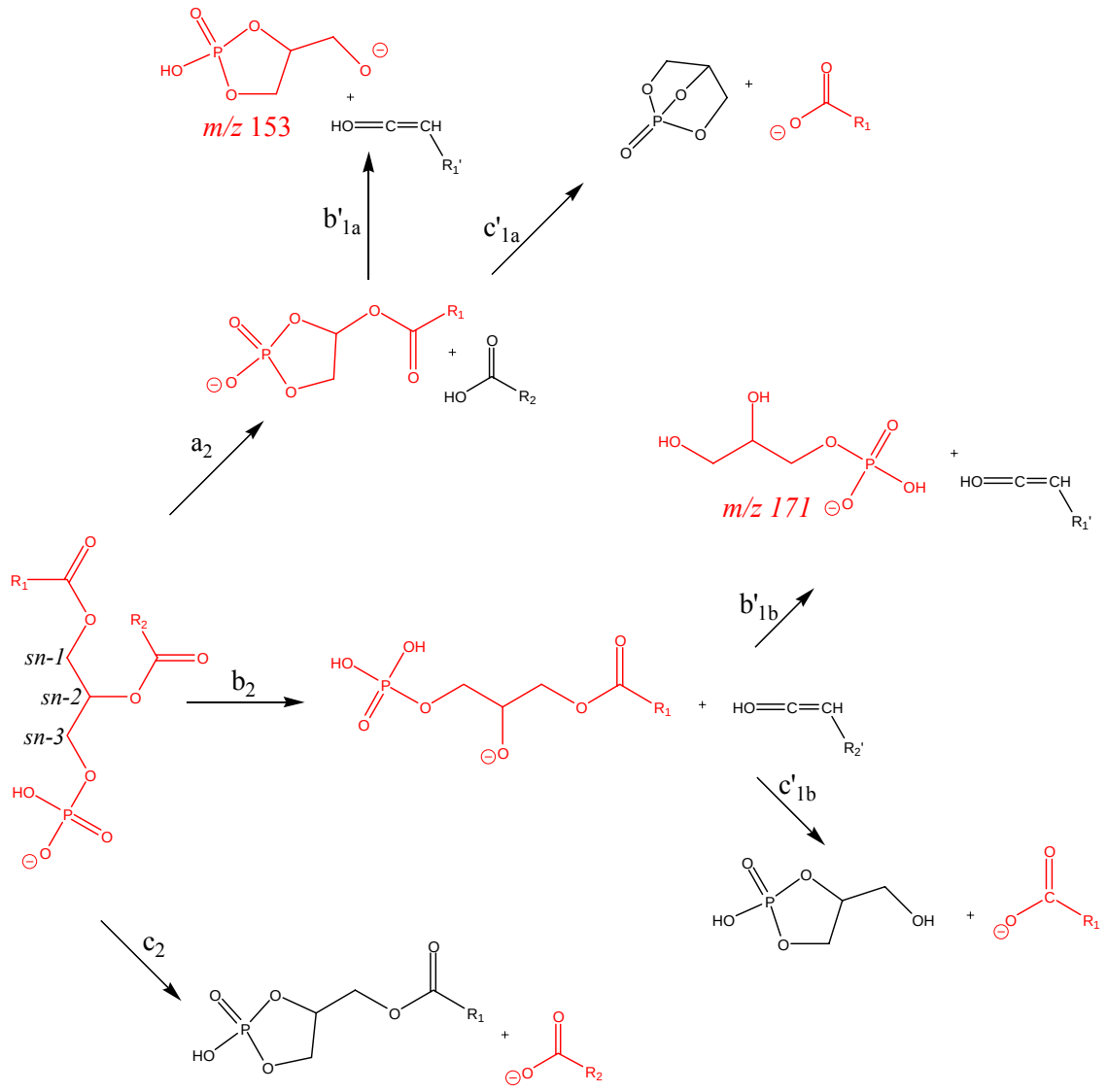


Figure 2-19: ***MS/MS spectrum obtained from the CID of PA(16:0,18:1) [M−H]¯ ions.***
The ion at m/z 255 is the sn-1 anion (palmitate) and at m/z 281 the sn-2 anion (oleate). The ions at m/z 391 and 417 represent the neutral loss of the two free fatty acids and at m/z 409 and 435 represent the neutral loss of the two fatty acids as ketenes. Adapted from Hsu & Turk (2000c).



Scheme 4: ***Proposed fragmentation pathways of deprotonated PA occurring at the sn-1 FA***



Scheme 5: ***Proposed fragmentation pathways of deprotonated PA occurring at the sn-2 FA***

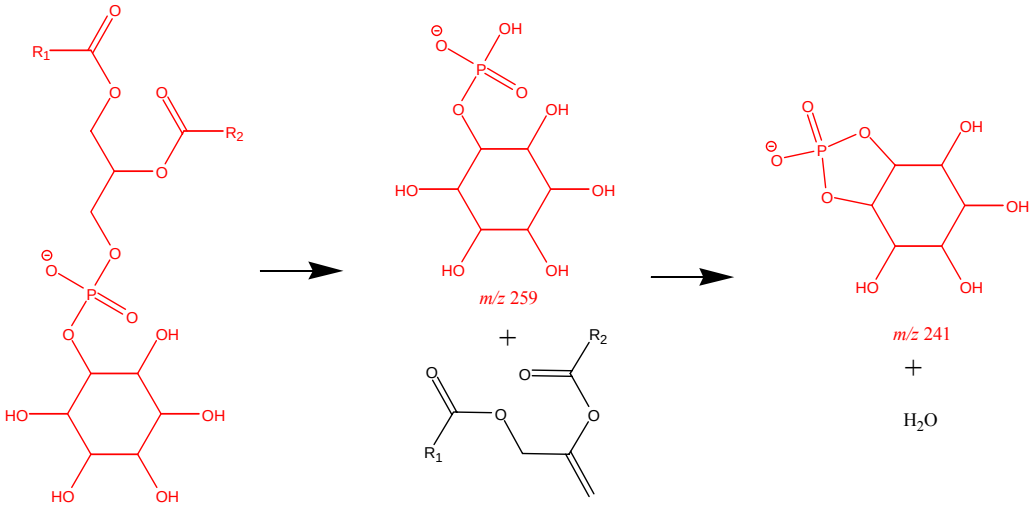
The CID of deprotonated PI molecular ions produces similar product ions to those seen for PA with additional dehydrated inositol phosphate ions observed at *m/z* 241 and $[M-H-R_xCOOH-162]^-$ ions produced from the neutral loss of dehydrated inositol from the $[M-H-R_xCOOH]^-$ ions as seen in Figure 2-20. The dehydrated head group ions may

be produced by the initial formation of the inositol phosphate ion (*m/z* 259) followed by cyclisation and dehydration as displayed in Scheme 6. Ion abundances for PI are also similar to PA with the exception of the carboxylate anions. A study by Hvattum (1998) found that PI had a comparable abundance of both the *sn*-1 and *sn*-2 FA carboxylate anions at collision energies up to 30 eV and a preferential formation of *sn*-1 anions at higher energies.

Hsu and Turk (2000b) have also presented data showing a similar fragmentation, however, it suggests that the abundance of the *sn*-1 anion does not exceed that of the *sn*-2 anion until the laboratory frame collision energy reaches 45 eV. An explanation for the slight difference in results between the two studies is the selection of molecular species used when studying a range of collision energies. Hvattum *et al.* (1998) analysed the MS/MS spectra of deprotonated PI(18:0,20:4) ions selected from a mixture of PI species obtained from human blood. While the selection of this molecular ion reduces the chance of positional isomers, the *sn*-2 carboxylate anion (arachidonate, *m/z* 303) is known to undergo secondary fragmentation to form an ion at *m/z* 259 through the loss of CO_2 (Kerwin *et al.*, 1996), with only 20% of the arachidonate ion surviving at a collision energy of 35 eV (Hsu & Turk, 2000b). In contrast, Hsu and Turk studied the fragmentation pattern of deprotonated synthetic PI(16:0,18:2). Their results suggest that when deprotonated PI ions are subject to low energy CID, carboxylate ions are produced predominately by the nucleophilic attack of the phosphate anionic charge site on the *sn*-1 and *sn*-2 FAs. At higher collision energies, the carboxylate ions may also arise from the secondary fragmentation of the $[M-H-R'_2CH=C=O]^-$ increasing the abundance of the *sn*-1 carboxylate ion. This is a similar event as described for PA (Scheme 5, pathway c'_{2b}), however; greater laboratory frame collision energy would be required for the analogous fragmentation of the more massive lyso-phosphatidylinositol species.

Please see print copy for Figure 2.20

Figure 2-20: ***MS/MS spectrum obtained from the CID of PI(16:0,18:2) [M−H]¯ ions.***
The ion at m/z 255 is the sn-1 anion (palmitate) and at m/z 279 the sn-2 anion (linoleate). The ion at m/z 241 is the characteristic inositolphosphate ion. The ions at m/z 553 and 577 represent the neutral loss of the two free fatty acids and at m/z 571 and 595 represent the neutral loss of the two fatty acids as ketenes. Adapted from Hsu & Turk (2000b).



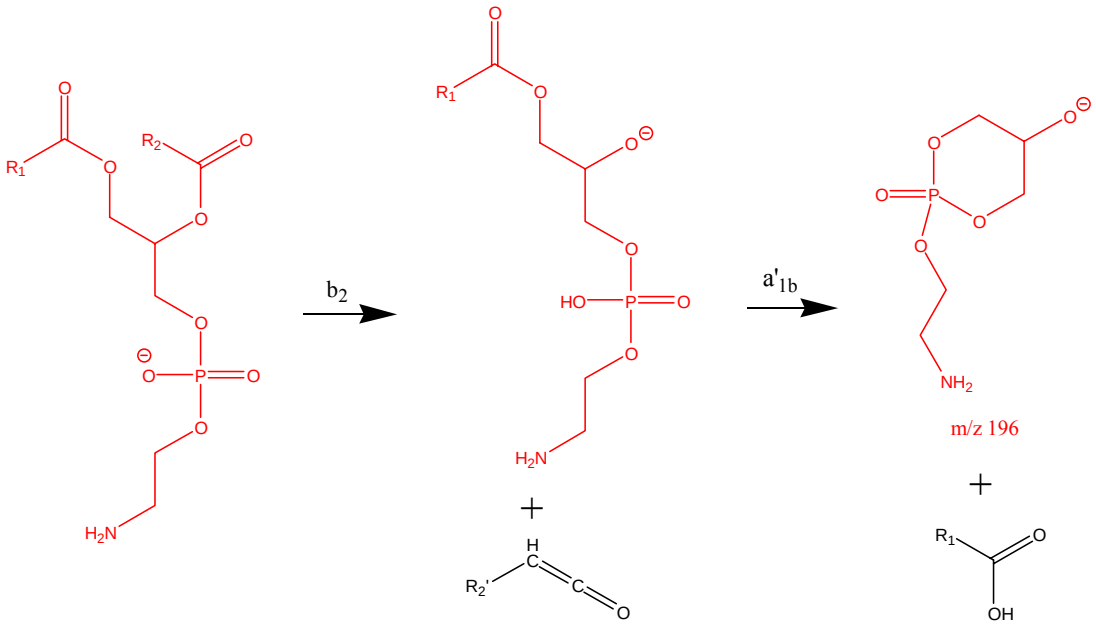
Scheme 6*: Proposed fragmentation pathway of deprotonated PI that produces the characteristic inositol phosphate anion*

As with the other more-acidic phospholipids (PA, PI, PS, PG) the major ions observed in the MS/MS spectra of deprotonated PE species are the two FA carboxylate anions when

collision energy is 40 eV (Han & Gross, 1995). A characteristic dilyso-phosphatidylethanolamine ion can also be observed at *m/z* 196 derived from the neutral loss of a FA ketene followed by the neutral loss of the other FA as shown in Scheme 7. An example MS/MS spectrum of PE(16:0,18:2) can be seen in Figure 2-21. PE is known to have a preferential loss of the *sn*-2 FA carboxylate ion at low collision energies whereas the loss of the *sn*-1 FA is favoured at collision energies above 40 eV (Hvattum *et al.*, 1998; Hsu & Turk, 2000d). This is a similar pattern to that observed for PI and may also be explained by the secondary fragmentation of the $[M-H-R'_xCH=C=O]^-$ increasing the abundance of the *sn*-1 carboxylate ion as collision energy increases (*c.f.*, the c'_{2b} pathway in scheme 5). The major difference seen in PE ions when compared to the more-acidic species is the preferential neutral loss of the ketene rather than that of the FA. The loss of these two species is a competitive process governed by the gas phase basicity of the precursor ion. Therefore, the PE phospholipids being more basic than the other species favour the loss of the ketene while the other more-acidic species favour the loss of the FA (Hsu & Turk, 2000d).

Please see print copy for Figure 2.21

Figure 2-21: ***MS/MS spectrum obtained from the CID of PE(16:0,18:2) $[M-H]^-$ ions.***
The ion at m/z 255 is the sn-1 anion (palmitate) and at m/z 279 the sn-2 anion (linoleate). The low abundance ion at m/z 196 is the characteristic dilyso-phosphoethanolamine ion. The ions at m/z 434 and 458 represent the neutral loss of the two free fatty acids and at m/z 452 and 476 represent the neutral loss of the two fatty acids as ketenes. This figure has been adapted from Hsu & Turk (2000d).

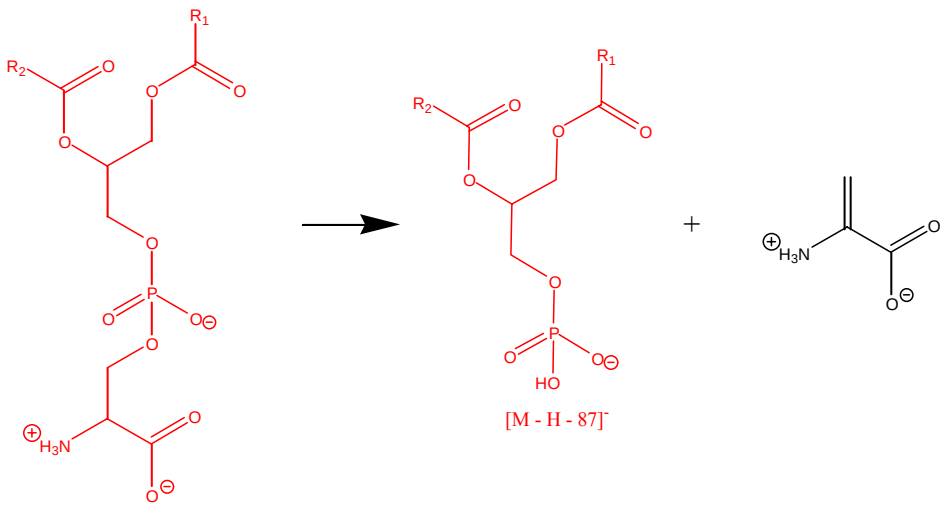


Scheme 7: ***Proposed fragmentation pathway of deprotonated PE that produces the characteristic dilyso-phosphoethanolamine anion***

The literature pertaining to the CID of deprotonated PS species is less extensive than that of other glycerophospholipid species. It appears that PS species undergo an initial loss of didehydroalanine (87 Da) as displayed in Scheme 8. They then follow a pattern similar to that of PA species producing $[M–H–C_3H_3NO_2–R_xCOOH]^-$, $[M–H–C_3H_3NO_2–R'_xCH=C=O]^-$ and $[R_xCO_2]^-$ ions as seen in Figure 2-22.

Please see print copy for Figure 2.22

Figure 2-22: ***MS/MS spectrum obtained from the CID of PS(18:0,18:1) [M−H]¯ ions.***
The ion at m/z 283 is the sn-1 anion (stearate) and at m/z 281 the sn-2 anion (oleate). The ion at m/z 701 is the characteristic neutral loss of didehydroalanine. The ions at m/z 419 represent the neutral loss of the sn-2 fatty acid and at m/z 437 the neutral loss of the sn-2 fatty acid as a ketene from the m/z 701.5 ions. This figure has been adapted from Brugger et al. (1997).



Scheme 8: ***Proposed fragmentation pathway of deprotonated PS that produces the characteristic neutral loss of didehydroalanine.***

The CID of deprotonated PG ions also follows the three pathways of fragmentation as described for PA (Schemes 4 and 5) (Hsu & Turk, 2001). However, PG being less acidic than PA, PI and PS has a preferential loss of the FAs as ketenes rather than free FAs in a similar manner to PE. In addition, the MS/MS spectrum of deprotonated PG ions also displays the neutral loss of glycidol (74 Da) as shown in Scheme 9. This ion then behaves as a deprotonated PA ion and undergoes further fragmentation through the three previously described pathways, producing a second set of neutral loss ions as can been seen at *m/z* 391, 409 and 415 in Figure 2-23.

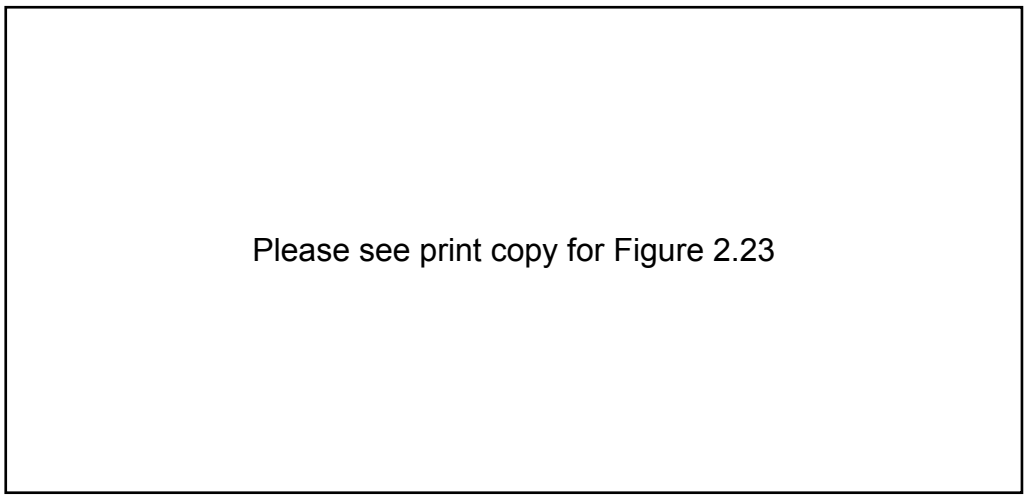
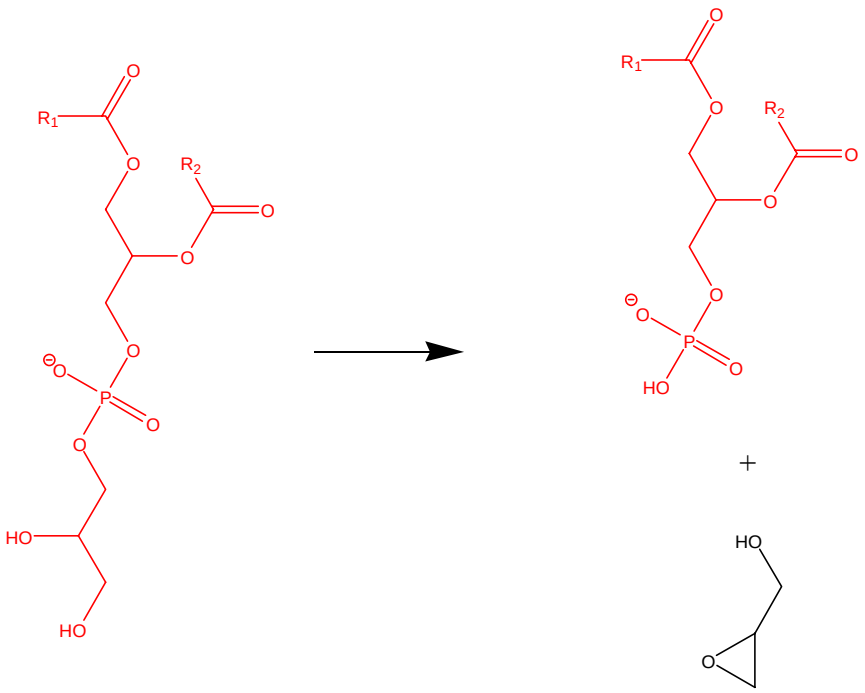


Figure 2-23: ***MS/MS spectrum obtained from the CID of PG(16:0,18:2) [M−H]¯ ions.***
The ion at m/z 255 is the sn-1 anion (palmitate) and at m/z 279 the sn-2 anion (linoleate). The ion at m/z 671 is the characteristic neutral loss of glycidol. The ions at m/z 465 and 489 represent the neutral loss of the two free fatty acids and at m/z 483 and 507 represent the neutral loss of the two fatty acids as ketenes. The ions at m/z 391,409 and 415 represent similar losses from the m/z 671 ions. This figure has been adapted from Hsu & Turk (2000a).



Scheme 9: ***Proposed fragmentation pathway of deprotonated PG that produces the characteristic neutral loss of glycidol.***

In summary, deprotonated acidic phospholipids fragment under CID through three major pathways. They are; i) the neutral loss of the two free FAs ($[M-H-R_xCOOH]^-$), ii) the neutral loss of the FAs as ketenes ($[M-H-R'_xCH=C=O]^-$) and, iii) the formation of the FA carboxylate anions ($[R_xCO_2]^-$). The degree of fragmentation occurring via each of these pathways differs depending on the head group and the degree of collision energy applied. In addition, the MS/MS spectra of deprotonated phospholipid species also display ions characteristic of their specific headgroup.

MS/MS of Lithiated Ions

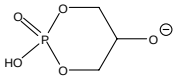
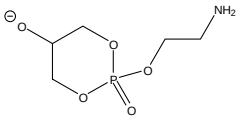
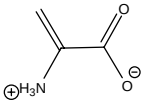
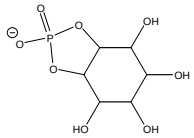
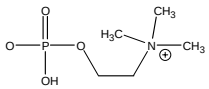
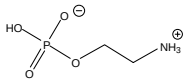
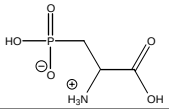
Lithium adducts of PE (Hsu & Turk, 2000e; Ho *et al.*, 2003), PG and PS (Ho *et al.*, 2003) have also been studied for structural characterisation using positive ion MS/MS. These

species, however, can be easily characterised by the CID of $[M-H]^-$ ions. This negates the need for such manipulations that can create multifarious spectra as cations compete for complexation. A study by Ho and colleagues investigating various alkali, alkaline earth and transitional metal adducts of these species found that the divalent metals, such as nickel and cobalt produced ions of greater abundance from the neutral loss of the two FAs ($[M+(Ni \text{ or } Co)-R_xCOOH]^+$) than the monovalent species (Ho *et al.*, 2003). This improves the ease with which the positive ion MS/MS spectra can be interpreted. Unfortunately, the divalent metals would not form complexes with PC species presumably because of their zwitterionic nature (Ho *et al.*, 2003).

2.3.2.c Precursor ion and neutral loss scanning

As mentioned above each of the different phospholipid head groups produce characteristic product ions after CID of both protonated and deprotonated molecular species. For example, CID of the $[M-H]^-$ ions of PE phospholipids produces characteristic ions at *m/z* 196 (dilyso-ethanolamine$-H_2O$), of PI phospholipids at *m/z* 241 (inositolphosphate$-H_2O$) and of PS species a loss of 87 Da (serine$-H_2O$). CID of PC and SM $[M+H]^+$ ions produce a product ion at *m/z* 184 (phosphocholine) while ions corresponding to the neutral loss of 141 Da (phosphoethanolamine) are observed from protonated PE and a neutral loss of 185 Da (phosphoserine) from protonated PS (Brugger *et al.*, 1997). Table 2-1 contains a list of these fragments and their likely structures.

Table 2-1: ***Characteristic headgroup fragments observed in the MS/MS spectra of $[M-H]^-$ and $[M+H]^+$ ions***

Phospholipid Species	Fragment or Neutral Structure	*m/z* / Neutral Loss
Negative ions		
All glycerophospholipids		*m/z* 153
PE		*m/z* 196
PS		87 Da
PI		*m/z* 241
Positive ions		
PC and SM		*m/z* 184
PE		141 Da
PS		185 Da

The typical product ions displayed in Table 2-1 have been exploited in order to separate species within a mixture by precursor ion or neutral loss scans (Brugger *et al.*, 1997; Schneiter *et al.*, 1999; Blom *et al.*, 2001; Koivusalo *et al.*, 2001). This method of separating phospholipid species was first introduced using FAB MS in the early 90s (Cole & Enke, 1991) and is particularly useful if one wishes to analyse a specific lipid in a complex mixture without additional separation steps such as HPLC or TLC. For example, a study by Lehmann *et al.* (1997) used this technique to characterise and quantify PC species in a total lipid extract from rat bile. Precursor ion/neutral loss scanning is also advantageous in reducing the complexity of negative ion MS spectra as it allows the

removal of any species with overlapping mass, *i.e.*, combinations of different head groups and FAs that have the same mass. Such analyses have traditionally been employed using triple quadrupole instruments that are ideally suited to such scans (Lehmann *et al.*, 1997; Hsu *et al.*, 2003). Nevertheless, recent advances in hybrid Q-ToF instruments that improve the poor duty cycle of ToF analysers when used in this mode (Chernushevich, 2000) have also seen such instruments used in this type of analysis (Ekroos *et al.*, 2002).

2.3.2.d Quantification

The quantification of phospholipids by ESI-MS is not straightforward as the relationship between ion intensity and analyte concentration is not linear across all species. A number of factors are known to influence the ion intensity of a particular phospholipid molecular species, such as acyl chain length and its degree of unsaturation, the latter having a greater effect as phospholipid concentration increases (Koivusalo *et al.*, 2001). The largest influence on instrument response, however, is the nature of the head group that has been shown to have a significant effect upon the ionisation efficiency of the phospholipid classes (Han & Gross, 1994; Koivusalo *et al.*, 2001; Zacarias *et al.*, 2002). There is also the likelihood of ion suppression effects (as discussed in section 2.1.1) where more efficiently ionised phospholipid classes scavenge charge from other species during the ionisation process. Consequently, peak intensities cannot be used for quantification between lipid classes. When using precursor ion or neutral loss scanning to separate and quantify phospholipid classes relative fragmentation efficiency presents a further variable. As would be expected, the effect of acyl chain length is even greater after CID as larger molecules will not fragment as efficiently and may be underrepresented (Brugger *et al.*, 1997).

In order to overcome these limitations a number of strategies have been employed. Isotopically labelled internal standards have been used to measure a particular component within a mixture (Harrison *et al.*, 1999), however, the application of this technique to

whole scale quantification of all phospholipids in a mixture is not practical. Unnatural synthetic phospholipids can also be utilised as internal standards. Han and Gross have undertaken a number of quantitative studies using a single synthetic PC molecular species as an internal standard to quantify all PC species, a single PE molecular species to quantify all PE species and a single PG molecular species for the quantification of all anionic phospholipid classes (Han *et al.*, 2000; Pike *et al.*, 2002). While the effect of acyl chain unsaturation is likely to be minimal at the concentrations used in these studies, this technique does not address this effect and does not compensate for the variation in ionisation efficiencies between PG, PA, PS and PI species. A recent study has suggested that a minimum of three internal standards of varying chain length (both saturated and unsaturated) for each head group be included (Koivusalo *et al.*, 2001). One report, using precursor ion scanning of *m/z* 184 to analyse PC species, used four saturated PC molecular species as internal standards with varying chain lengths to normalise the MS/MS data (Brugger *et al.*, 1997). Another approach for overcoming the non-linearity between concentration and ion intensity involves the calculation of a response factor from a calibration curve of the log of observed ion intensity versus the log of the analyte concentration (Zacarias *et al.*, 2002).

While chromatographic techniques such as HPLC and TLC followed by MS may also be used for quantification and characterisation further sample handling is required. Furthermore, a recent study by DeLong *et al.* (2001) demonstrated that both HPLC and TLC result in the preferential loss of phospholipid species with three or less double bonds, possibly owing to strong, non-specific binding to silica gel and compounded by the higher solubility of the more polyunsaturated phospholipids in the mobile phase. This results in an overestimation of phospholipid unsaturation.

2.3.2.e Research Applications

In recent years a number of studies have been published in the scientific literature that have employed ESI-MS to study the effects of disease states on phospholipid content in a variety of tissues. A study on grey and white matter in post-mortem human brain used ESI-MS to demonstrate reductions in a number of plasmenyl PE molecular species in both of these tissues in the brain of Alzheimer's disease sufferers when compared to age-matched controls (Han *et al.*, 2001). Recent work by Williams *et al.* (2000) analysing the effect of myocardial ischemia and reperfusion on the phospholipid profile of rat cardiac myocyte nuclear membranes found that total PC and PE content was reduced after ischemia and reperfusion. This reduction was partially reversed by the inhibition of calcium-independent PLA_2 suggesting a controlled cleavage of the *sn*-2 FA, possibly to act in signal transduction as second messengers. Yet another recent study on rat myocardium suggest that there are large alterations in the abundance of several phospholipid molecular species in the heart of streptozotocin-induced diabetic rats (Han *et al.*, 2000). This was particularly apparent for PE(18:0,20:4) that displayed a 22 % decrease in the myocardium of the diabetic rats compared to controls.

A current area of interest in membrane biology is the presence of phase-separated lipid rafts (Simons & Ikonen, 1997; Brown & London, 1998; Pike, 2003; Pike, 2004). These regions have been linked to insulin signalling (Bickel, 2002), protein and lipid trafficking (Ikonen, 2001) and disease (Simons & Ehehalt, 2002; Takeda *et al.*, 2003). The common method of isolating lipid rafts is to exploit their lack of solubility in detergent, however, they may also be isolated by co-fractionation with small grape-like membrane invaginations termed caveolae (Smart *et al.*, 1995). ESI-MS studies are particularly applicable to this field and have already demonstrated a large variability in the lipid profile of rafts when isolated by different detergents (Schuck *et al.*, 2003). Another ESI-MS study examining caveolae co-fractionated rafts suggests that they are enriched in plasmenyl PE molecular species containing arachidonate (20:4 n-6) (Pike *et al.*, 2002).

It should be noted that while ESI-MS is gaining popularity in the analysis of phospholipid molecular species it is an emerging field and the implications of many of the findings are still undetermined.

2.3.3 MALDI-MS

The initial report on MALDI analysis of whole phospholipid species (Marto *et al.*, 1995) appeared shortly after the initial ESI papers (Han & Gross, 1994; Kerwin *et al.*, 1994; Kim *et al.*, 1994; Han *et al.*, 2000). The MALDI technique has not enjoyed the same rapid rise in popularity in this field as ESI did. Nevertheless, MALDI-MS analysis of phospholipids has also begun to gain popularity in recent years (Petkovic *et al.*, 2001; Ishida *et al.*, 2002; Al-Saad *et al.*, 2003a; Al-Saad *et al.*, 2003b). At present the mechanisms of MALDI ionisation of phospholipids is the major focus of research, however, techniques for rapid analysis of biological samples are also being developed (Ishida *et al.*, 2002). Fragmentation by post source decay (PSD) can also provide useful structural information and has been shown to produce similar fragmentation ions as seen in the CID of phospholipid molecular ions produced by FAB and ESI (Al-Saad *et al.*, 2003a; Al-Saad *et al.*, 2003b). MALDI has the advantage of low sensitivity towards impurities, such as buffer salts, making it well suited to the analysis of biological samples. Another interesting feature of MALDI analysis of phospholipids is its unique ability to detect all species in positive ions (Petkovic *et al.*, 2001; Schiller *et al.*, 2002). Unfortunately, molecular species form complex adducts with various numbers and combinations of various cations, *e.g.* $[M+H]^+$, $[M+H_n]^+$, $[M+H_n+Na_n]^+$, $[M+H_n+K_n]^+$, meaning even relative comparisons between treatments (as performed in the current study) are not possible. Furthermore, the suppression by PC on the detection of other molecular ions (Petkovic *et al.*, 2001) is problematic when analysing biological samples, particularly if attempting quantification.

Chapter 3

Development of ESI-MS Techniques for Analysing Phospholipids Using a Hybrid Quadrupole Time-of-Flight Mass Spectrometer

3.1 Introduction

Methods for analysing phospholipids using ESI-MS have been in practice since the mid 1990s (Han & Gross, 1994; Kerwin *et al.*, 1994; Kim *et al.*, 1994). Traditionally these methods have employed triple quadrupole instruments due to their cost effectiveness as a platform for low-resolution ESI-MS and ESI-MS/MS experiments. Triple quadrupole instruments have performance characteristics that differ from Q-ToF instruments (see Chapter 2) and as a result, the existing techniques for phospholipid analysis were assessed for the suitability of the Q-ToF instrument for achieving the aims of the project.

The first step was to optimise the Q-ToF 2 instrument settings to achieve the greatest signal in ESI-MS experiments. Once this was achieved, CID spectra were analysed to ensure accurate identification and characterisation of phospholipid molecular species. These spectra were compared to previous data obtained from triple quadrupole instruments for verification. In addition, no definitive data describing the fragmentation of plasmenyl PE or cardiolipin molecular species have been presented in the scientific literature. Accordingly, the CID spectra and fragmentation pathways of these lipids required clarification to ensure their accurate characterisation. Various scanning techniques, such as neutral loss and precursor ion scans, were also evaluated and compared with standard MS experiments to assess their effectiveness in separating lipid classes using the Q-ToF 2. Once all techniques were assessed, a standard method of producing and analysing the spectral data was then developed for use in subsequent studies. This method was then validated against established GC procedures.

In this chapter, the results of these analyses will be presented and discussed. The methods devised after assessment of these results will be reported in the methods section at the end of this chapter. In addition, novel data will be presented describing the fragmentation pathways of plasmenyl PE and cardiolipin phospholipids.

3.2 Results/Discussion

3.2.1 Optimisation of instrument settings

The first step in the development of techniques for MS analysis of a particular compound is to specifically 'tune' or optimise the instrument for the best possible combination of sensitivity and resolution. It is also important to impart enough energy to the analyte so that efficient ionisation can occur without excessive source fragmentation. Figure 3.1 displays a comparison between two negative ion ESI-MS spectra of the same lipid extract (containing all lipids, including free FAs and phospholipids) using two different cone voltages. When a cone voltage of 30 V was used (Figure 3.1, top panel) it is apparent that ionisation of the phospholipid molecular species was poor compared to the free FAs. In fact, the most abundant ion in the *m/z* range at which phospholipid molecular ions are observed (*m/z* 650 – 920) was 320 times less abundant than the most abundant FA ion. When the cone voltage was increased to 70 V (Figure 3.1, bottom panel) the spectrum obtained over the *m/z* 650 – 920 range has a greater signal-to-noise ratio. Furthermore, the most abundant anion in this range is more than 4 times more intense in this spectrum compared to the one obtained when a cone voltage of 30 V was used (in relation to the most abundant FA carboxylate anion). This suggests that excessive source fragmentation was not occurring at this cone voltage as the ratio of phospholipid molecular ions to FA carboxylate ions would decrease if this was the case. A range of cone voltages was tested from 30 V to 90 V with the greatest ratio of phospholipid to FA ions being observed at a cone voltage of 70 V. In positive ions very little difference in ion abundance was observed over a range of cone voltages and so a relatively low voltage (30 V) was chosen in order to ensure minimal in-source fragmentation. The instrument parameters found to best achieve these aims on the Q-ToF 2 are summarised in the methods section at the end of this chapter.

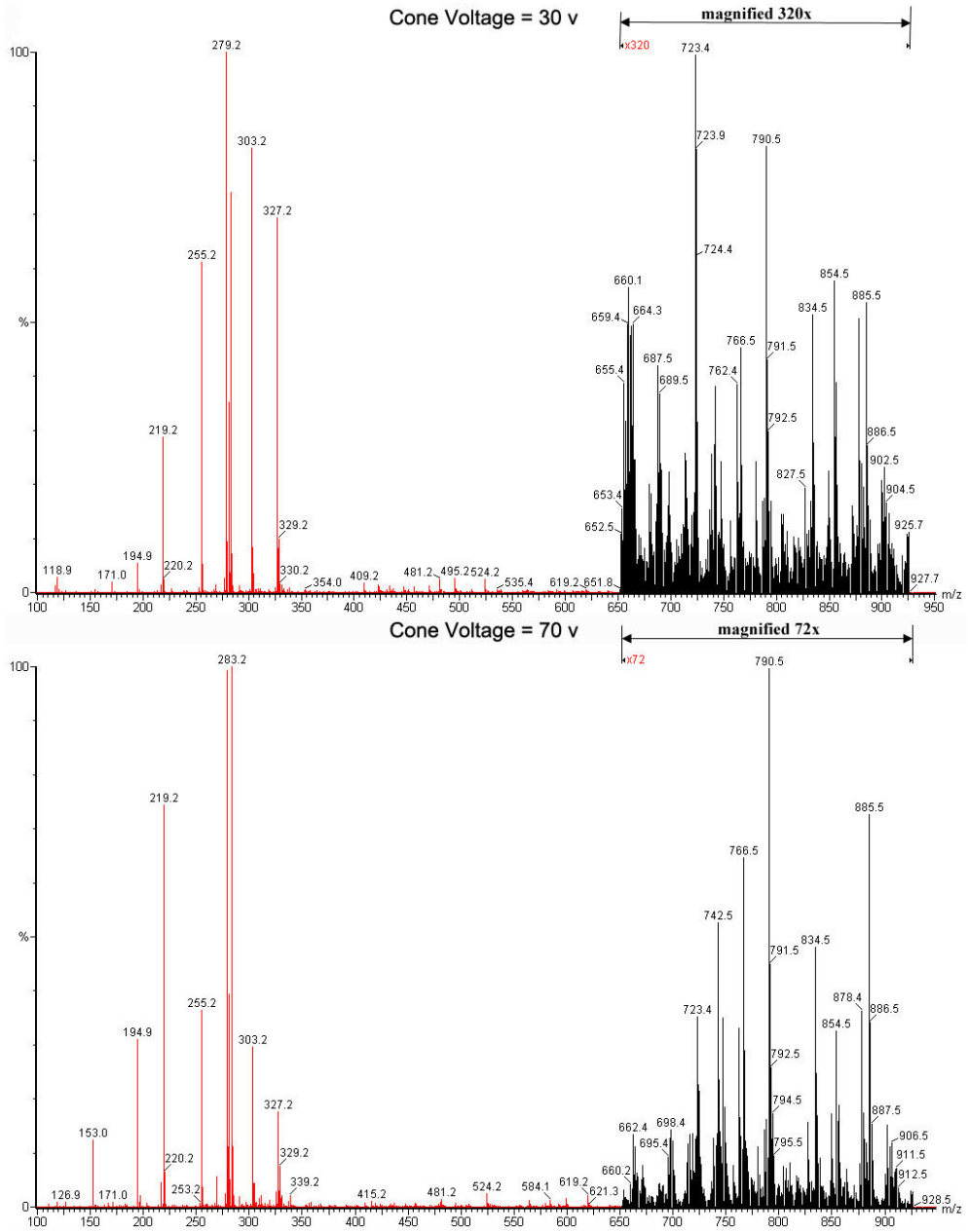


Figure 3-1: ***A comparison of negative ion ESI-MS spectra of the same total lipid extract from rat skeletal muscle using a cone voltage of 30 V (top) and 70 v (bottom).***

The m/z range at which whole phospholipid species are observed (650-920, shown in black) has been amplified in each spectrum until the most abundant ion in this range was ~ 100 % of the base peak. This amplification was 320 x for the top spectrum and 72 x for the bottom spectrum.

3.2.2 Positive ion MS

An example of a positive ion MS spectrum of a phospholipid extract from rat red vastus lateralis muscle is displayed in Figure 3-2. In this spectrum both protonated and sodiated ions are present for each molecular species creating a tortuous spectrum. A number of protonated PC species ranging in *m/z* from PC34:2 (*m/z* 758.7) to PC38:4 (*m/z* 810.6) were observed. Sodiated (+22 Da) cations were also observed for each of the PC species.

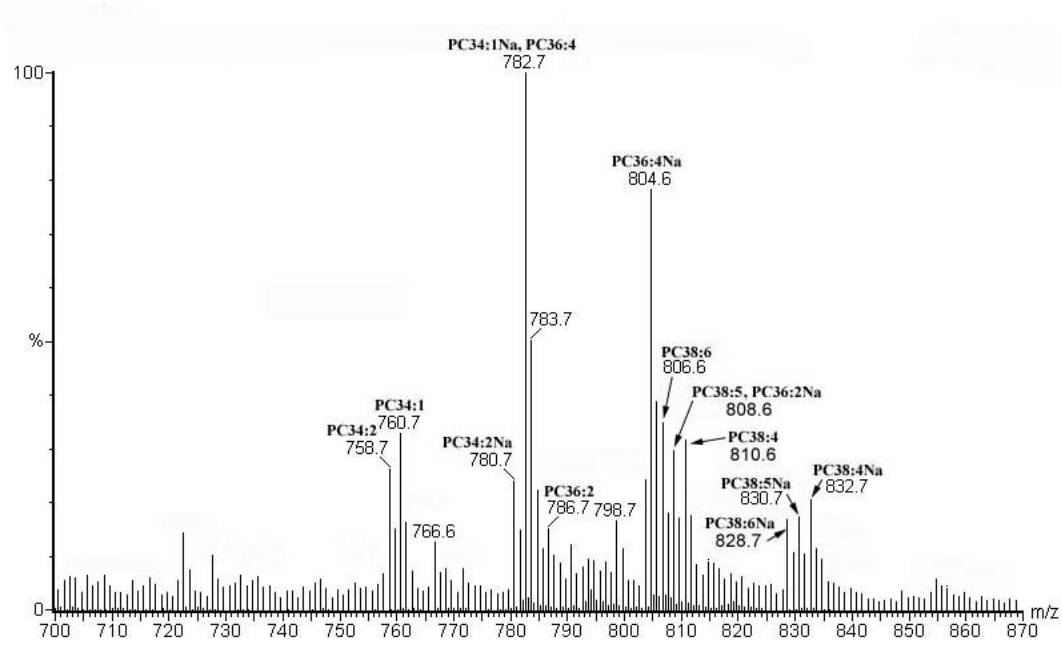


Figure 3-2: ***Positive ion mass spectrum of protonated and sodiated phospholipids extracted from red vastus lateralis muscle of a rat fed a high fat diet.***

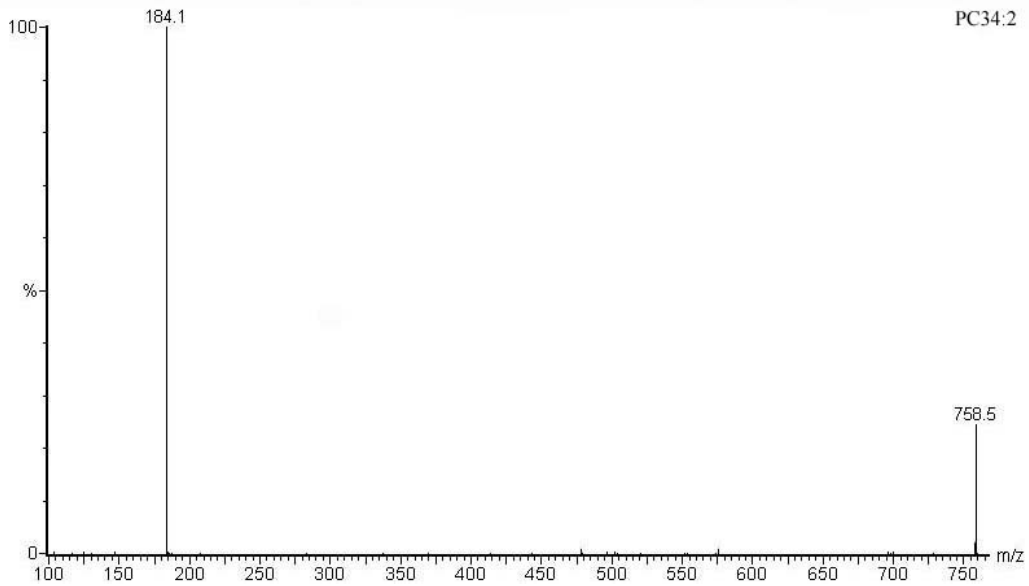


Figure 3-3: ***Positive ion MS/MS spectrum of [PC34:2+H]$^+$ ions (m/z 758 in Figure 3-1).***
Laboratory-frame collision energy was set to 32 eV.

An MS/MS spectrum obtained from the CID of [PC34:2+H]$^+$ ions (*m/z* 758.7) is shown in Figure 3.3. This spectrum contains little structural information with the only product ion observed at *m/z* 184 corresponding to phosphocholine (Figure 3.4 A) as has previously been described (see section 2.3.2a).

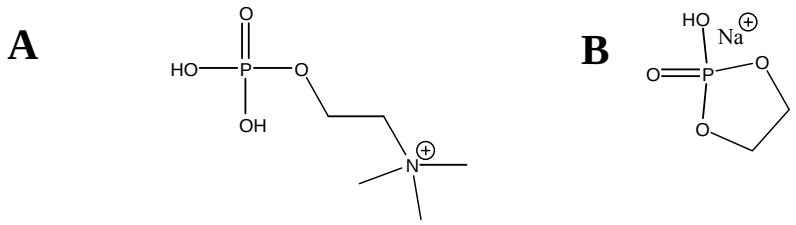


Figure 3-4: ***Phosphocholine cation formed during the CID of protonated PC molecular ions (A) and sodiated O,O'-dimethylenephosphoric acid cation formed during the CID of sodiated PC molecular ions (B).***

In contrast, Figure 3-5 displays a mass spectrum produced by the CID of $[PC34:2+Na]^+$ ions (*m/z* 780) that contains a number of structurally informative product ions. The most abundant ions are observed at *m/z* 721.5 and 597.5 and are produced by the neutral loss of trimethylamine ($[M+Na-59]^+$) followed by the secondary neutral loss of O,O'-dimethylenephosphoric acid ($[M+Na-183]^+$). The secondary fragmentation of the $[M+Na-59]^+$ ions may also result in the neutral loss of sodiated O,O'-dimethylenephosphoric acid ($[M+Na-205]^+$) producing the ions observed at *m/z* 575.5. A typical product ion produced by sodiated phospholipids at *m/z* 147 is also present, corresponding to the charged form of sodiated O,O'-dimethylenephosphoric acid (Figure 3-4 B).

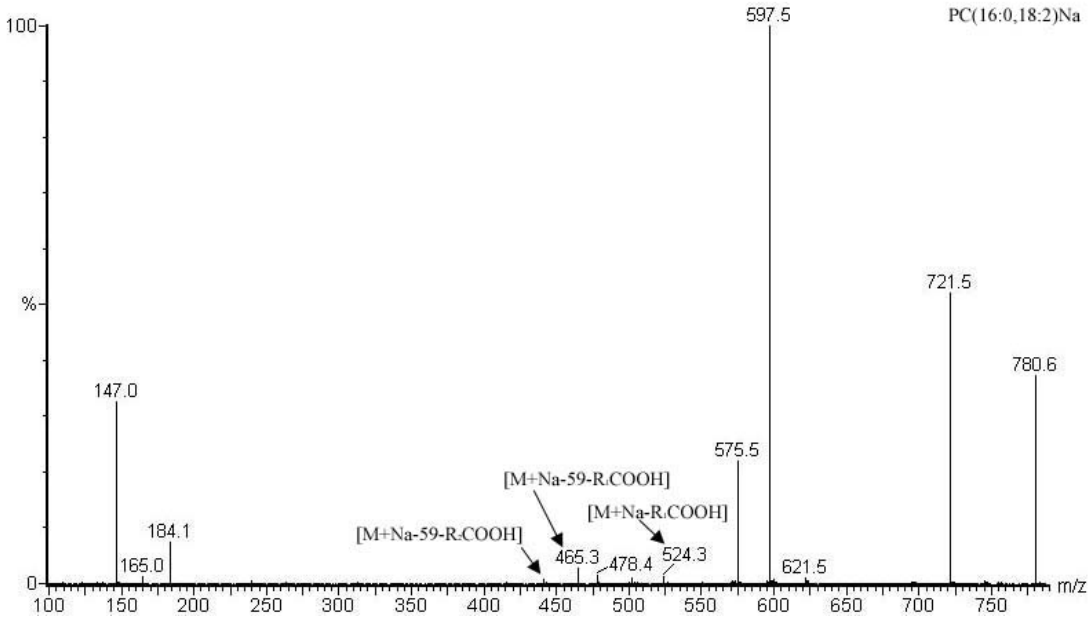


Figure 3-5: ***Positive ion MS/MS spectrum of $[PC34:2+Na]^+$ ions (m/z 780 in Figure 3-1).***
Laboratory-frame collision energy was set to 34 eV.

The most informative product ions, however, are of relatively low abundance namely, *m/z* 524.3, 500.3, 465.3 and 441.3. The ions at *m/z* 524.3 ($[M+Na-16:0]^+$) and 500.3 ($[M+Na-18:2]^+$) are formed from the neutral loss of the two FAs from the sodiated molecular ion (*m/z* 780.6) while those at *m/z* 465.3 and 441.3 are formed by the analogous losses from the *m/z* 721.5 ion, *i.e.* $[M+Na-59-16:0]^+$ and $[M+Na-59-18:2]^+$ respectively. Each of these CID fragments have been previously observed by FAB-MS (Domingues *et al.*, 2001). It is possible that a small number of protonated PC36:5 ions are present as a low abundance of phosphocholine ions (*m/z* 184.1) are also observed.

The occurrence of phospholipid species with overlapping masses, *i.e.* a protonated and a sodiated PC molecular species with the same *m/z*, further complicates the assignment of molecular ions in the positive ion spectrum in Figure 3-2. Figure 3-6 presents the MS/MS spectrum of one such example. In this spectrum the typical product ions formed from the CID of sodiated PC species (described above) are present along with the typical phosphocholine cation produced from the CID of protonated PC species.

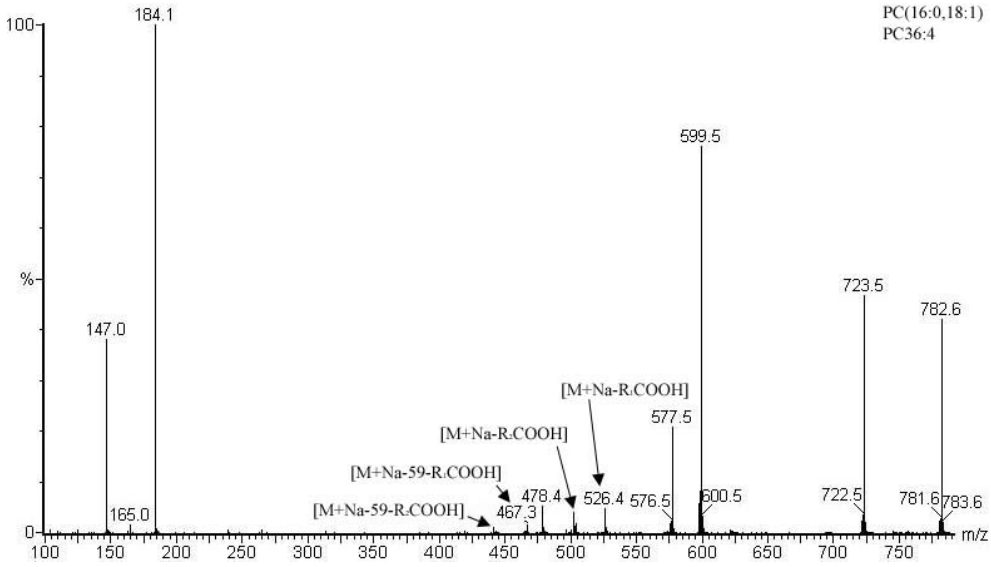


Figure 3-6: ***Positive ion MS/MS spectrum of the isobaric ions $[PC34:1+Na]^+$ and $[PC36:4+H]^+$ (m/z 782 in Figure 3-2).***
Laboratory frame collision energy was set to 34 eV.

The presence of both protonated and sodiated species presents a clear problem when attempting to analyse differences in the relative abundance of molecular ions between samples. One possible solution would be to increase the concentration of sodium in the phospholipid extract so that only sodiated ions would be formed. This would also provide an additional advantage in that the CID of sodiated phospholipids is known to produce product ions characteristic of the two FAs. It was our experience, however, that these ions were often of very low abundance, especially those produced from the neutral loss of the *sn*-2 FA (See Figures 3-5 and 3-6) and thus structural characterisation was often difficult. Previous studies have used lithiated PC molecular ions for structural characterisation by MS/MS (Hsu *et al.*, 1998; Ho *et al.*, 2003). The advantages of lithiation are (i) high affinity of PC for lithium cations and thus the ability to displace protons and sodium cations and (ii) the greater abundance of structurally characteristic product ions in MS/MS experiments.

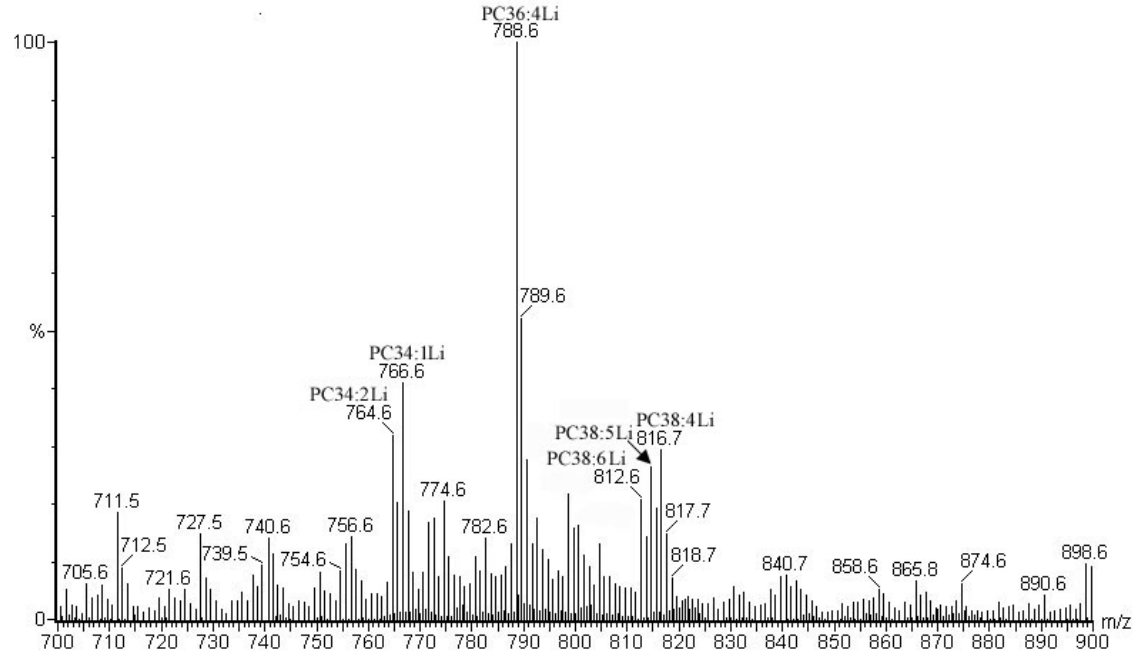


Figure 3-7: ***Positive ion mass spectrum of lithiated phospholipids extracted from red vastus lateralis muscle of a rat fed a high fat diet.***

An example positive ion spectrum of a phospholipid extract from the red vastus lateralis muscle of a rat displaying $[PC+Li]^+$ ions is shown in Figure 3-7. Lithium iodide was added at a ratio of 800:1 (lithium iodide:phospholipid) in order to produce solely $[PC+Li]^+$ ions during the electrospray process. The concentration of lithium iodide added was found to be critical, as at low concentrations sodiated and protonated ions were also observed whereas too high a concentration resulted in the formation of lithium iodide ion clusters that dominated the mass spectrum (see Equation 3, Section 2.1.1a). With one molecular ion for each molecular species present, changes in relative abundance can be more easily assessed.

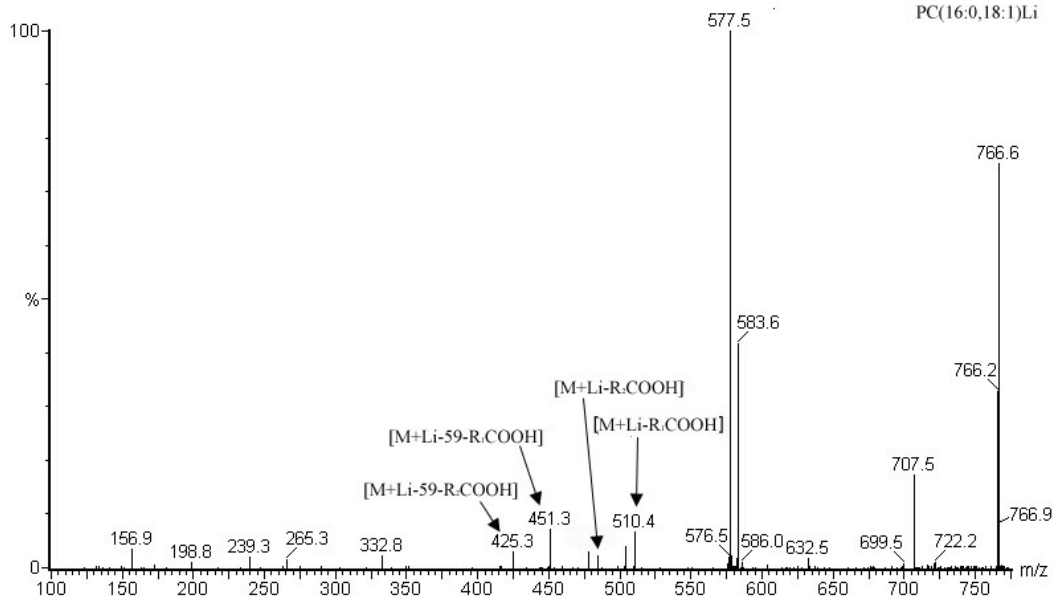


Figure 3-8: ***Positive ion MS/MS spectrum of [PC34:1+Li]$^+$ ions (m/z 766 in Figure 3-6).***
Laboratory-frame collision energy was set to 37 eV.

The MS/MS spectrum obtained from the CID of $[PC34:1+Li]^+$ (*m/z* 766.6) is displayed in Figure 3-8. The product ions observed in the CID of this lithiated species are formed by the analogous neutral losses to those observed for sodiated species (*c.f.* Figure 3-5). While

the ions generated by the neutral loss of the *sn*-2 FA (18:1) are still of low abundance they are more apparent than those observed for the sodiated species, particularly from the $[PC34:1+Li-59]^+$ ion (*m/z* 425.3). These data confirm the twofold advantages of lithiation and as a consequence, lithium iodide was added to all phospholipid extracts prior to positive ion ESI-MS analysis.

3.2.3 Negative ion MS

In contrast to the positive ion MS spectra, negative ion ESI-MS analyses of lipid extracts were found to produce clean spectra with acidic phospholipids observed as single $[M-H]^-$ ions. An initial problem observed during negative ESI-MS of lipid extracts was the lack of PE ions present in the spectrum. This was remedied by the addition of ammonia to increase the pH to approximately 10 as has previously been achieved using lithium hydroxide (Han & Gross, 1995). A negative ion mass spectrum of a phospholipid extract from red vastus lateralis muscle of a rat is shown in Figure 3-9.

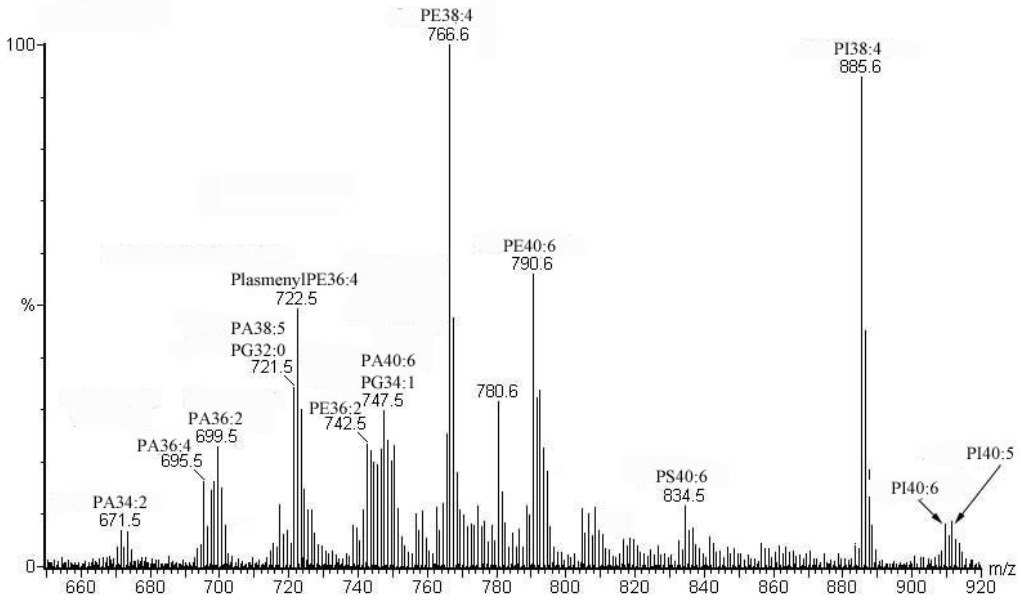


Figure 3-9: ***Negative ion mass spectrum of deprotonated phospholipids extracted from red vastus lateralis muscle of a rat fed a high fat diet.***

Increasing the pH to 10 helped to produce abundant deprotonated PE species *e.g.*, plasmenyl PE34:2 (*m/z* 698.5), plasmenyl PE36:4 (*m/z* 722.5), PE36:2 (*m/z* 742.5), PE38:6 (*m/z* 762.5), PE38:4 (*m/z* 766.6) and PE40:6 (*m/z* 790.6) (Figure 3-9). A number of other deprotonated phospholipid species where also observed, including PA34:2 (*m/z* 671.5), PA36:4 (*m/z* 695.5), PA36:2 (*m/z* 699.5), PA38:5 (*m/z* 721.5), PA40:6 (*m/z* 747.5), PG32:0 (*m/z* 721.5), PG34:1 (*m/z* 747.5), PS34:0 (*m/z* 762.), PS40:6 (*m/z* 834.5), PI38:4 (*m/z* 885.6), PI40:6 (*m/z* 909.6) and PI40:5 (*m/*z 911.6).

Negative ion ESI-MS/MS spectra were obtained for each phospholipid observed in Figure 3-9. The CID of $[M-H]^-$ ions produced a number of product ions useful in the structural characterisation of the phospholipid molecular species. These data were consistent with literature reports (as discussed in chapter 2) and allowed the structural characterisation of all phospholipids observed in this sample. Examples of each phospholipid class are

discussed briefly below in order to demonstrate how identification of each phospholipid molecular species was made in later chapters. Several examples, namely cardiolipin and plasmenyl PE have not appeared in the literature previously and are therefore discussed in more detail.

Phosphatidic Acid

Figure 3-10 shows a typical MS/MS spectrum obtained from the CID of deprotonated PA ions.

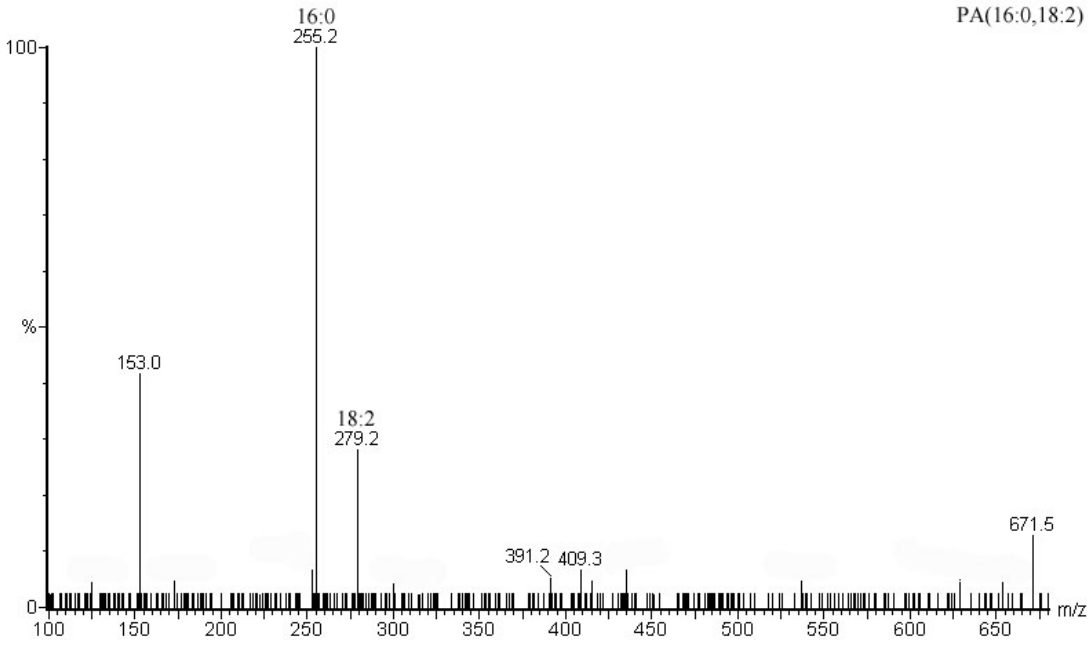


Figure 3-10: ***Negative ion MS/MS spectrum of $[PA34:2-H]^-$ ions (m/z 671 in Figure 3-9).***
Laboratory-frame collision energy was set to 34 eV.

Abundant product ions corresponding to the two FA carboxylate ions, 16:0 (*m/z* 255.2) and 18:2 (*m/z* 279.2) are present allowing the characterisation of the molecular species as

PA(16:0,18:2). An abundant glycerophosphate ion (*m/z* 153) is also present confirming that this is a phospholipid. As discussed in chapter 2 ions produced from the neutral loss of both FAs and/or the FA ketenes are generally observed for phospholipids and, although of low abundance, these ions are present in Figure 3-10 (*m/z* 391, 409, 415, 433). The 34 eV laboratory-frame collision energy used in this CID experiment was sufficient for significant secondary fragmentation of these ions. This resulted in an increase in the relative abundance of the carboxylate ions effectively increasing the ease of identification of the two FA moieties as required in subsequent studies.

Phosphatidylserine

A typical MS/MS spectrum obtained from the CID of deprotonated PS ions is displayed in Figure 3-11.

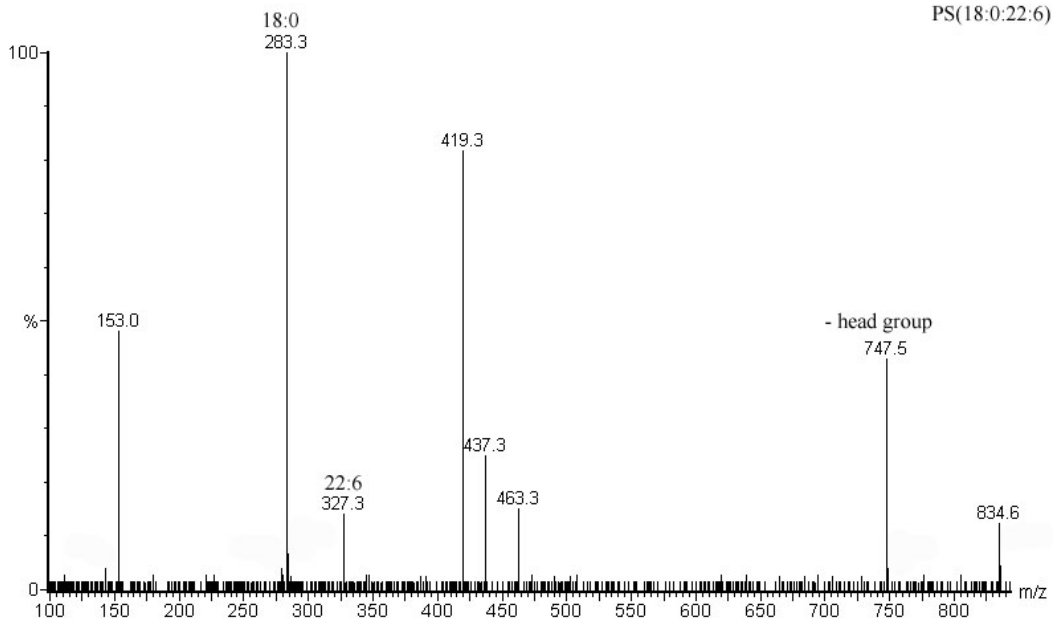


Figure 3-11: ***Negative ion MS/MS spectrum of [PS40:6−H]⁻ ions (m/z 834 in Figure 3-9).***
Laboratory-frame collision energy was set to 34 eV.

The $[M-H-87]^-$ ion, produced by the neutral loss of didehydroalanine is clearly visualised at *m/z* 747.5 and the two FAs can be identified as 18:0 (*m/z* 283.3) and 22:6 (*m/z* 327.3). These data allow the phospholipid molecular species to be clearly identified as PS(18:0,22:6). The further neutral losses of 22:6 as a free FA (*m/z* 419.3) and as a ketene (*m/z* 437.3) from this ion are also clearly apparent, as is the loss of 18:0 as a free FA (*m/z* 463.3). These ions are far more abundant in this spectrum than the equivalent neutral losses for PA shown in Figure 3-10 even though the same laboratory-frame collision energy was used. This is presumably due to two reasons; (i) these ions are secondary product ions created from the CID of the $[M-H-87]^-$ ion and therefore tertiary fragmentation is required for their dissociation; and (ii) the PS phospholipid is a larger molecule and a larger laboratory-frame collision energy is required to achieve the same amount of internalised energy (see equation 8, chapter 2).[6] Even though this results in a decrease in the abundance of the FA carboxylate ions, particularly 22:6, increased laboratory-frame collision energy would result in greater fragmentation of the ion corresponding to the neutral loss of the head group (*m/z* 747.5) and decrease the ease of molecular species characterisation.

Phosphatidylinositol

The MS/MS spectrum of a typical PI species observed in the experiments of this thesis is shown in Figure 3-12

[6] This could also be due to the different combination of FAs between the PA in Figure 3-10 and PS in Figure 3-11 however this contribution is most likely extremely minor.

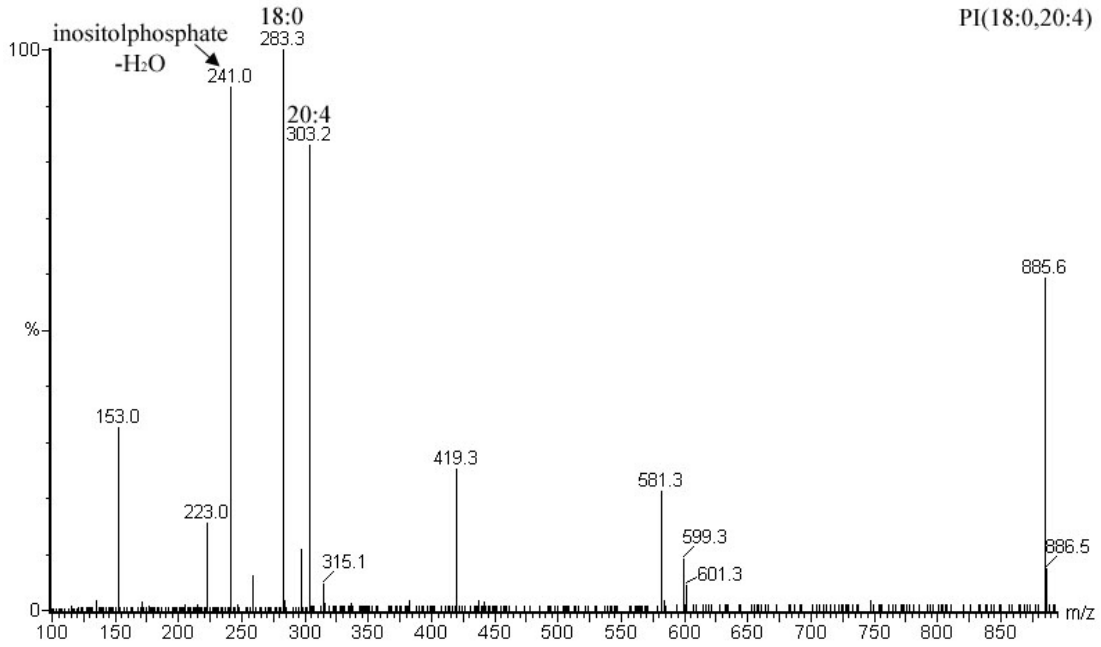


Figure 3-12: ***Negative ion MS/MS spectrum of [PI38:4−H]¯ ions (m/z 885 in Figure 3-9).***
Laboratory-frame collision energy was set to 40 eV.

This figure displays the spectrum obtained from the CID of the ion found at *m/z* 885.6 in Figure 3.8 (PI38:4). The identity of this species is readily apparent, as the three most abundant ions are structurally characteristic. The ion at *m/z* 241 is the dehydrated inositolphosphate anion that is indicative of PI phospholipids. The ions at *m/z* 283.2 and 303.2 identify the two constituent FAs as 18:0 and 20:4 respectively. Other structurally characteristic ions are also observed as discussed in Chapter 2, *e.g.,* glycerophosphate at *m/z* 153 and the ions resulting from the neutral loss of the two FAs at *m/z* 601.3 and 581.3. The ion observed at *m/z* 419 is produced by the neutral loss of dehydrated inositol (168 Da) from the ion seen at *m/z* 581.

Phosphatidylethanolamine

Deprotonation of neutral PE lipids was achieved at elevated pH (as discussed above). The MS/MS spectrum of a typical deprotonated PE molecular species is shown in Figure 3-13.

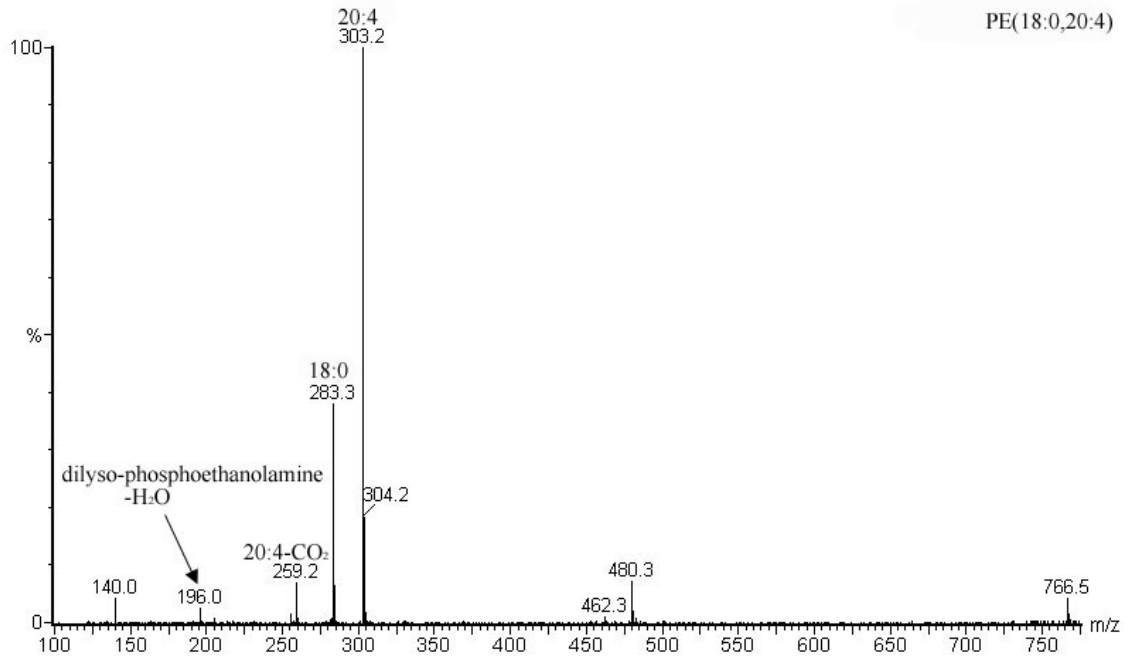


Figure 3-13: ***Negative ion MS/MS spectrum of [PE38:4−H]¯ ions (m/z 766 in Figure 3-9).***
Laboratory-frame collision energy was set to 35 eV.

This spectrum shows that the formation of FA carboxylate ions at *m/z* 303 (20:4) and *m/z* 283 (18:0) are the major fragmentation products at laboratory-frame collision energy of 35 eV. In contrast to the acidic phospholipids discussed previously the abundance of the unsaturated carboxylate product ion is greater than the saturated. This has been observed previously at collision energies up to 40 eV (Hvattum *et al.*, 1998; Hsu & Turk, 2000d) and has been attributed to preferential fragmentation from the *sn*-2 position. The two carboxylate ions and the dilyso-phosphoethanolamine ion at *m/z* 196 identifies this

molecular species as PE(18:0,20:4). Observations during this thesis have indicated that the dilyso-phosphoethanolamine ion is generally of very low abundance and often not detected (see Chapter 2, scheme 7 for fragmentation pathway). In such cases the identification of the two FAs and the mass of the molecular ion was sufficient for identification.

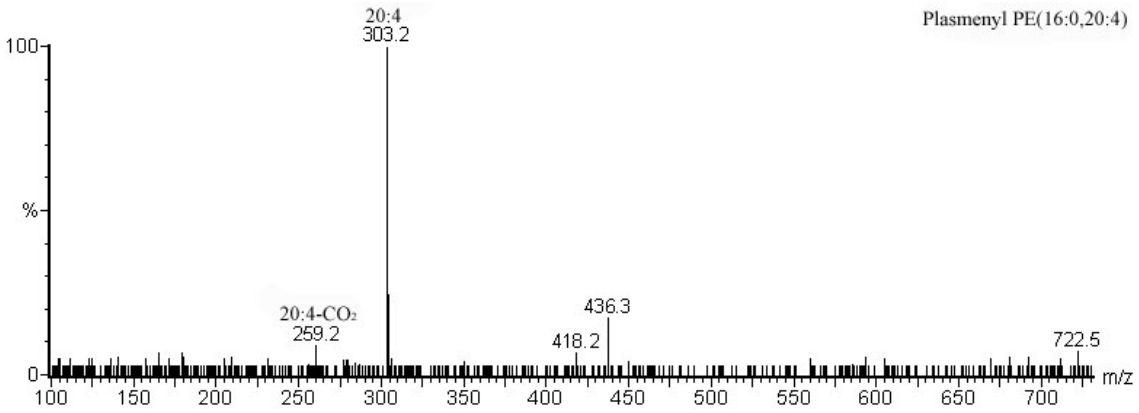


Figure 3-14: ***Negative ion MS/MS spectrum of [plasmenyl PE20:4−H]⁻ ions (m/z 722 in Figure 3-9).***
Laboratory-frame collision energy was set to 34 eV.

Plasmenyl PE differs from PE in that the radyl at the sn-1 postion is bound via a vinyl ether linkage rather than the more common ester. An example of a negative ion MS/MS spectrum of a typical plasmenyl PE is displayed in Figure 3-14. The most notable contrast in the spectra shown in Figures 3-13 and 3-14 is that only a single carboxylate ion, *m/z* 303 (20:4), appears in the latter. There are also low abundance ions produced from the neutral loss of this fragment as a FA (*m/z* 418) and a ketene (*m/z* 436). This suggests either (i) the phospholipid has 20:4 attached via ester linkages at both the *sn*-1 and *sn*-2 positions, or (ii) the mass selected ion consists of a PE species with 20:4 at the *sn*-2 position and a vinyl ether linked FA at the *sn*-1 position. Nonetheless, a phospholipid with two 20:4 FAs is unlikely and a molecular ion with an *m/z* of 698 suggests that this

phospholipid is plasmenyl PE(16:0,20:4). Consequently, the second possibility is likely the correct one. It should be pointed out, however, that to this point no model studies have been conducted to confirm this hypothesis and thus synthetic plasmenyl PE(16:0,20:4) was analysed. The MS/MS spectrum of this synthetic phospholipid, shown in Figure 3-15, displays analogous product ions to the native phospholipid in Figure 3-14 confirming the assigned identification.

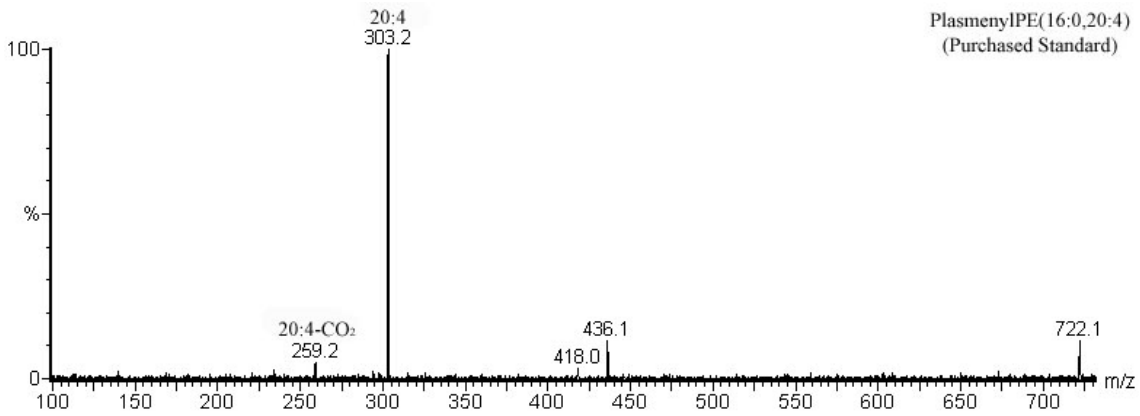
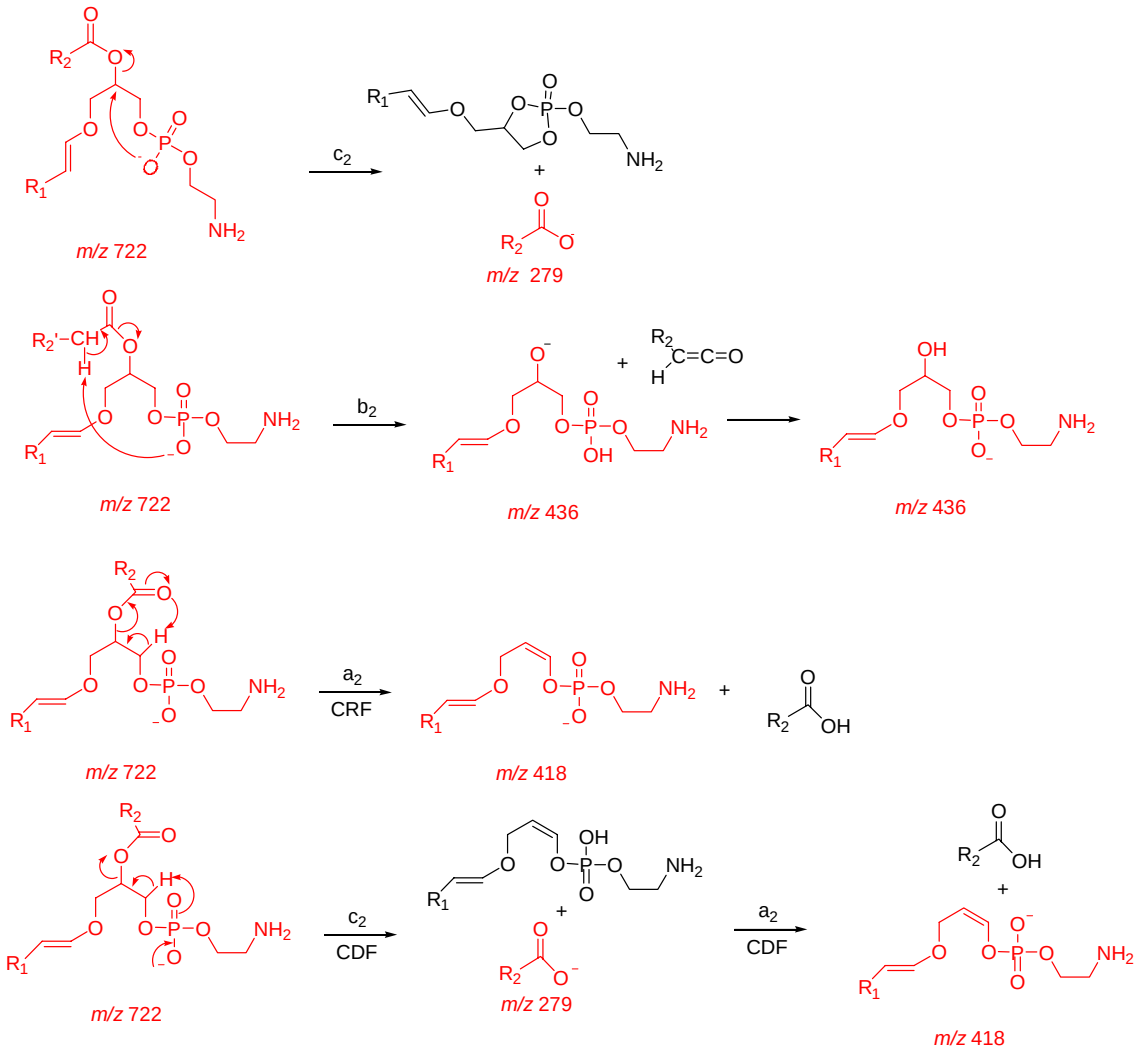


Figure 3-15: ***Negative ion MS/MS spectrum obtained from synthetic [plasmenyl PE20:4−H]⁻ ions***
Laboratory-frame collision energy was set to 34 eV.

Scheme 10 displays the possible fragmentation pathways of the plasmenyl PE molecular ion. This phospholipid again follows the three pathways observed for all deprotonated phospholipids (described in Chapter 2) with the exception that these fragmentations are exclusive to the *sn*-2 FA. Given that the carboxylate moiety at the sn-2 position ($R_2CO_2^-$) is a substantially better leaving group than the vinyl oxide ($R_1CHCH_2O^-$) the exclusive observation of the former as a product ion appears logical.



Scheme 10: Proposed fragmentation pathways of plasmenyl PE(16:0,20:4)

Cardiolipin

Cardiolipin (diphosphatidylglycerol, DPG) has a molecular weight greater than that of the other glycerophospholipids as it is essentially heterodimer of PG and PA (see Figure 1-5). It was detected in the *m/z* range analysed, however, as it contains two negatively charged phosphate groups and is therefore identified by 0.5 *m/z* spacing between isotopic ions.

When performing CID of a doubly charged molecular ion it is prudent to extend the mass range up to the singly charged *m/z* value as fragmentations can occur that result in singly charged product ions with an *m/z* greater than that of the doubly charged precursor ion. Such fragmentation can be seen in the sample cardiolipin spectrum shown in Figure 3-16.

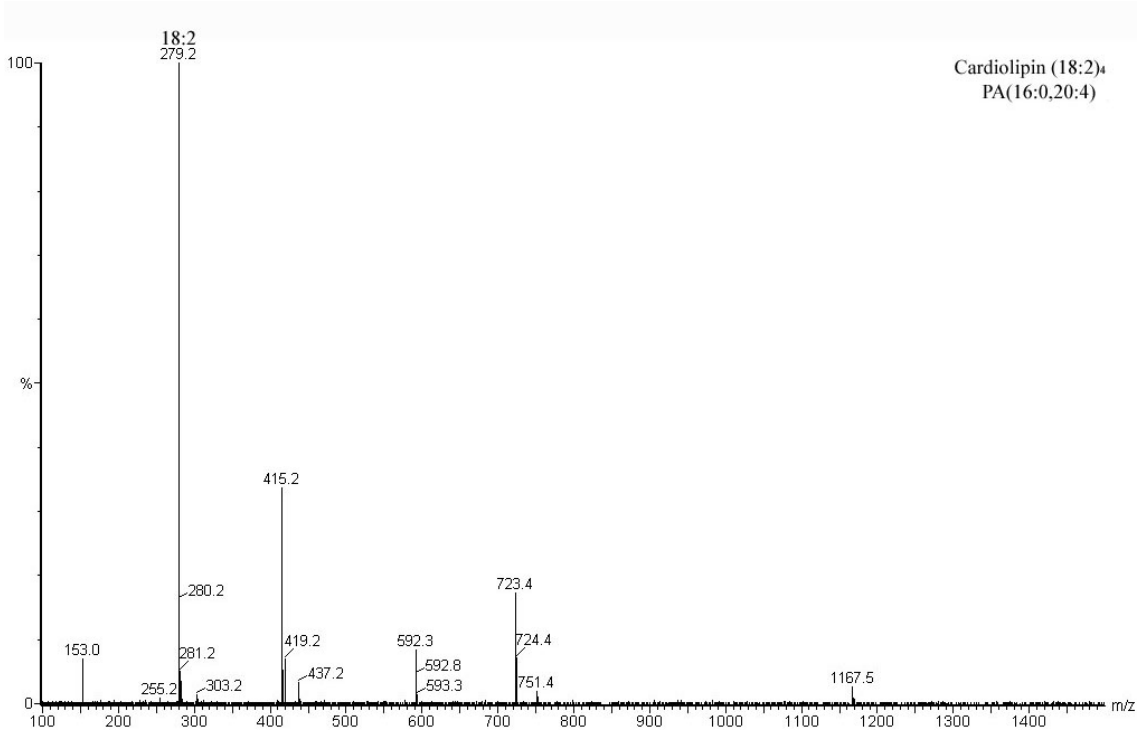


Figure 3-16: ***Negative ion MS/MS spectrum of [Cardiolipin72:8−2H]$^{2-}$ ions from rat heart.***
Laboratory-frame collision energy was set to 34 eV.

In this figure, a doubly charged molecular ion with an *m/z* of 723.4 was subjected to CID at a laboratory-frame collision energy of 34 eV. The high abundance ion at *m/z* 279.2 (18:2) in combination with the ion at *m/z* 1167.5 corresponding to the loss of 18:2 anions suggest the molecular ion is predominately cardiolipin(18:2)$_4$. Furthermore, the ion at *m/z* 751.5 is a dehydrated PG(36:4) ion created by the neutral loss of lyso PA(18:2) from the

ion at *m/z* 1167.5 indicating a symmetrical FA distribution at either end of the molecule. The doubly charged ion seen at *m/z* 592.3 is produced by the neutral loss of 18:2 as a ketene and the relatively high abundance ion at *m/z* 415.2 corresponds to lyso PA(18:2). There are also product ions present in this spectrum that indicate the presence of PA(18:0,20:4). Carboxylate ions at *m/z* 283.2 and 303.2 corresponding to 18:0 and 20:4 respectively are observed, as are ions at *m/z* 419 and 437 corresponding to the loss of 20:4 as a free FA and as a ketene from a singly charged ion at *m/z* 723.4. As with the plasmenyl PE phospholipids no studies have been published describing the fragmentation pathways of cardiolipins. Furthermore, the spectrum shown in Figure 3-16 is complicated by the presence of PA(18:0,20:4) product ions. Accordingly, a synthetic cardiolipin was analysed to confirm the assigned identification of the cardiolipin molecular species. The MS/MS spectrum obtained from the CID of synthetic cardiolipin$(18:2)_4$ is shown in Figure 3-17. The inset spectra in Figure 3-17 demonstrate the 0.5 *m/z* spacing between isotope ions at *m/z* 461.3 and 592.4 indicating that they are doubly charged fragments. In this spectrum two additional low-abundance ions are observed, namely; (i) the singly charged ion observed at *m/z* 905.5 produced from the neutral loss of the FA ketene from the ion at *m/z* 1167.8, and (ii) the doubly charged ion observed at *m/z* 461.3 produced from the equivalent fragmentation from the doubly charged ion at *m/z* 592.4. The presence of these ions is a result of the increased concentration of the cardiolipin in the standard when compared to the native sample allowing the observation of these lower abundance product ions. The matching product ions observed in this spectrum and the one in Figure 3-16 confirm the assigned identification of the native molecular species to be accurate.

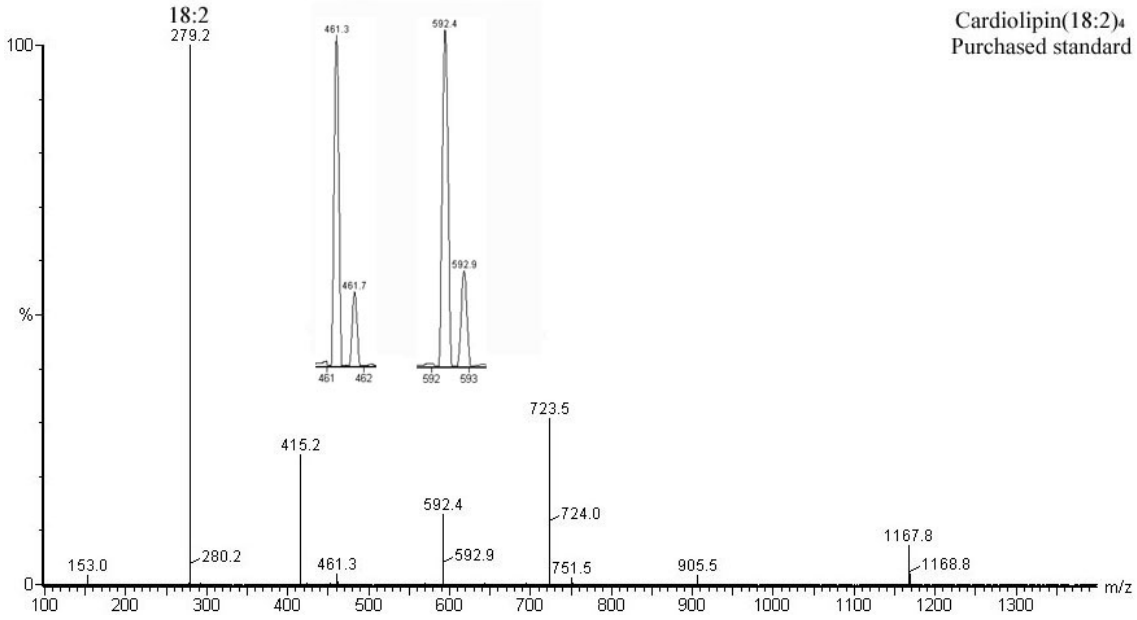
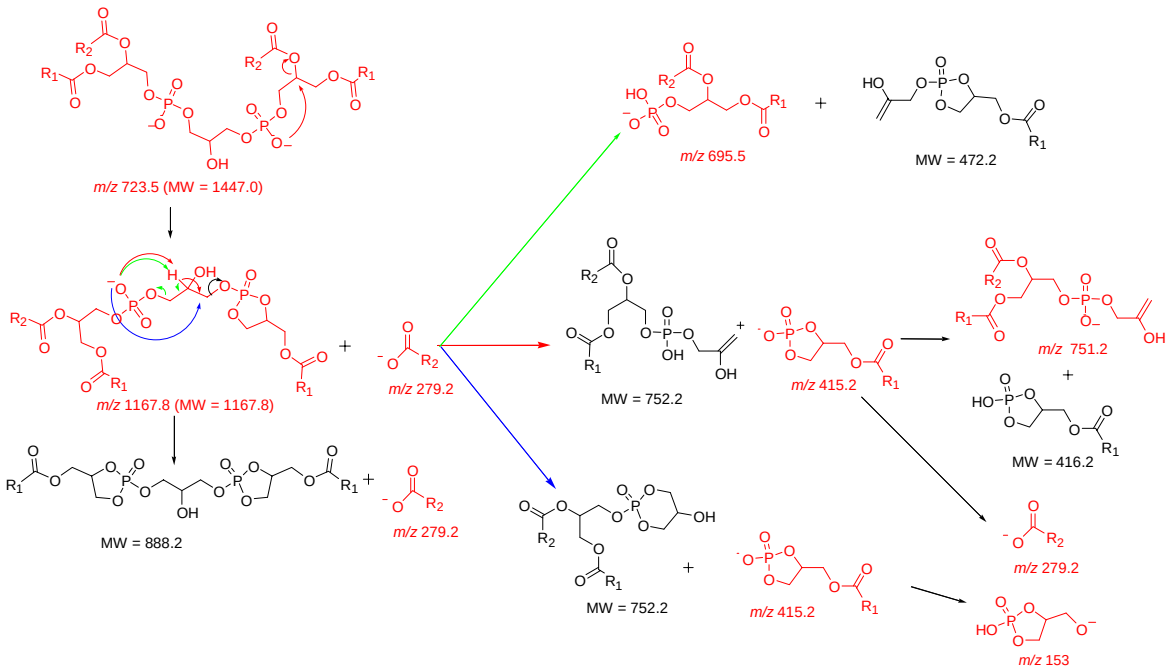
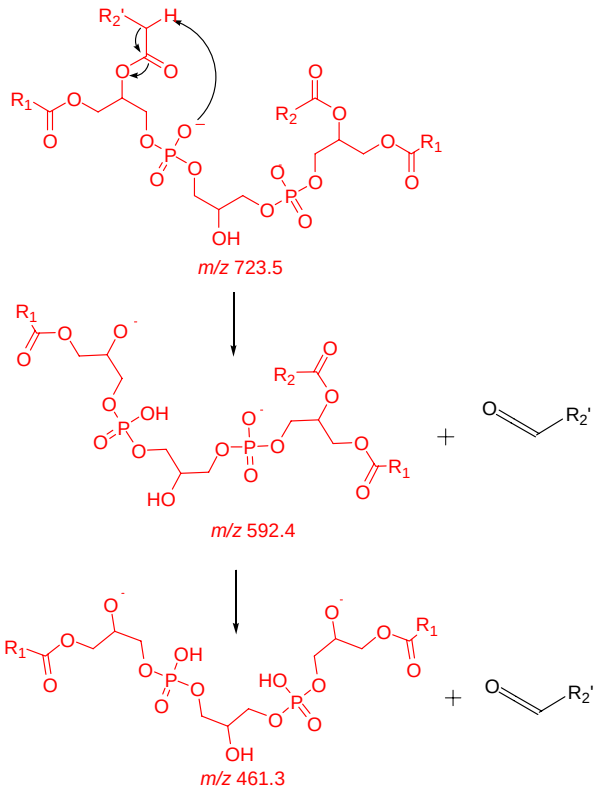


Figure 3-17: ***Negative ion MS/MS spectrum of synthetic [Cardiolipin72:8−2H]$^{2-}$ ions.***
Laboratory-frame collision energy was set to 23 eV. Inserts show magnifications of the ions at m/z 461.3 and 592.4. The 0.5 m/z difference between isotope ions indicate that they are doubly charged ions.

The proposed fragmentation mechanisms and pathways for doubly charged cardiolipin molecular ions are presented in schemes 11 and 12. It appears that this cardiolipin follows two initial fragmentation pathways. The first, shown in scheme 11 results in the formation of a FA carboxylate ion (*m/z* 279) and the corresponding decarboxylated cardiolipin ion. The second, shown in scheme 12 produces the doubly charged ion seen at *m/z* 592. This ion is produced by the neutral loss of the FA as a ketene.



Scheme 11: ***Proposed fragmentation pathways of cardiolipin with a primary fragmentation resulting in the production of a carboxylate ion***



Scheme 12: ***Proposed fragmentation pathways of cardiolipin with a primary fragmentation resulting in the loss of a ketene.***

One of the problems encountered when analysing a mixture of phospholipid molecular species extracted from a biological sample using negative ion MS is the occurrence of two or more molecular species with the same mass (often referred to as isobaric interference). This is a separate problem from the one discussed earlier where both sodiated and protonated PC molecular species were observed. The molecular species in this case are all deprotonated and therefore the addition of salts to the sample to produce adduct ions would only result in a equal mass shift for each phospholipid and consequently not solve the problem. Isobaric interference is problematic for two reasons, (i) characterisation is more difficult and, (ii) any attempt to quantify or analyse changes in the relative abundance of an individual molecular species is not possible. Isobaric interference may

occur when two different combinations of FAs that have the same total number of carbons and double bounds are bound to a particular head group as displayed in Figure 3-18.

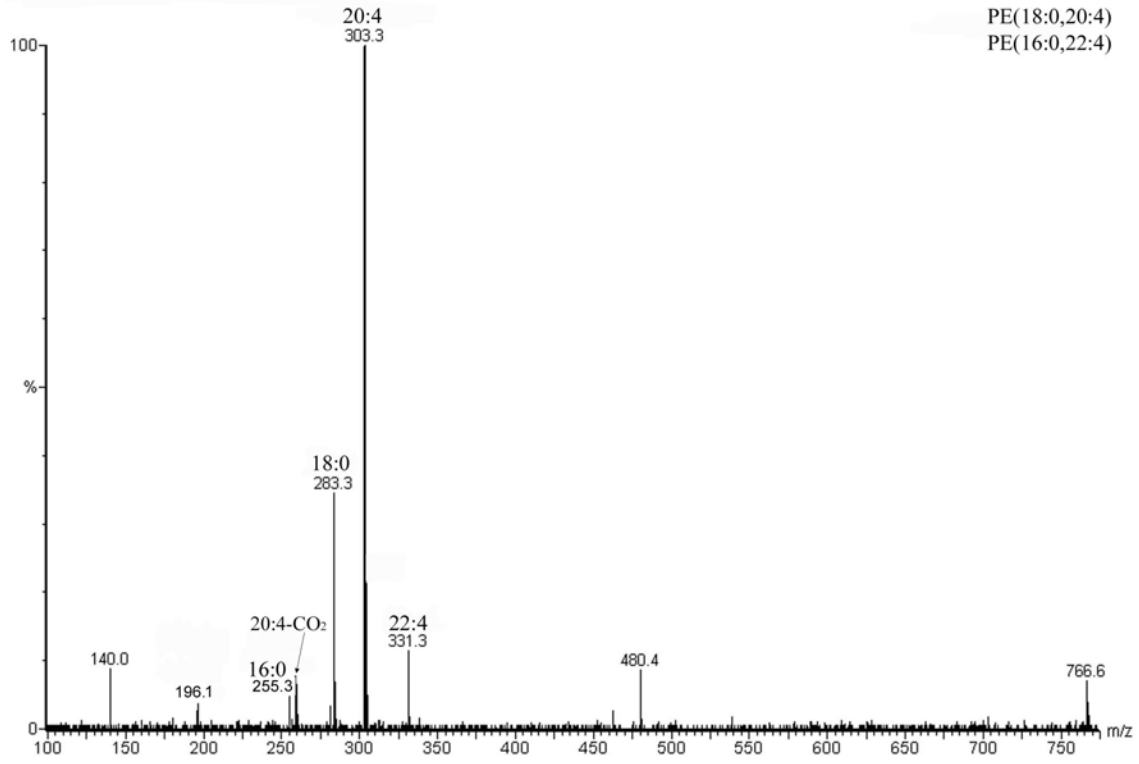


Figure 3-18: ***Negative ion MS/MS spectrum of [PE38:4−H]¯ ions from a rat brain total lipid extract.***
Laboratory-frame collision energy was set to 34 eV.

This figure shows the MS/MS spectrum obtained from the CID of deprotonated PE(38:4) ions in a total lipid extract from a whole rat brain. In contrast to the analogous spectrum obtained from rat skeletal muscle (Figure 3-13), there are four carboxylate ions present. Moreover, there are two possible combinations that have the same total mass, *i.e.* 18:0/20:4 and 16:0/22:4 and therefore it is apparent that two molecular species of PE [PE(16:0,22:4) and PE(18:0,20:4)] are present.

Isobaric interferences may also arise when different combinations of head group and FAs have a coincident mass, as is the case in Figure 3-19.

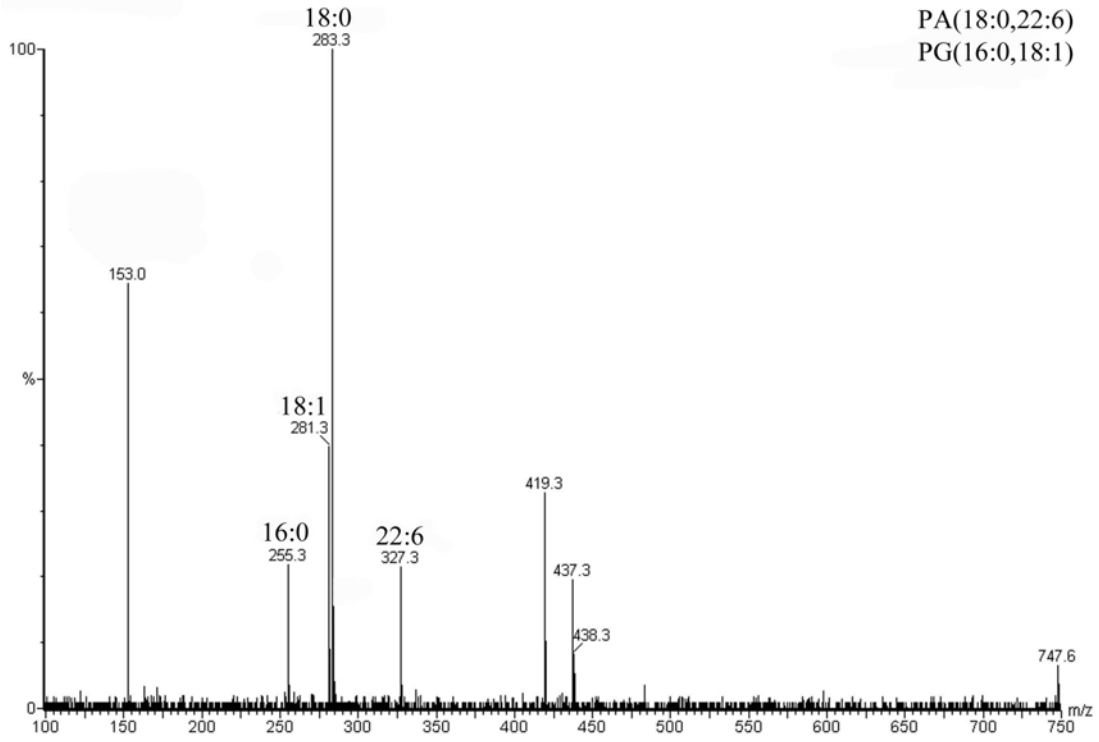


Figure 3-19: ***Negative ion MS/MS spectrum of overlapping [PA40:6−H]¯ and [PG34:1−H]¯ ions (m/z 747 in Figure 3-8).***
Laboratory-frame collision energy was set to 32 eV.

In this figure, four separate FA carboxylate anions are observed. In this instance, however, no combination of two FAs can be combined to yield the same mass as the remaining two. This means that the head groups must also be different. No head-group specific product ions are present in this spectrum allowing PS and PI to be excluded from consideration. Therefore by taking combinations of FAs (usually one saturated with one unsaturated) and subtracting the masses from *m/z* 747.6 the contributing phospholipids can be identified as PA(18:0,22:6) and PG(16:0,18:1).

While it is extremely difficult to separate isobaric phospholipid molecular ions using MS, chromatographic techniques such as TLC and HPLC can be used to separate phospholipids into head group classes. These techniques are extremely laborious and difficult and tend to result in the overestimation of long chain PUFAs (see section 2.3.2.d) (DeLong *et al.*, 2001). Accurate mass measurements may also be a possible method of separating some isobaric phospholipid molecular ions. In the example in Figure 3-19, however, the PA and PG molecular ions have a mass difference of only 0.003 % and the resolution of the Q-ToF 2 is not sufficient to resolve these ions. Another way to separate phospholipids into head group classes and remove some of the molecular species 'overlap' is to exploit some of their characteristic product ions by performing precursor ion and neutral loss scans (see section 2.3.2.c).

3.2.4 Precursor ion / neutral loss scans

Precursor ion and neutral loss scans are extremely useful tools for separating phospholipids according to their head group when using triple quadrupole mass spectrometers. Unfortunately, the same properties of a ToF analyser that increase its efficiency and speed in conventional tandem MS experiments, namely simultaneous detection of all ions (see section 2.1.2.b), preclude this instrument from performing precursor ion and neutral loss scans. In these experiments, the instrument does not benefit from the simultaneous detection of all ions and suffers from a decrease in sensitivity, mostly due to the low duty cycle of an orthogonal ToF when operated in these modes.[7] Despite these difficulties, neutral loss and precursor ion experiments were attempted using the Q-ToF 2 to assess their usefulness in the current analysis. While a true precursor ion/neutral loss scan cannot be undertaken on this instrument the Micromass Q-ToF 2 does allow for a post-accumulation reconstruction of precursor ion or neutral loss spectra

[7] Although recent development of Q-ToF instruments has shown that the duty cycle can be improved to 100% by trapping, gating and bunching ions before they enter the ToF (Chernushevich, 2000) we did not have access to such an instrument.

from data accumulated by the ToF while scanning the quadrupole over a selected mass range. As might be anticipated this is an extremely laborious process requiring accumulation of data for several seconds at each *m/z* in the range to be analysed. The quantity of data accumulated is also very large. Figure 3-20 displays the ion chromatogram of precursor ions that produce a phosphocholine product ion with an *m/z* of 184 in a total lipid extract from a whole rat brain.

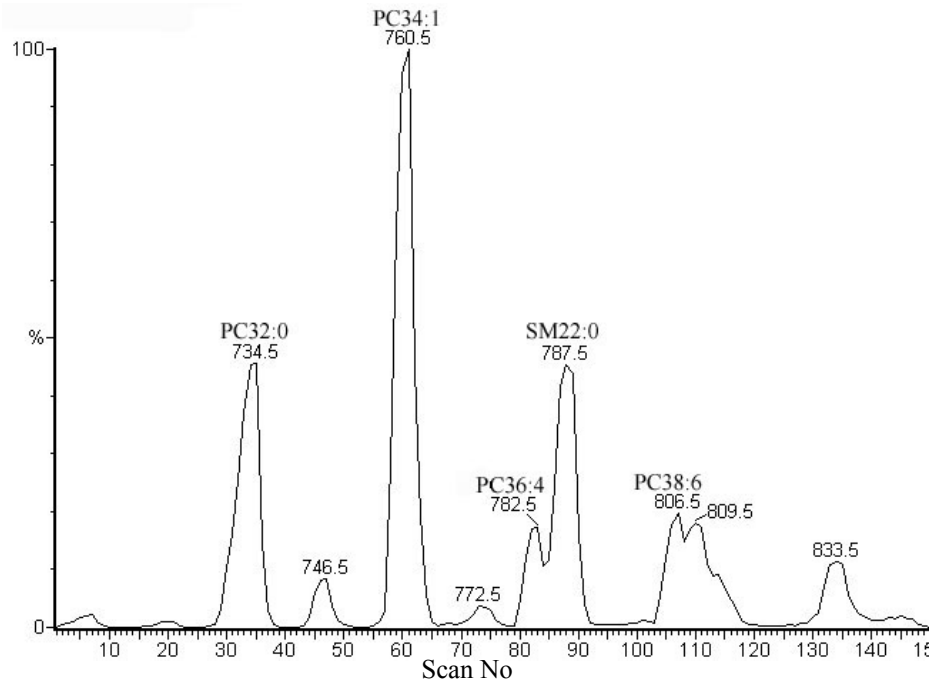


Figure 3-20*: Positive ion chromatogram of protonated PC and SM ions in a total lipid extract from rat brain obtained by a precursor ion scan of the product ion m/z 184 (phosphocholine).*
Laboratory-frame collision energy was set to 34 eV. Each scan number represents a 1 m/z step of the quadrupole, with step 0 equal to m/z 700. Each m/z step was analysed for 3.9 sec.

A number of PC molecular species are apparent, as is one SM species. In addition, other peaks present do not correspond to the mass of any PC or SM species. It is also apparent that the resolution in this chromatogram is quite poor when compared to an MS or a true

precursor ion spectrum obtained on a triple quadrupole (see section 2.3.2.c). This is particularly problematic when analysing a phospholipid mixture as molecular species are often observed with only a 2 Da mass difference, owing to the presence of one additional double bond. When compared to the MS spectrum obtained from the same lipid extract (Figure 3-21) the precursor ion scan (Figure 3-20) does not detect a number of molecular species, presumably as a result of the decreased sensitivity (discussed above), and furthermore there are some inaccuracies in the displayed masses due to poor peak shape and low resolution.

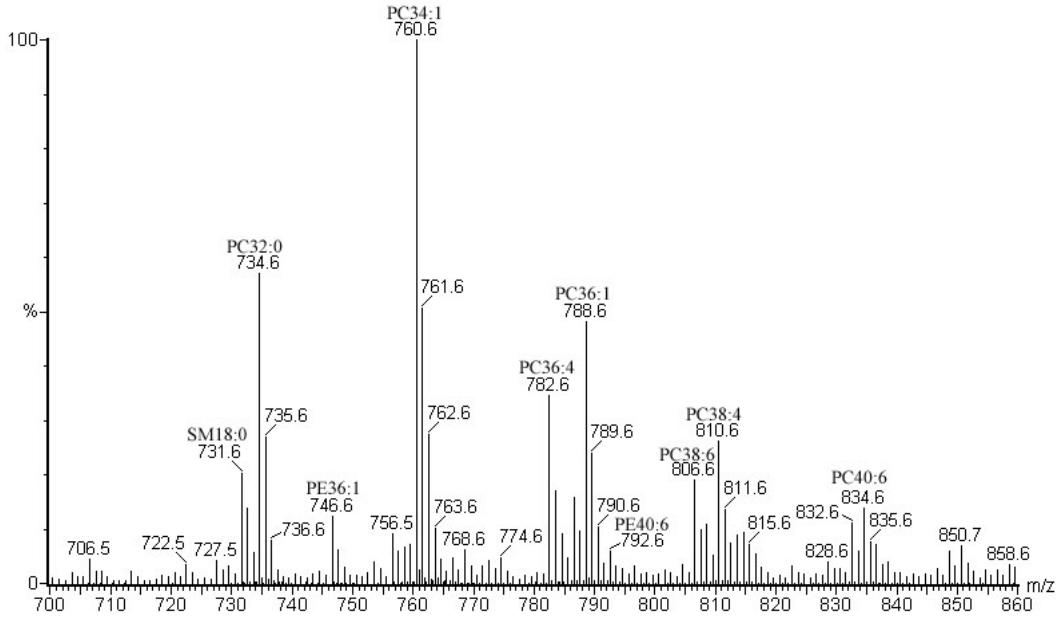


Figure 3-21: ***Positive ion mass spectrum of protonated phospholipids from rat brain total lipid extract.***
This is the same lipid extract used in the precursor ion and neutral loss scans displayed in Figures 3-20, 3-22 and 3-23. Molecular species were identified by manually performing MS/MS for each molecular ion.

It should also be noted that, while not apparent in Figures 3-20, 3-22 and 3-23, the ion count obtained from the MS was much greater than that of the precursor ion scan. This is

because only a limited time can be spent collecting data for each *m/z* step of the quadrupole when performing "pseudo" precursor ion/neutral loss scans.

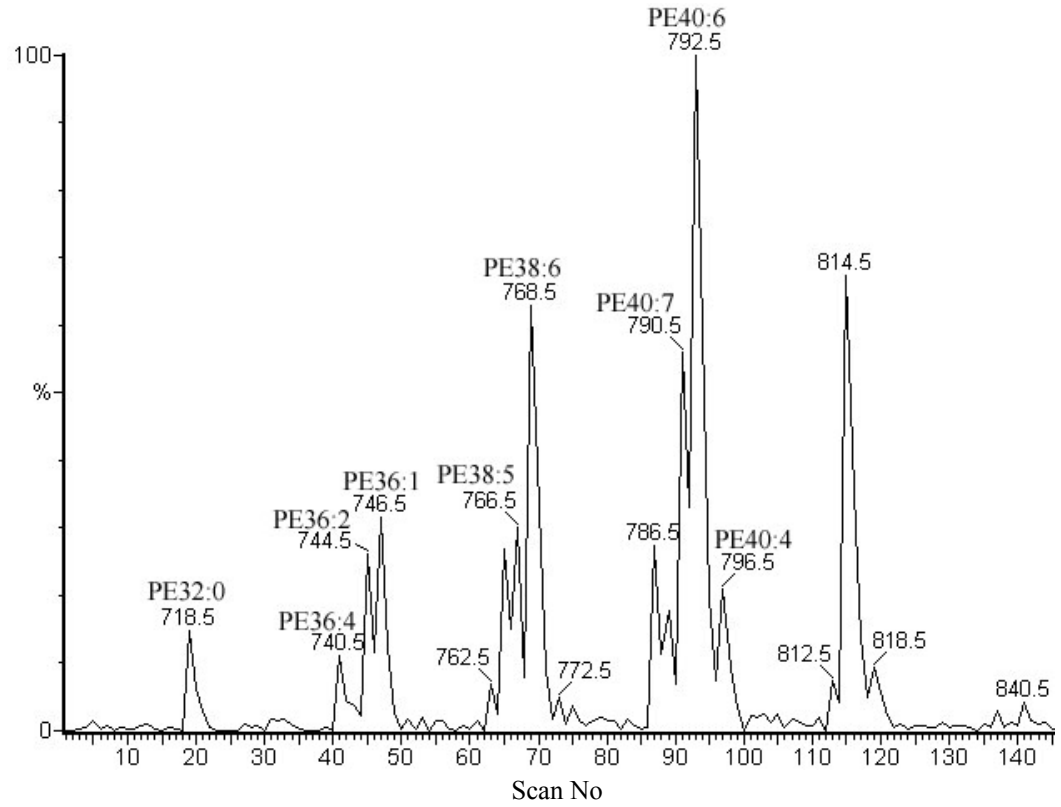


Figure 3-22: ***Positive ion chromatogram of protonated PE ions in a total lipid extract from rat brain obtained by a neutral loss scan of 141 Da (phosphoethanolamine).***
Laboratory-frame collision energy was set to 34 eV. The quadrupole was scanned in 1 m/z steps from m/z 700 to 900. Each m/z step was analysed for 3.9 sec.

Figure 3-22 displays the ion chromatogram produced when a neutral loss scan of 141 Da is extracted from the same data set as used for the *m/z* 184 precursor ions in Figure 3-20. This neutral loss is characteristic of PE phospholipids. Again, the resolution is relatively poor compared to the MS spectrum (Figure 3-21) and the ion count extremely low making the assignment of molecular ions difficult. Non-PE species also appear in this

spectrum at relatively high abundance, *e.g.*, the ion at *m/z* 814.5 that does not correspond to any PE molecular species and remains unidentified.

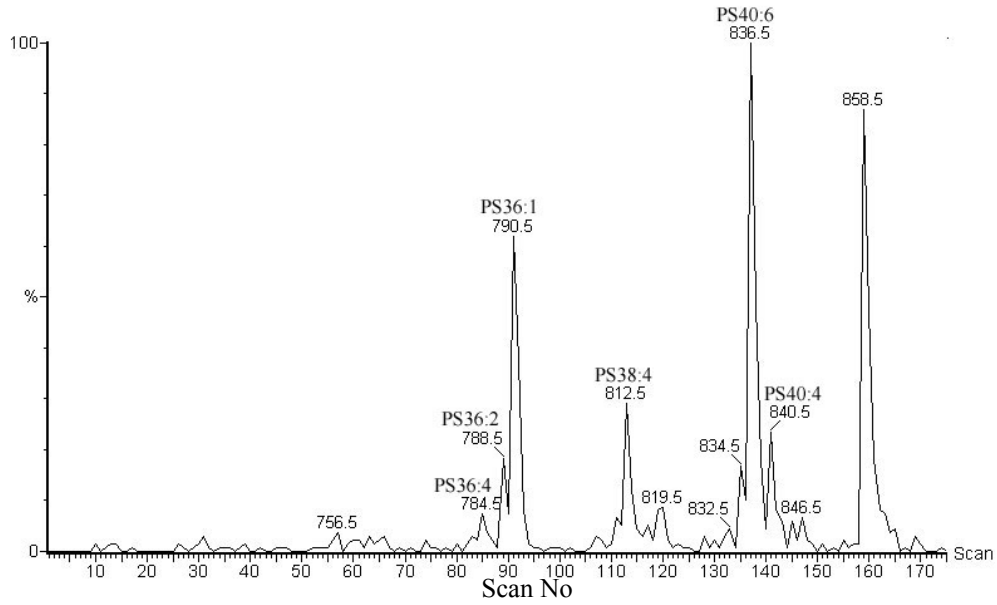


Figure 3-23: ***Positive ion chromatogram of protonated PS ions in a total lipid extract from rat brain obtained by a neutral loss scan of 185 Da (phosphoserine).***
Laboratory-frame collision energy was set to 34 eV. *Each scan number represents a 1 m/z step of the quadrupole, with step 0 equal to m/z 700. Each m/z step was analysed for 3.9 sec.*

The accurate identification of PS molecular species in the ion chromatogram produced from the neutral loss scan of 185 Da (Figure 3-23) is also difficult. The MS/MS spectrum obtained by the ToF from scan 142, corresponding to an *m/z* of 842 (the 142nd 1 *m/z* step of the quadrupole starting from *m/z* 700) is presented in Figure 3-24. The mixture of ions observed in this spectrum, both molecular and fragment, indicate the presence of a number of possible species and demonstrates the level of uncertainty in determining molecular species observed in this neutral loss scan. Again, the poor duty cycle resulted in a very low ion count (only 7 for the most intense ion) and extraneous ions that do not

correspond to any PS molecular species, *e.g.*, *m/z* 858.5 are observed at relatively high abundance.

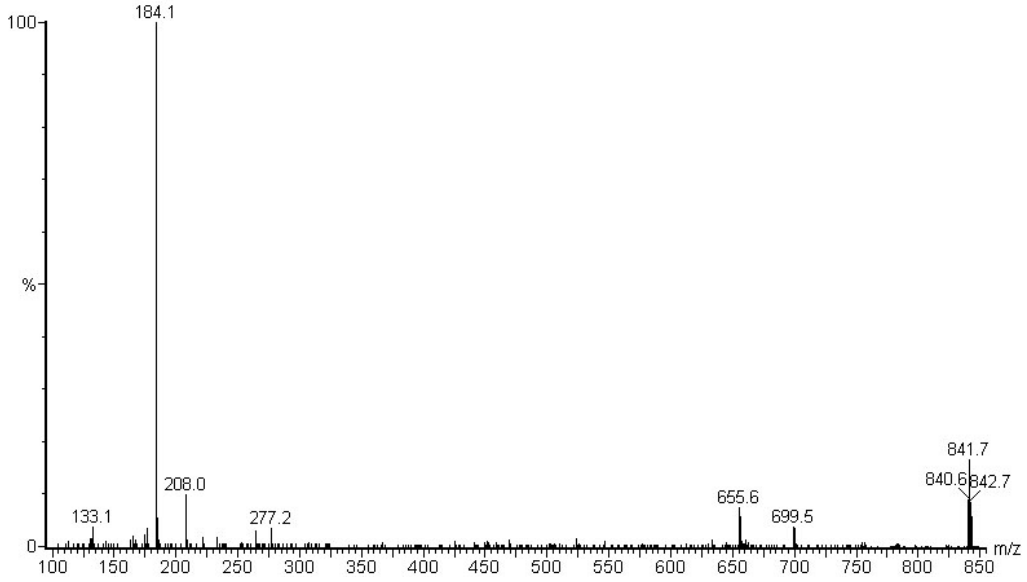


Figure 3-24: ***The MS/MS spectrum produced at scan 142 (labelled mass 840.5) in Figure 3-18.***
Laboratory-frame collision energy was set to 34 eV. This scan should correspond to a precursor ion of m/z 842

When performing precursor ion and neutral loss scans in negative ion mode, the response was inferior to the positive ion results. In an attempt to improve the quality of the reconstructed precursor ion and neutral scans, the scan time on the quadrupole was extended to 45 seconds for each *m/z* step. Unfortunately, this did not result in a significant improvement in the quality of the spectra and only the most intense precursor ion (*m/z* 885, PI40:4, data not shown) was able to be determined with certainty. This is presumably because negative ions are less intense overall, even in standard MS experiments.

In summary, an attempt was made to examine the potential of both precursor ion and neutral loss scans to separate phospholipids into headgroup classes on the Q-ToF 2 in

order to overcome some of the isobaric interference problems. After this examination, however, it was apparent that such experiments would not be suitable for the separation of phospholipid classes in a complex biological mixture. Therefore, positive and negative ion MS was used to determine molecular ion mass and conventional MS/MS CID experiments used for phospholipid identification in the current study.

3.2.5 Reproducibility

When attempting to analyse relative differences between groups (*e.g.*, tissues, exercise or diet) using any methodology, the measures must be reproducible. In order to test whether the MS spectra produced from a phospholipid extract were reproducible, samples were analysed at different times of the same day and on different days. Figure 3-25 (A) displays two positive ion spectra obtained from the same phospholipid extract at different times on the same day (each spectrum is the average of 20-40 scans). The two spectra have been overlaid and have an x-axis offset of 1% for ease of comparison. It is clear from these spectra that the positive ion spectrum obtained is highly reproducible. The equivalent comparison for negative ions is displayed in Figure 3-25 (B). Again, the spectra obtained on both occasions are virtually identical.

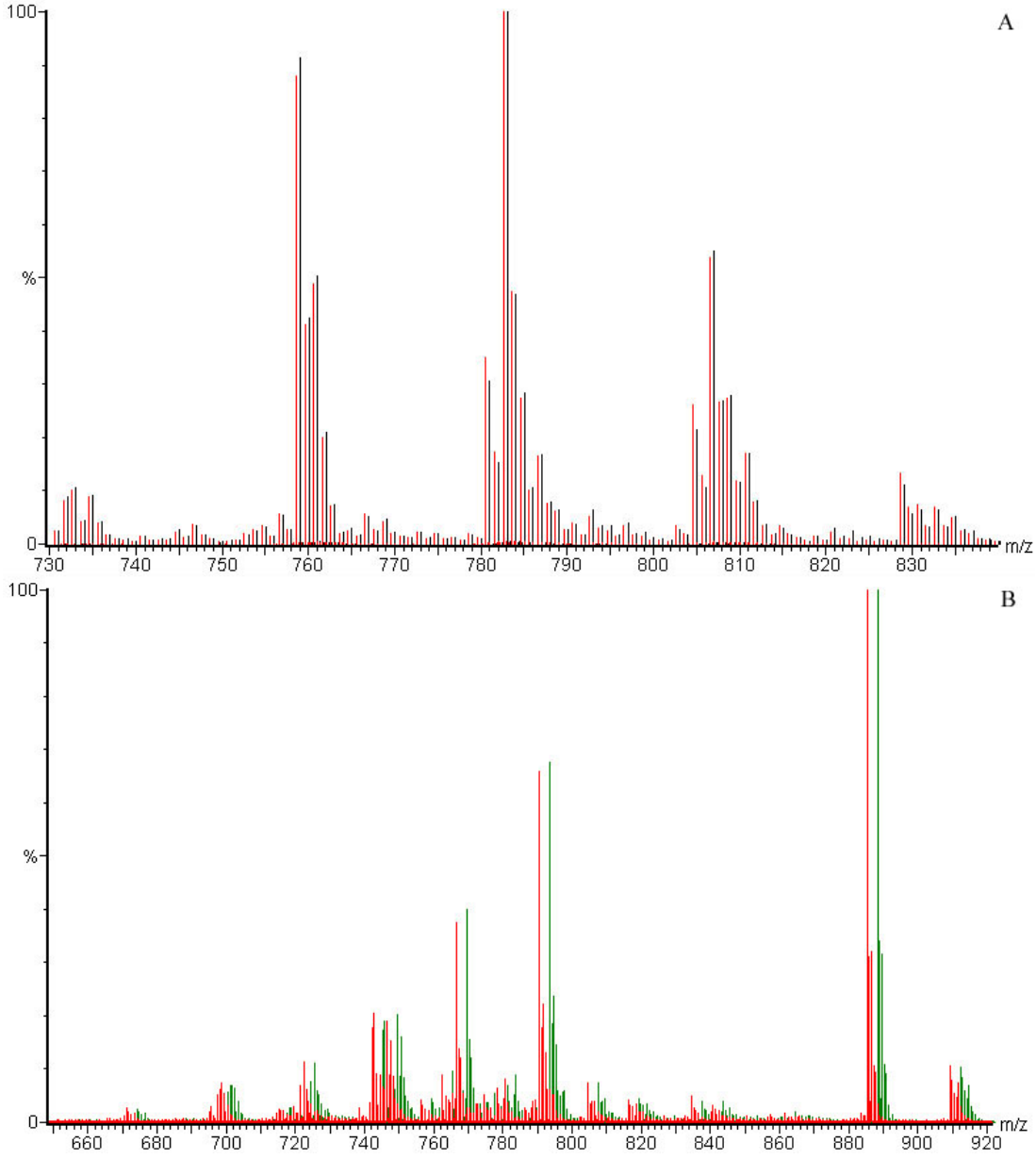


Figure 3-25: ***A comparison of two MS analyses of the same WVL phospholipid extract of a rat fed a high carbohydrate chow diet in (A) positive ions and (B) negative ions performed on the same day.***
Each positive ion spectrum was obtained from the average of approximately 20 and negative ion spectrum approximately 40, 5 sec acquisitions. The spectra have been overlayed with an x-axis offset of 1% with one shown in red and the other in black for positive ions or green for negative ions. WVL, white vastus lateralis muscle.

Very small variations in negative ion data could be due to small variations in the concentration of added ammonia and hence pH. Variation in the abundance of particular phospholipids with pH changes, such as PE has been observed in this and other labs (Han & Gross, 1995). Figure 3-26 displays two negative ion spectra obtained from the same phospholipid extract approximately one month apart. These spectra indicate that the mass spectral data presented here are highly reproducible and that furthermore, the lipid extracts themselves are stable when stored for protracted periods at low temperature (-80 °C). This means that variation between experimental groups, *e.g.*, trained vs sedentary could be assessed with confidence as the data would not be affected by variable instrument response.

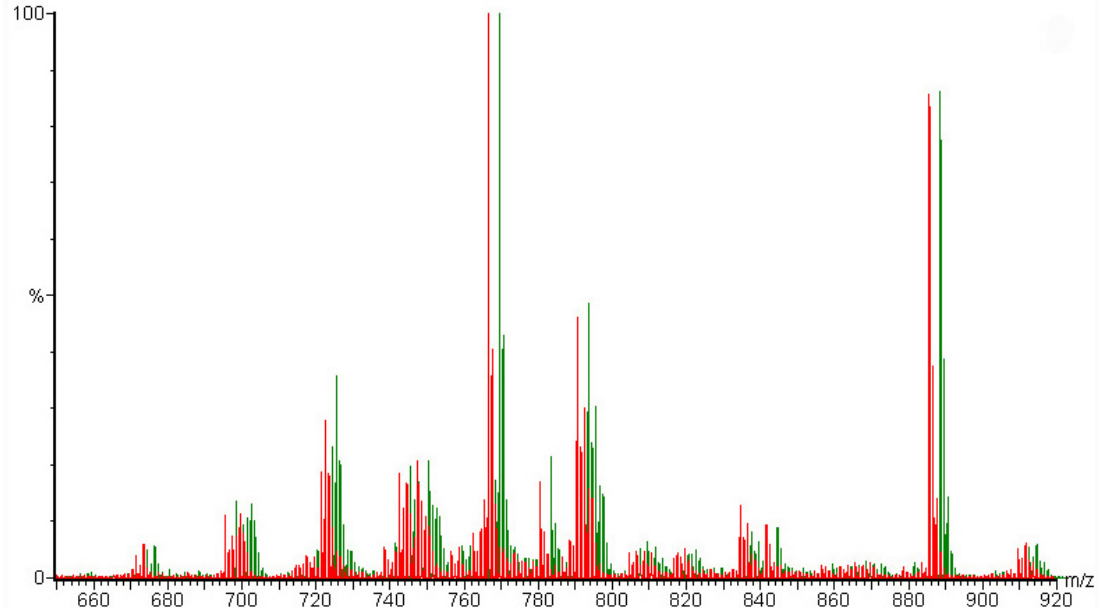


Figure 3-26: ***A comparison of two negative ion MS analyses of the same WVL phospholipid extract of a rat fed a high fat diet performed approximately one month apart.***
Each spectrum was obtained from the average of approximately 40, 5 sec acquisitions. The spectra have been overlayed with an x-axis offset of 1% with one shown in red and the other green. WVL, white vastus lateralis muscle.

3.2.6 Isotope correction and data normalisation

In order to compare between samples, the data obtained from separate MS experiments require normalisation. This was achieved by presenting the ion abundance of each phospholipid molecular species as a percentage of the total ion abundance of all identified phospholipids within the *m/z* range analysed. This procedure is akin to the one used to normalise FA data obtained from GC. Before performing such normalisation procedures it is crucial that any contribution of isotopic ions of one phospholipid molecular species to the observed abundance of another phospholipid molecular species is taken into account. To perform this correction a model spectrum displaying the percentage abundance of isotopic ions was created for each phospholipid molecular species that was within 2 *m/z*, on the low-mass side of another molecular species. This model spectrum was created using Masslynx software, version 3.5 (Micromass, Manchester, U.K.). The calculated isotope ion abundances were then subtracted from the ion abundance of the molecular ion with which they overlapped. This normalisation procedure was used in the following section, comparing MS to GC data, and in the studies described in subsequent chapters.

3.2.7 Method validation: A comparison with gas chromatography

To test the accuracy of the ESI-MS technique in the comparative analysis of phospholipid molecular species the results obtained by this method were compared to those obtained using the established technique of GC. This was achieved by comparing the results obtained by each technique from the *same phospholipid extract*. As discussed in Chapter 1, diet is known to have a large effect upon the phospholipid FA profile of skeletal muscle (Ayre & Hulbert, 1996; Andersson *et al.*, 2002). Accordingly, skeletal muscle phospholipid extracts taken from (i) rats on a high n-3 PUFA (n-3) diet and (ii) rats on a high saturated fat (SF) diet were analysed by both ESI-MS and GC. The FA composition of the two diets was determined by GC as described in the methods section of this chapter and is presented in Table 3.1.

Table 3-1: ***Fatty acid composition of the two diets***

Fatty acid	% Total	
	n-3	SF
16:0	12.7	15.0
16:1 (n-7)	2.4	17.5
17:0	ND	16.5
18:0	3.4	16.1
18:1(n-9)	19.8	29.4
18:2 (n-6)	39.6	5.4
18:3 (n-3)	5.8	ND
20:4 (n-6)	0.9	ND
20:5 (n-3)	2.5	ND
22:5 (n-3)	1.7	ND
22:6 (n-3)	11.3	ND

n-3, high n-3 diet; SF, high saturated fat diet; ND, not detected. Values are expressed in mol %

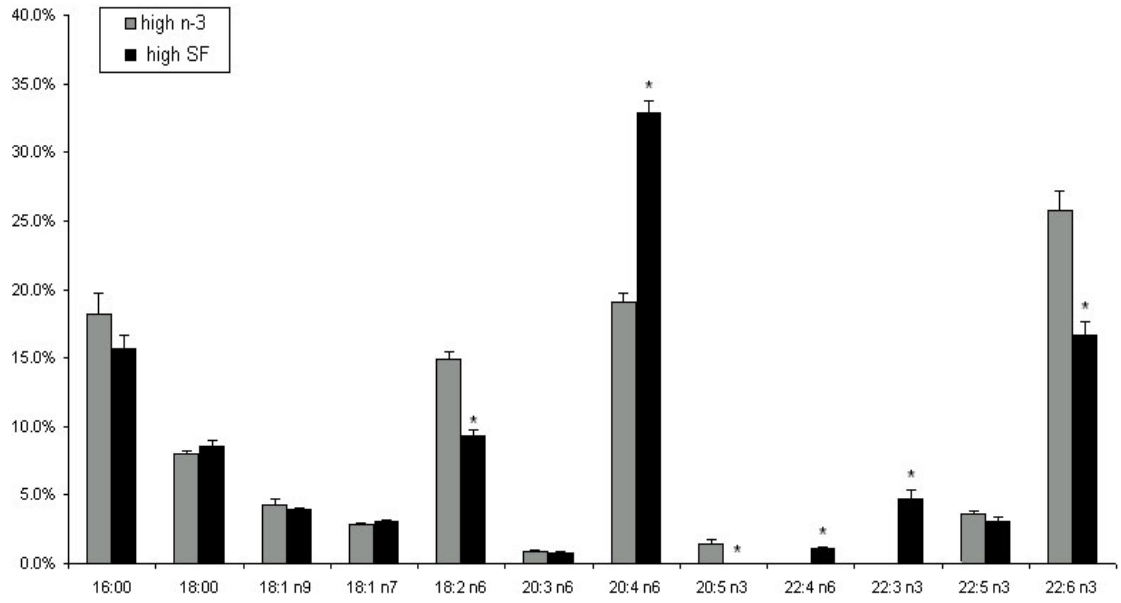


Figure 3-27: ***Comparison of the FA profile of rat skeletal muscle after a high n-3 or high saturated fat diet.***
*Values are expressed as mean percentage ± S.E.M. (n=6 per group). n-3, high n-3 fat diet; SF, high saturated fat diet *Significantly different from n-3 diet (P<0.05).*

Figure 3-27 displays the FA profile of rat vastus lateralis muscle obtained from GC analysis. The most notable difference in the skeletal muscle FA profile between the animals fed each diet was the relative proportion of the long-chain PUFAs. The muscle from the rats fed the high n-3 diet (shown in grey) had a greater level of 22:6 n-3 while those on the high saturated fat diet (shown in black) demonstrated elevated levels of 20:4 n-6. This is not surprising as the high n-3 diet was high in 22:6 n-3 while the saturated fat diet was completely devoid of any essential n-3 FAs (table 3.1). Even though the n-3 diet was also higher in essential n-6 FAs than the saturated fat diet, the partiality for n-3 PUFAs (discussed in Chapter 1, section 1.3.2) meant they were preferentially incorporated into the phospholipids. Conversely, the saturated fat diet contained only a relatively small amount of n-6 PUFAs and no 20:4 n-6. The only essential FA present in

the diet was 18:2 n-6 (Table 3.2), which was presumably elongated and desaturated to form 20:4 n-6, and incorporated into the vastus lateralis phospholipids at a relatively high level.

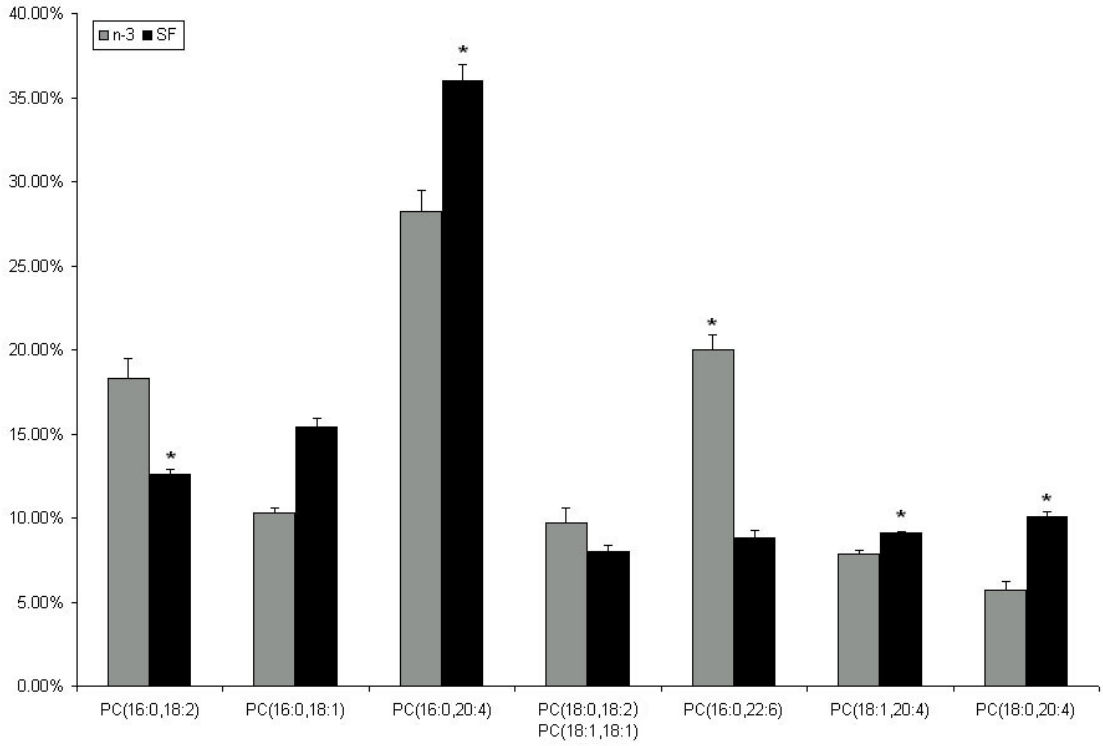


Figure 3-28: ***Comparison of the neutral phospholipid molecular species profile of rat skeletal muscle after a high n-3 or high saturated fat diet.***
Values are expressed as percentage mean normalised ion intensity ± S.E.M. (n=6 per group). n-3, high n-3 fat diet; SF, high saturated fat diet.
**Significantly different from n-3 diet (P<0.05).*

A comparison of neutral phospholipid molecular species (PC) present in the vastus lateralis of rats fed the two diets is presented in Figure 3-28. The procedures used in obtaining and presenting these data are described in the methods section of this chapter. The PC molecular species observed were dominated by the presence of 20:4 n-6 at the *sn*-

2 position with each of these species being significantly (P<0.05) more abundant in the skeletal muscle of rats fed the saturated fat diet (shown in black). Only one PC species containing 22:6 n-3 was observed, *i.e.* PC(16:0,22:6), and this was significantly (P<0.05) more abundant in the vastus lateralis of the rats fed the high n-3 diet (shown in grey).

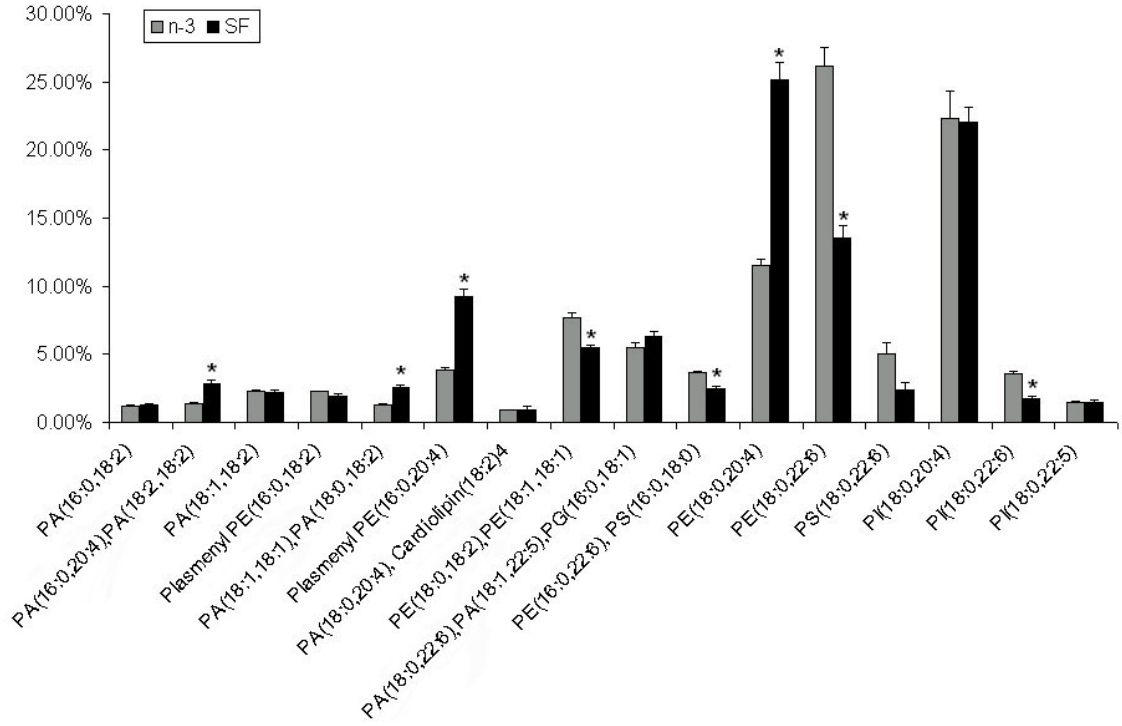


Figure 3-29: ***Comparison of the acidic phospholipid molecular species profile of rat skeletal muscle after a high n-3 or high saturated fat diet.***
*Values are expressed as percentage mean normalised ion intensity ± S.E.M. (n=6 per group). n-3, high n-3 fat diet; SF, high saturated fat diet. *Significantly different from n-3 diet (P<0.05).*

A similar distribution of acidic phospholipid molecular species containing 20:4 n-6 and 22:6 n-3 FAs was observed (Figure 3-29). One very interesting exception to this distribution was PI(18:0,20:4) ions where the abundance was almost identical in both

diets. This phospholipid is known to be an important molecule in a number of intracellular signalling pathways (Stryer, 1995) and may, therefore, be closely regulated.

The GC data and the results obtained from the ESI-MS analysis of the same phospholipid extracts demonstrate similar trends between diet groups. In particular, both methods show that molecular species containing 22:6 n-3 were of greater abundance in the muscle of rats that consumed a high n-3 diet while species containing 20:4 n-6 were of higher abundance in those that consumed the saturated fat diet. Additionally, these two FAs were the most abundant observed by both techniques.

3.3 Conclusions

The initial phases of this study, described in this chapter, involved testing various MS techniques and instrument settings on the Q-ToF 2 to determine the best methods for the differential analysis of biological phospholipid extracts and whether the results obtained would be reproducible and valid.

Initial analysis revealed that both protonated and sodiated PC species were present in positive ion MS studies, creating a problem when trying to analyse relative differences between treatments. The addition of lithium iodide to the sample was able to solve two problems. First, when the experimentally determined concentration of 800:1, lithium iodide:phospholipid was added only lithiated phospholipid species were observed and second, these species could then be definitively characterised by MS/MS.

After the pH of the sample was increased to approximately ten by the addition of ammonia (in order to deprotonate PE species), abundant deprotonated PE, PA, PG, PS and PI phospholipids were observed in negative ion mode. The CID of these species produced MS/MS spectra analogous to those observed in previous studies (discussed in Chapter 2, Section 2.3.2b). In addition, several new characteristic fragmentations were

also observed and mechanisms proposed. It was found that high laboratory-frame collision energies (~30 - 40 eV) caused substantial secondary fragmentation. This served to increase the abundance of the carboxylate ions and aid in the characterisation of the molecular species.

A number of different species with isobaric interference were observed in negative ion MS. While it is possible to perform 'pseudo' precursor ion and neutral loss scans on the Q-ToF 2 the results obtained from these analyses could not be used as they were under resolved and ambiguous. Accordingly, it was decided not to separate any group of ions with isobaric interference and to analyse them as a group. It is acknowledged that this may have a diluting effect upon any differences seen between groups in the following studies and the effect of the treatment (exercise in the case of this thesis) upon an individual phospholipid molecular species affected by isobaric interference cannot be determined. Nevertheless, any attempts to quantify the proportion of each species by comparing the abundance of product ions in MS/MS spectra would be inaccurate owing to the different amounts of secondary fragmentation occurring as some species require less energy for this to occur than others.

The results obtained from repeated ESI-MS analysis of the same lipid extracts demonstrate a high level of reproducibility. This was true for both positive and negative ions and across different days. Therefore, any variations in the relative ion abundances between groups would not be a function of instrument variability. In addition, the comparison of ESI-MS results to those obtained from GC indicates that ESI-MS is able to accurately detect changes in phospholipid speciation of a biological extract between treatment groups.

The details of the standard MS methods that were determined following these investigations are given in detail in section 3.4.3 below. These methods were then applied to the studies described in the following chapters.

3.4 Methods

3.4.1 Animal care

Sprague-Dawley rats were obtained from the Animal Resources Centre, Monash University, Melbourne, Victoria, Australia and the Animal Resource Centre, Perth, WA, Australia. Rats were housed two per cage and kept at 22 ± 1 °C and 50 ± 2 % relative humidity, under a 12 hour light-dark cycle (light 0700-1900 h). All experimental procedures were approved by the Animal Experimentation Ethics Committee at either the University of Wollongong or RMIT University.

3.4.2 Lipid extraction

Animals were euthanased by heart removal under anaesthesia (intraperitoneal injection of sodium pentobarbital at 60 mg per kg of body weight) and various tissues, *e.g.,* brain, heart and skeletal muscle dissected out. The tissues were frozen in liquid nitrogen and stored at –80 °C until required. Tissue lipids were extracted by standard methods (Folch *et al.*, 1957). Briefly, tissues (100-200 mg) were homogenised in 4 mL of ultra-pure grade chloroform:methanol (2:1, v/v) by hand using a glass tissue homogeniser and then left on a tube rotator overnight at 4 °C. This was followed by the addition of two millilitres of 1 M H_2SO_4 and the mixture spun at 400 g in a Sorvall® RT 6000D centrifuge (Du Pont, Wilmington, U.S.A) for 10 min. The bottom layer was retained and the procedure repeated. A small amount of sodium sulphate was added to absorb H_2O and then removed by filtration through silane treated glass wool. Lipid extracts were then dried under nitrogen and resuspended in 5 mL of hexane. Phospholipids were separated by solid phase extraction on Strata SI-2 silica cartridges (Phenomenex, Pennant Hills, NSW, Australia) using 3 x 10 mL methanol washes. All solvents used for lipid extraction contained butylated hydroxytoluene (0.01%, w/v) as an antioxidant. Total phospholipid

concentration of extracts was determined by phosphorous assay (Mills *et al.*, 1984). Phospholipid extracts were dried under nitrogen, resuspended in 0.8 mL of 72 % (w/w) perchloric acid and heated to 190 °C for 45 minutes. They were then placed on ice and 5 mL of water added. This was followed by the addition of 500 μL of the colour reagents ammonium molybdate (8 %, w/v) and stannous chloride (0.005 % dilution of 40 % (w/v) $SnCl_2$ in HCl). The solution was then made up to 10 mL by the addition of water and colour allowed to develop for 10 minutes. Absorbance was then read at 680 nm in a Shimadzu UV 1601 spectrophotometer (Shimadzu Scientific Instruments, Colombia, U.S.A.). The concentration of phosphorous associated with the phospholipid extract was quantified by comparison of absorbance values with a standard reference curve. The standard used was KH_2PO_4 at 20 μg mL^{-1} and the curve constructed using 1, 2, 5,and 10 μg of phosphorous.

Phospholipid content was calculated using equation 18:

$$phospholipid\ content = \frac{\mu g\ \ phosphorous \times 780}{30.97} \qquad \text{Equation 18}$$

Where 780 is the assumed average mass of phospholipids in grams and 30.97 is the molecular weight of phosphorous in grams.

3.4.3 Mass spectrometry

ESI-MS analyses were performed on a Micromass Q-ToF2 (Micromass, Manchester, U.K.) equipped with an electrospray ion source and controlled by Micromass Masslynx version 3.5 software operated on a Compaq professional workstation AP200. The instrument conditions used are summarised in Table 3.1.

Table 3-2*: Q-ToF 2 instrument conditions used to analyse phospholipids*

	Positive Ions	Negative Ions
Capillary voltage (V)	2800	-2800
MCP (V)	2300	2300
Cone Voltage (V)	30	-70
Source Temperature (ºC)	80	80
Desolvation Temperature (ºC)	120	120

Phospholipids were diluted to100 pmol μL^{-1} for negative ions and 25 pmol μL^{-1} for positive ions using ultra-pure grade methanol:chloroform 2:1 v/v. For negative ion analysis, the pH of the sample was increased to 10 by the addition of ammonia. To facilitate the formation of lithium adducts for positive ion characterisation, lithium iodide was added (~20 nmol μL^{-1}). Positive ion analysis was used to monitor neutral phospholipids (PC), while negative ion analysis was used for acidic phospholipids (PA, PG, PS, PI and PE). Samples were infused (20 μL min^{-1}) using a Harvard syringe pump and phospholipids were detected in the *m/z* range of 650 to 920. Tandem mass spectra (ESI-MS/MS) were obtained using argon as the collision gas at laboratory-frame energies ranging from 32 to 45 eV. The laboratory-frame collision energies were chosen to attain the maximum abundance of the carboxylate product ions without causing excessive secondary fragmentation of any characteristic head group product ions. This allows for the simple and accurate identification of phospholipid molecular species.

Typically, 100 - 120 spectra were averaged for each phospholipid extract. Each mass spectrum was normalised as a percentage of the total phospholipid species observed within the mass range (*m/z* 650 - 920) after correction for isotope contributions, *i.e.* the total ion abundance for each species is presented as a percentage of the total ion

abundance of all identified phospholipid molecular species. This procedure is analogous to the method used for normalisation of FA data obtained by GC.

3.4.4 Gas chromatography

FA acid analysis of phospholipid fractions was analysed by gas chromatography as described previously (Pan & Storlien, 1993). Phospholipid fractions were transmethylated by incubation in 14 % (w/v) boron trifluoride in methanol for 1 hr. The methylated FAs were then extracted using 5 x 2 mL hexane washes. A small amount of sodium sulphate was added to absorb H_2O and then removed by filtration through silane treated glass wool. FA methyl esters were purified by elution from florisil seppack columns (Phenomenex, Pennant Hills, NSW, Australia) using 7 mL of 5 % (v/v) diethyl ether in petroleum spirit. They were then separated by gas-liquid chromatography on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) with a 30 m x 0.25 mm internal diameter fused silica capillary column. Individual FAs were identified by comparing each peak's retention time to those of external standards. The relative amount of each FA (as a percentage of the total FA) was determined by integrating the area under the peak and dividing the result by the total area for all FAs. All solvents used for the preparation of FA methyl esters contained butylated hydroxytoluene (0.01 %, w/v) as an antioxidant.

3.4.5 Statistical analysis

Data analysis was performed using a Student's paired T-test. Significance was accepted at the level of $P<0.05$ and results are presented as means ± S.E.M. All statistical analyses were performed using JMP version 4.0 statistical software (SAS Institute Inc., Cary, USA). Figures 3-27, 3-28 and 3-29 were produced using Microsoft Excel 2000 all other figures were produced using Masslynx versions 3.5 and 4.0 (Micromass, Manchester, U.K.) and Adobe Photoshop version 6.0 (Adobe Systems Inc., U.S.A.).

Chapter 4

The Effect of Exercise on Skeletal Muscle Phospholipid Molecular Species Profile in Rats Fed a Laboratory-Chow Diet

4.1 INTRODUCTION

Phospholipids are the major structural component of biological membranes, and as such have a significant influence on their physical properties. Alterations in membrane phospholipid composition are known to influence a diverse range of cellular functions, from the properties of membrane-bound enzymes to cell growth (Spector & Yorek, 1985; Hulbert & Else, 1999). Furthermore, it has been established that the fatty acid (FA) composition of skeletal muscle phospholipids is linked to metabolic disorders such as obesity (Pan *et al.*, 1994; Storlien *et al.*, 1998) and insulin resistance (Borkman *et al.*, 1993; Helge *et al.*, 1998b). Overall, these findings have shown that saturated FAs are associated with insulin resistance whereas polyunsaturated FAs (PUFAs), particularly n-3 PUFAs, improve insulin stimulated glucose uptake in skeletal muscle (Storlien *et al.*, 1991; Sohal *et al.*, 1992; Borkman *et al.*, 1993; Clandinin *et al.*, 1993).

The FA composition of skeletal muscle phospholipids is altered by diet (Pan & Storlien, 1993; Ayre & Hulbert, 1996; Andersson *et al.*, 2002) and, to a lesser extent, by exercise training (Andersson *et al.*, 1998; Helge *et al.*, 1999; Andersson *et al.*, 2000; Helge *et al.*, 2001). It has also been established that alterations in the concentration of phospholipid head group classes occur in response to endurance training (Gorski *et al.*, 1999). A recent study in healthy humans by Helge and Dela (2003) found that exercise training was associated with minor increases in long-chain polyunsaturated fatty acid content in skeletal muscle phospholipid that were correlated with leg glucose uptake during a hyperinsulinemic clamp ($r = 0.57$, $p < 0.04$, $n = 8$). Clore *et al* (1998) have also reported correlations between the PUFA content of PC (but not PE) and glucose disposal during a hyperinsulinemic-euglycemic clamp in vastus lateralis of healthy humans ($r = 0.58$, $P < 0.01$, $n = 27$). Yet another recent report has established that a large number of insulin-sensitive changes in phospholipid molecular species occur in diabetic rat myocardium (Han *et al.*, 2000). Collectively, the results of these studies suggest that changes in muscle

membrane lipid composition, particularly phospholipid molecular species, may be an important determinant of alterations in insulin sensitivity.

Data presented in a recent report suggests that no significant exercise-induced changes are observed in the total skeletal muscle FA profile of chow-fed rats when analysed by GC (Turner *et al.*, 2004). Despite the constancy of the FA profile, extensive membrane remodelling (*i.e.* changes in phospholipid speciation) cannot be ruled out. Nevertheless, a search of the current literature failed to uncover any data describing changes in whole phospholipid molecular species in response to exercise training in skeletal muscle.

Recent advances in the analysis of phospholipid molecular species derived from biological sources by ESI-MS (Han & Gross, 2003; Pulfer & Murphy, 2003) now provides the capacity to rapidly screen for relative changes in specific phospholipid populations brought about by environmental factors such as diet or exercise (Pulfer & Murphy, 2003; see Chapter 2 for more detail). For the rapid analysis of phospholipid mixtures positive ion ESI-MS may be used to study neutral phospholipids (PC) and negative ion analysis for acidic phospholipids (PA, PS, PG, PI and PE). ESI-MS has a number of advantages over traditional methods of membrane lipid analysis such as; (i) high sensitivity; (ii) minimal sample handling; and (iii) the ability to structurally characterise phospholipid molecular species. The results of the investigations discussed in Chapter 3 also demonstrate this methodology to be both reproducible and accurate. In this chapter, ESI-MS is used to provide the first detailed analysis of changes in phospholipid molecular species associated with exercise training in rodent skeletal muscle.

The scope of this study has been extended by analysing both glycolytic (white vastus lateralis, WVL) and oxidative (red vastus lateralis, RVL) muscle fibres (Figure 4.1). Previous reports have demonstrated differences in the phospholipid FA profile of these muscle fibre types, with the insulin sensitive type I and type IIa (oxidative) fibres containing a greater proportion of long-chain PUFAs (Kriketos *et al.*, 1995) and a higher ratio of stearate (18:0) to palmitate (16:0) (Gorski *et al.*, 1998) than insulin resistant type

IIb (glycolytic) fibres. There is also evidence that exercise-induced changes in phospholipid head group classes differ in oxidative and glycolytic fibres, with increases in total PE content in the oxidative and total PI content in the glycolytic fibres (Gorski *et al.*, 1999). By adding these parameters to the study, this work will provide the first set of data describing the relative differences in the profile of phospholipid molecular species between muscle fibre types.

It is reasonable to expect a differing response to exercise intensity in the two muscle types as glycolytic fibres are primarily activated during more intense exercise while oxidative fibres are activated at lower intensity levels (Gollnick *et al.*, 1974; Armstrong *et al.*, 1987; Russell *et al.*, 2003). Accordingly, two exercise intensities (high vs. low) were also examined to determine if different levels of muscle activation result in differential alterations in the profile of phospholipid molecular species in the two fibre types.

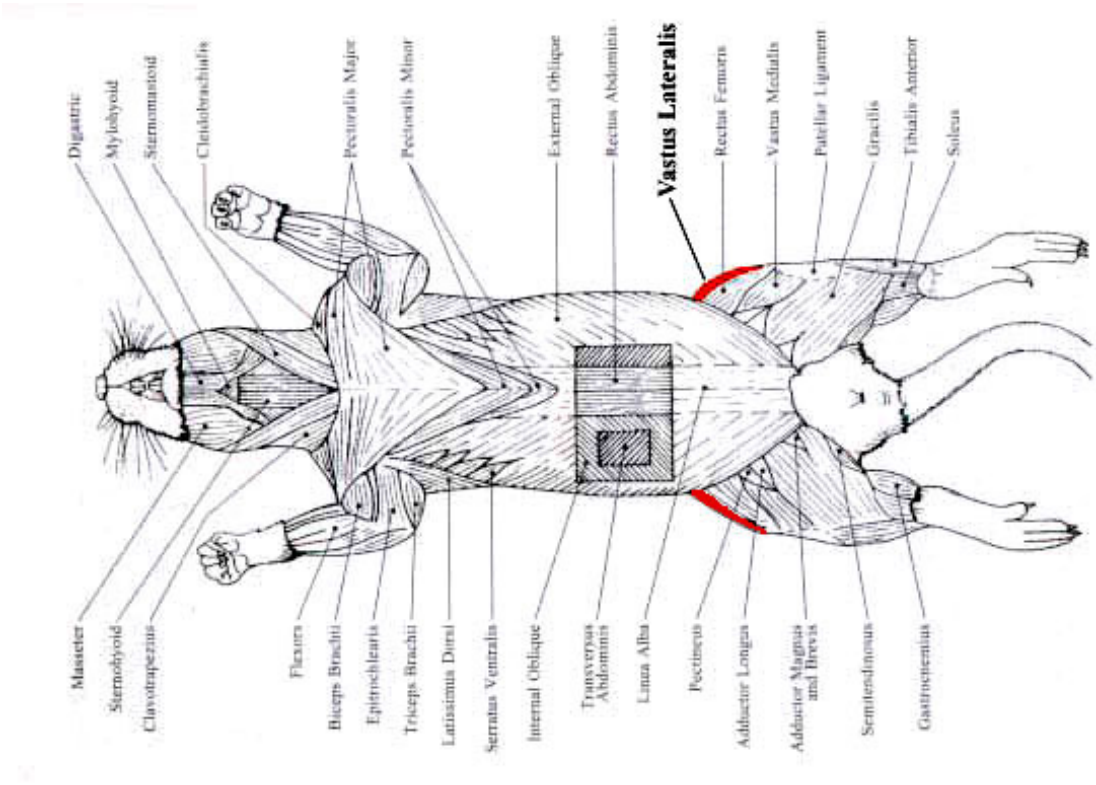


Figure 4-1: ***Ventral view of the rat superficial muscles.***
The vastus lateralis has been highlighted in red. The white fibres of the vastus lateralis are found superficially while the red fibres are found deeper within the muscle belly.

4.2 METHODS

4.2.1 Animal care, dietary treatments and exercise-training program

Muscle tissue for this study and the one described in Chapter 5 was provided by Professor John Hawley and his Exercise Metabolism Group in the School of Medical Sciences, RMIT University, Melbourne, Victoria, Australia. Diet and exercise interventions were designed and performed by this group.

Eighteen female Sprague-Dawley rats with an initial body mass of 90-100 g were obtained from the Animal Resource Centre, Monash University, Melbourne, Victoria, Australia and housed two per cage. Rats were kept at a constant 22 ± 1 °C and 50 ± 2 % relative humidity, with a 12 hour light-dark cycle (light 0700-1900 h). The rats were fed a standard laboratory chow diet (64 percent of energy (E%) carbohydrate, 20 E% protein and 16 E% fat) (Ridley Agriproducts Pty, Ltd., Victoria, Australia). The FA composition of the diet was determined using GC as previously described (Pan & Storlien, 1993) and is presented in Table 4.1. A more detailed explanation of the GC method is described in the methods section of the previous chapter (section 3.2.4). All experimental procedures were approved by the Animal Experimentation Ethics Committee of RMIT University.

Table 4-1: ***Fatty acid composition of the laboratory chow diet***

Fatty acid	% Total
16:0	12.7
16:1 (n-7)	2.4
18:0	3.4
18:1(n-9)	19.8
18:2 (n-6)	39.6
18:3 (n-3)	5.8
20:4 (n-6)	0.9
20:5 (n-3)	2.5
22:5 (n-3)	1.7
22:6 (n-3)	11.3

Values are expressed in mol % and also appear in Table 3-1

In the first week of the training protocol, all animals were familiarised with exercise by running on a motorised treadmill at 16 m min^{-1} for 10 min day^{-1} on a custom-built eight

lane motorised treadmill in the hours before dark. The treadmill was not equipped with any form of electric shock device. After one week, the rats were divided randomly into three groups, a control sedentary group (SED, n=6) that performed no specific training, a low intensity group (LOW, n=6) and a high intensity group (HIGH, n=6). The sedentary group performed 10 min of treadmill running at a speed of 16 m min^{-1}, 2 days wk^{-1} for 8 weeks for familiarisation purposes. The low and high intensity groups both had exercise intensity gradually increased over 4 weeks until they could complete 1000 m day^{-1} of treadmill running 4 days wk^{-1}. The low intensity group then performed 125 min of running at 8 m min^{-1}, 4 days wk^{-1} and the high intensity group 36 min at 28 m min^{-1}, 4 days wk^{-1} for the next four weeks. The LOW program was chosen because previous investigations have shown this speed (8 m min^{-1}) to elicit ~45 % of maximal O_2 uptake in rats, an intensity at which lipid oxidation predominates over carbohydrate oxidation (Divine-Patch & Brooks, 1980). This was an important factor in an original study examining the effect of high fat and high carbohydrate diets on endurance and substrate utilisation (Lee *et al.*, 2001). The metabolic adaptations induced by a running program that all animals could complete without the use of external motivation (*e.g.*, electric shock) was examined. Furthermore, this stimulus was maximized so that the effects of the two distinct training programs could be examined. In determining the appropriate speed for the HIGH group, pilot testing revealed that all rats could run at a speed of 28 m min^{-1} (a velocity 3.5 times faster than the speed of running in LOW), which corresponds to ~75 % of maximal O_2 uptake (Divine-Patch & Brooks, 1980). A summary of the training parameters for the final 4 weeks of the training period is provided in Table 4-2.

Table 4-2: Exercise training parameters for each group of rats during the last four weeks of the exercise intervention

Training Group	Duration of training session (min)	Treadmill speed (m min^{-1})	% VO_{2max}	Total distance per training session (m)	Training sessions per week	Total distance run per week (m)
SED	10	16	-	160	2	320
LOW	125	8	~ 45	1000	4	4000
HIGH	36	28	~ 75	1000	4	4000

SED, sedentary; LOW, low intensity training; HIGH, maximal voluntary running intensity; VO_2max, maximal oxygen uptake

4.2.2 Animal sacrifice and tissue preparation

After the 8 wk training program, animals were euthanased by heart removal under anaesthesia (intraperitoneal injection of sodium pentobarbital at 60 mg per kg body weight) 48 h after their last training bout. The muscles from the right hind limb were exposed and the red vastus lateralis (RVL; 16% type I, 33% type IIa, 50% type IIb fibres) and white vastus lateralis (WVL; 100% type IIb fibres) (Delp & Duan, 1996) were dissected out, frozen in liquid nitrogen and then stored at –80 °C until analysed.

4.2.3 Phospholipid extraction

All solvents used in the lipid analysis were of ultra-pure grade, purchased from Merck Pty Ltd (Kilsyth, Vic, Australia) and Crown Scientific (Moorebank, NSW Australia). Analytical grade butylated hydroxytoluene was purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Skeletal muscle lipids were extracted by standard methods (Folch *et al.*, 1957) that are described in more detail in Chapter 3. Phospholipids were separated by solid phase extraction on Strata SI-2 silica cartridges (Phenomenex, Pennant Hills,

NSW, Australia). A small amount (50 μL) of the phospholipid extract was used for MS analysis as outlined in this study. The remaining phospholipid extract was taken for FA extraction and analysed by GC as previously reported (Turner *et al.*, 2004).

4.2.4 Mass spectrometry

ESI-MS was performed using the methods developed in chapter three as described in section 3.4.3.

4.2.5 Statistical analysis

Data analysis was performed using a two-way analysis of variance (ANOVA) with training and fibre type as fixed factors. Where ANOVA revealed a significant effect, Tukey's post hoc test was administered to identify differences between training groups. Significance was accepted at the level of $P<0.05$ and results are presented as means ± S.E.M. All statistical analyses were performed using JMP version 4.0 statistical software (SAS Institute Inc., Cary, USA). Figure 4-1 was produced using Microsoft Excel 2000.

4.3 RESULTS

4.3.1 Compliance with training programs and anthropometric adaptations

All animals in the exercise training groups completed each of the prescribed training sessions. There were no significant differences in the body mass of animals between the three groups at the end of the 8 wk experimental period (248 ± 11 g). The effect of exercise training intensity on endurance running capacity and enzyme activity for these rats has been reported previously (Lee *et al.*, 2001). Briefly, endurance running capacity was increased by ~ 234 % following training at the low exercise intensity ($P < 0.05$) and ~ 332 % at the high exercise intensity ($P < 0.05$) compared to SED. Changes in the

activities of the enzymes citrate synthase, β-hydroxy-acyl-CoA dehydrogenase, and carnitine palmitoyl transferase, however, were minimal (Lee *et al.*, 2001).

4.3.2 Phospholipid molecular species composition

A comparison between exercise training and the observed phospholipid profile is presented in Tables 4-3 and 4-4 (neutral and acidic phospholipids, respectively). A comparison of significant training-induced changes in phospholipid profile between the glycolytic and oxidative fibres is displayed in Figure 4-2.

4.3.2.a Training comparisons

No novel molecular species were observed in glycolytic or oxidative muscles after exercise training. Nevertheless, a number of significant changes in the overall profile of both neutral (Table 4-3, column T) and acidic (Table 4-4, column T) phospholipids were observed following exercise training. Increases in the abundance of PA(16:0,18:2) (P<0.05), PA(18:1,18:2) (P<0.001), plasmenyl PE(16:0,18:2) (P<0.005), PI(18:0,22:5) (P<0.01) and PC(16:0,18:2) (P<0.01) ions were all observed with training. Exercise training was also associated with decreases in the abundance of PE(18:0,22:6) (P<0.001), PC(16:0,20:4) (P<0.005), and PC(16:0,22:6) (P<0.05) ions.

Table 4-3: *Neutral phospholipid molecular species*

Molecular Species	*m/z*	SED		LOW		HIGH		*P* Value		
	$[M+Li]^+$	WVL	RVL	WVL	RVL	WVL	RVL	T	F	TxF
PC(16:0,18:1)	766	10.7±0.3	10.3±0.3	10.9±0.2	11.4±0.4	10.2±0.3	11.8±0.2*†	NS	NS	<0.05
PC(16:0,18:2)	764	19.5±0.9	18.3±1.2	22.9±0.5	20.4±1.0	21.8±0.9	20.9±0.6	<0.01	NS	NS
PC(16:0,20:4)	788	29.7±1.1	28.2±1.3	27.1±0.4	25.3±0.4	26.9±0.6	25.7±0.3	<0.005	NS	NS
PC(16:0,22:6)	812	18.3±1.2	20.0±0.9	16.3±0.4	18.1±0.3	16.7±0.5	18.6±0.5	<0.05	NS	NS
PC(18:0,18:2) PC(18:1,18:1)	792	6.9±0.4	9.7±0.9	7.8±0.3	10.1±1.4	8.7±0.7	8.6±0.7	NS	<0.05	NS
PC(18:0,20:4)	816	6.9±0.5	5.7±0.5	7.4±0.7	6.8±0.4	8.1±0.9	6.6±0.3	NS	NS	NS
PC(18:1,20:4)	814	8.0±0.3	7.8±0.3	7.7±0.2	7.8±0.4	7.7±0.2	7.9±0.3	NS	NS	NS

m/z, mass-to-charge ratio; M, molecular mass; Li, lithium; T, training; SED, sedentary; LOW, low intensity training (8 m min^{-1}); HIGH, maximal voluntary running intensity (28 m min^{-1}); F, fibre; WVL, white vastus lateralis; RVL, red vastus lateralis; NS, not significant. *Significantly different from SED ($P<0.05$). †Significantly different from WVL ($P<0.05$). Values are expressed as mean normalised ion intensities ± S.E.M. after correction for isotope effects (n=6 per group).

Table 4-4: *Acidic phospholipid molecular species*

Molecular Species	*m/z*	SED		LOW		HIGH		*P* Value		
	$[M+Li]^+$	WVL	RVL	WVL	RVL	WVL	RVL	T	F	TxF
PA(16:0,18:2)	671	1.7±0.2	1.2±0.0	2.3±0.1*	1.3±0.1†	2.5±0.3*	1.3±0.1†	<0.05	<0.05	NS
PA(16:0,20:4) PA(18:2,18:2)	695	1.9±0.3	1.4±0.1	2.2±0.1	1.4±0.1†	2.3±0.2	1.3±0.1†	NS	<0.05	NS
PA(18:0,20:4) Cardiolipin$(18:2)_4$	723	0.8±0.2	0.9±0.1	0.8±0.2	1.1±0.1	0.8±0.1	1.1±0.1	NS	NS	NS
PA(18:0,22:6) PA(18:1,22:5) PG(16:0,18:1)	747	6.9±0.6	5.5±0.4	6.5±0.6	6.1±0.2	6.8±0.8	5.9±0.2	NS	NS	NS
PA(18:1,18:1) PA(18:0,18:2)	699	1.6±0.1	1.3±0.1	1.9±0.1	1.5±0.1	2.0±0.2	1.4±0.1	NS	NS	NS
PA(18:1,18:2)	697	3.1±0.3	2.3±0.0	4.3±0.2*	2.7±0.1†	4.4±0.3*	2.5±0.1†	<0.001	<0.05	<0.05
Plasmenyl PE(16:0,18:2)	698	3.1±0.3	2.2±0.0	4.4±0.3*	2.6±0.1†	4.5±0.2*	2.3±0.1†	<0.005	<0.05	<0.05
Plasmenyl PE(16:0,20:4)	722	6.3±0.6	3.9±0.2†	7.0±0.3	3.9±0.2†	7.6±0.4	3.5±0.2†	NS	<0.0001	NS
PE(16:0,22:6), PS(16:0,18:0)	762	4.2±0.3	3.6±0.1	4.2±0.3	3.8±0.2	4.1±0.1	3.8±0.1	NS	<0.05	NS
PE(18:0,18:2) PE(18:1,18:1)	742	7.9±0.7	7.6±0.4	8.0±0.4	8.2±0.4	8.4±0.2	7.4±0.4	NS	NS	NS
PE(18:0,20:4)	766	13.2±1.2	11.5±0.5	12.1±0.9	11.5±0.3	12.5±0.5	10.0±0.4	NS	NS	NS
PE(18:0,22:6)	790	19.4±0.9	26.2±1.4†	15.5±0.7*	24.0±0.9†	15.4±0.7*	23.0±0.4†	<0.001	<0.0001	NS
PS(18:0,22:6)	834	3.8±1.1	5.0±0.9	5.6±1.3	4.6±0.4	4.6±0.9	5.5±0.7	NS	NS	NS
PI(18:0,20:4)	885	21.5±2.6	22.3±2.0	20.7±1.2	22.1±1.5	19.7±1.7	25.6±2.0	NS	NS	NS
PI(18:0,22:5)	911	1.5±0.1	1.5±0.1	1.5±0.1	1.7±0.1	1.7±0.0	1.8±0.1	<0.01	NS	NS
PI(18:0,22:6)	909	3.2±0.3	3.6±0.2	2.9±0.1	3.5±0.1	2.9±0.2	3.7±0.2	NS	NS	NS

m/z, mass-to-charge ratio; M, molecular mass; Li, lithium; T, training; SED, sedentary; LOW, low intensity training (8 m min^{-1}); HIGH, maximal voluntary running intensity (28 m min^{-1}); F, fibre; WVL, white vastus lateralis; RVL, red vastus lateralis; NS, not significant. *Significantly different from SED ($P<0.05$). †Significantly different from WVL ($P<0.05$). Values are expressed as mean normalised ion intensities ± S.E.M. after correction for isotope effects (n=6 per group).

Many of the main training effects were observed to occur in the white vastus lateralis, all at a significance level of $P<0.05$ (Figure 4-2). The largest effect of exercise was a significant decrease in PE(18:0,22:6) ions. This decrease was seen in both the low and high intensity training groups compared to the sedentary animals (Table 4-4). Conversely, increases were observed in PA(16:0,18:2), PA(18:1,18:2), and plasmenyl PE(16:0,18:2) in both the low and high intensity groups.

Similar trends to those found in white vastus were also observed in red vastus, however, only one significant training-induced alteration in the phospholipid profile was observed (Figure 4-2). This was an increase in the abundance of PC(16:0,18:1) ($P<0.05$) ions in the high but not in the low intensity training regime when compared to the sedentary animals. Even though a number of differences were observed between the sedentary group and the two training groups, no statistically significant difference was observed between the two training intensities.

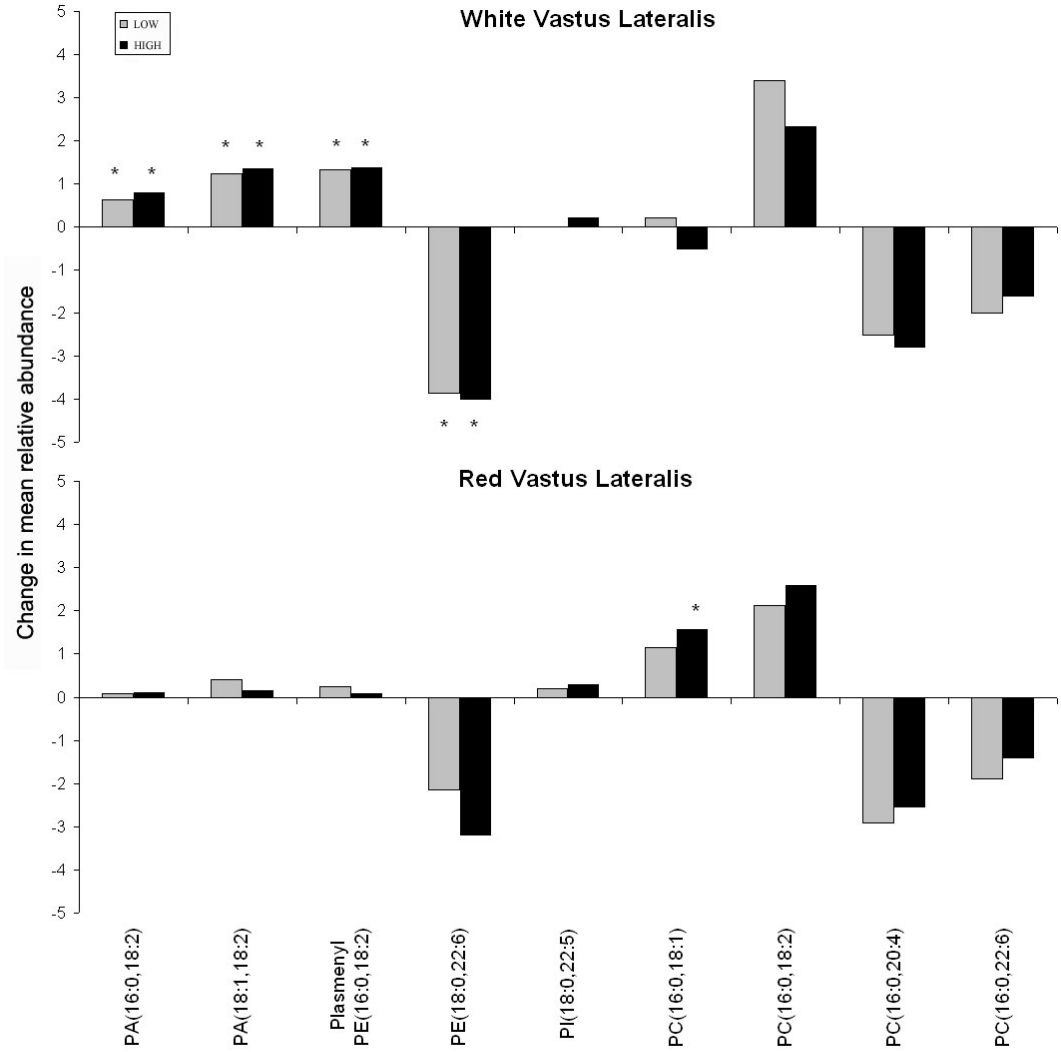


Figure 4-2: ***Change in mean relative abundance of phospholipid molecular species after exercise training in White (top) and Red (bottom) Vastus Lateralis.***

Only species with a significant overall effect of exercise training are displayed (see Tables 4-3 and 4-4 column T). LOW, low intensity training (8 m min^{-1}); HIGH, maximal voluntary running intensity (28 m min^{-1}). PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Values are expressed as the absolute change in mean normalised ion intensity from the SED group (n=6 per group).

**Significant change from SED group of same muscle type (P<0.05).*

4.3.2.b Comparison of oxidative and glycolytic fibres

A number of differences in the overall phospholipid profile between muscles were observed in the acidic phospholipids (Table 4-4, column F) with only one difference observed in the neutral phospholipids (Table 4-3, column F). The largest difference between the two muscles was a decrease in plasmenyl PE(16:0,20:4) (P<0.0001) and an increase in PE(18:0,22:6) (P<0.0001) in the oxidative (RVL) compared to the glycolytic (WVL) muscle. The red vastus also had a lower abundance of PA(16:0,18:2), PA(16:0,20:4), PA(18:2,18:2), PA(18:1,18:2), plasmenyl PE(16:0,18:2) ions and the isobaric PE(16:0,22:6) and PS(16:0,18:0) ions (P<0.05 for each). A higher abundance of isobaric PC(18:0,18:2) and PC(18:1,18:1) ions (P<0.05) was also observed in the red vastus than in the white vastus.

Only two significant differences in phospholipid speciation between WVL and RVL were observed in the sedentary animals. That is, a lower level of plasmenyl PE(16:0,20:4) ions and a higher level of PE(18:0,22:6) ions in the oxidative muscle fibres. The number of differences in acidic phospholipid species between the two muscles increased dramatically in the low intensity training group with no further differences in the high intensity group (Table 3). The only difference observed in the neutral phospholipid species within training groups was a slight increase in the abundance of PC(16:0,18:1) ions in the oxidative fibres of the high intensity training group (Table 2). This divergence between the two muscles with exercise, particularly in acidic phospholipid species, highlights the fibre specific effects of training on the phospholipid molecular species profile.

4.3.2.c Interaction of training and fibre type

The interaction of training and fibre type was found to have a significant effect upon PA(18:2,18:2), plasmenyl PE(16:0,18:2) and PC(16:0,18:1) (P<0.05 for each). Both PA(18:2,18:2) and plasmenyl PE(16:0,18:2) ions increased in abundance with exercise

training in WVL but not RVL while a decrease in abundance was apparent for PC(16:0,18:1) ions also in the white vastus but not the red vastus.

4.4 DISCUSSION

The FA composition of skeletal muscle phospholipids is altered in both humans (Andersson *et al.*, 1998; Andersson *et al.*, 2000; Helge *et al.*, 2001) and rats (Kriketos *et al.*, 1995; Helge *et al.*, 1999; Turner *et al.*, 2004) after chronic (≥ 4 wk) exercise training. This study provides the first data describing the effect of exercise training on skeletal muscle phospholipid molecular species profile. Specifically, it has characterised relative changes in the phospholipid molecular species profile of both oxidative (RVL) and glycolytic (WVL) muscle fibres with low and high intensity exercise training. Differences in the phospholipid molecular species profile between these two muscle fibre types have also been characterised for the first time.

Previous studies have reported that long chain n-3 PUFAs (22:6, n-3) are higher in the insulin sensitive oxidative (type I and type IIa) fibres (Kriketos *et al.*, 1995) compared to the glycolytic type II fibres and that type I fibres have a preference for stearic acid (18:0) over palmitic acid (16:0) (Blackard *et al.*, 1997). The results of the present study reveal that these differences in phospholipid FA composition are manifest in phospholipid PA and PE species. Even though the phospholipid molecular species profile was found to be similar in both the oxidative and glycolytic muscles, a number of differences in the abundance of particular molecular species were observed. The largest differences being a higher level of PE(18:0,22:6) and a lower level of plasmenyl PE(18:0,20:4) in oxidative fibres. There is also some level of similarity between the phospholipid profile of the skeletal muscles reported here and that of rodent cardiac muscle reported previously (Han *et al.*, 2000). That is, the most abundant phospholipid molecular species, *e.g.,* PC(16:0,20:4), PE(18:0,22:6) and PI(18:0,20:4), are similar in each muscle with the

exception of cardiolipin and PC(18:0,20:4) that were found in higher abundance in cardiac muscle than in the skeletal muscles examined here.

An important finding of the present study is that exercise training is associated with alterations in the relative abundance of a number of phospholipid molecular species in rat skeletal muscle. The results also demonstrate that exercise-induced changes in skeletal muscle membranes are not facilitated through the creation of novel molecular species but rather through changes in abundance of molecular species already present in the membrane. These data suggest this membrane rearrangement is occurring predominantly in PC, PE and PA species. The greatest effect of exercise training on an individual phospholipid molecular species was a reduction in the relative amount of PE(18:0,22:6) in glycolytic fibres. Interestingly, analogous reductions in this same specific phospholipid have been observed with aging (Barcelo-Coblijn *et al.*, 2003) and thermal adaptation (Buda *et al.*, 1994; Kitajka *et al.*, 1996) in the brain, indicating that this molecule is regularly targeted in association with physiological adaptation to a variety of stressors.

The observed changes in phospholipid molecular species profile may be partially explained by exercise-induced changes in muscle morphology such as mitochondrial biogenesis, changes in fibre type and hypertrophy. It is unknown if mitochondrial biogenesis was a major contributor to the changes observed in this study although, increases in mitochondrial content would result in an increase in the relative abundance of cardiolipin, found exclusively in the mitochondria of eukaryotes (Gurr *et al.*, 2002). An increase in cardiolipin was not observed in this study. Indeed, previous work on these rats demonstrated only small increases in the activities of several mitochondrial enzymes (citrate synthase, β-hydroxy-acyl-CoA dehydrogenase and carnitine palmitoyl transferase) with little or no additive effect of the more intense training protocol (Lee *et al.*, 2001). Furthermore, if exercise training had induced a shift from type IIb fibres to type IIa fibres it would be expected that the phospholipid composition of the two muscles would become more alike, when in fact the opposite occurred. This suggests that fibre type changes may

also not have been a major contributor to the observed effect of training. Although there was no significant difference in body mass between the exercised and sedentary rats the lean mass was not measured and therefore the synthesis of phospholipids to accommodate increases in muscle mass cannot be ruled out as a significant contributor to the observed changes in phospholipid molecular species profile. Nevertheless, while this would definitely increase the total number of phospholipid molecular species there is no evidence to suggest it would have an effect on their overall profile.

Glycolytic and oxidative muscles are know to be differentially activated by the level of exercise intensity (Gollnick *et al.*, 1974; Armstrong *et al.*, 1987; Russell *et al.*, 2003). Phospholipid molecular species profile, however, did not appear to reflect this differential activation in either fibre type, in spite of the fact that exercise training itself had a large effect upon skeletal muscle phospholipid species composition (Tables 4.3 and 4.4). Nevertheless, this lack of difference between exercise intensities is similar to that observed for a number of other muscle parameters (*e.g.* enzyme activity) in these same animals (Lee *et al.*, 2001). It is possible therefore, that the stimulus exerted by the higher-intensity training protocol may have been insufficient to exert additional effects above those achieved through the low-intensity regimen. It is also possible, however, that the magnitude of the expected differences was reduced owing to the fibre composition of the red vastus (~50 % type IIb fibres). A comparison between the white vastus and an oxidative muscle with less type IIb fibres, such as soleus, may demonstrate a more differential change in phospholipid molecular species profile in association with exercise intensity.

The large number of exercised-induced changes in phospholipid molecular species observed in the current study are in contrast with previous reports of only minor alterations in FA profile with exercise training (Kriketos *et al.*, 1995; Helge *et al.*, 1999; Turner *et al.*, 2004). In a previous report using GC analysis of these same lipid extracts it was found that exercise training resulted in a small (non significant) increase in linoleic

acid (18:2 n-6) content and slight reductions in the content of both docosahexaenoic acid (DHA, 22:6 n-3) and arachidonic acid (20:4 n-6) (Turner *et al.*, 2004). The finding of the present study, that we can detect changes in whole phospholipids but not in FAs (when examined by GC) at first appears contradictory but may be rationalised by the serious dilution effect of grouping all FAs for GC analysis. Using such a procedure, the level of sensitivity is reduced and consequently none of the trends observed in the previous report were strong enough to reach statistical significance. In concert with the FA changes, however, the concentrations of numerous 18:2 containing phospholipids were significantly increased after exercise training, *e.g.*, PA(18:1,18:2), plasmenyl PE(16:0,18:2) and PC(16:0,18:2). The data also display reductions in the major 22:6 n-3 and 20:4 n-6 containing phospholipid molecular species. Furthermore, the most abundant FAs observed by GC were 16:0, 18:2 n-6, 20:4 n-6 and 22:6 n-3. They were also the most abundant FAs observed when analysing phospholipid molecular species by ESI-MS. This agreement with the GC data was also demonstrated in the diet comparison described in Chapter 3 (Section 3.3.6). Together, these results indicate that the ESI-MS technique is both a sensitive and precise method to rapidly screen for relative changes in phospholipid populations brought about by environmental factors such as exercise and diet.

It should be noted that several of the exercise-induced changes in phospholipid populations were observed in phospholipid molecular ions of relatively low abundance, *e.g.,* PA(16:0,18:2) with a maximum normalised abundance of 2.5 (in the white vastus HIGH group), which constitutes only 2.5% of the acidic phospholipid profile. Although a direct comparison between species concentration would not be entirely accurate, owing to differences in ionisation efficiency between head groups, recent work (Koivusalo *et al.*, 2001) has demonstrated a bias towards PA species over other head groups when phospholipids were analysed under similar conditions as those used in the current study. This suggests that the contribution of this species to the total pool of membrane phospholipids may in fact be even lower than measured. Similarly, the abundance of PI(18:0,22:5) ions detected was a very low proportion of the total molecular ions.

Consequently, the observed increases in the abundance of these two molecular species may play only a small mechanistic role in the adaptation of skeletal muscle membranes to exercise training. That aside, PA is the precursor for the synthesis of all other phospholipids and triacylglycerol through the Kennedy pathway (Voelker, 2003) and it is possible that the changes observed in this PA species are occurring in the endoplasmic reticulum as new phospholipids are synthesized in response to exercise. The other significant changes were found to occur in PC and PE species, the two most abundant phospholipids found in rat skeletal muscle membranes (Blackard *et al.*, 1997). These phospholipids are distributed asymmetrically within the plasma membrane, PC being the most abundant species in the exofacial leaflet and PE the cytofacial leaflet (Rothman & Lenard, 1977). Thus, the observed changes in the relative concentration of these two species may be the consequence of a specific response by each membrane leaflet to exercise training.

The observed reductions in phospholipids containing long chain PUFAs is potentially the result of lipid peroxidation as previous data have shown an increase in free radical and hydroperoxide production in rodent skeletal muscle mitochondria during exercise (Mataix *et al.*, 1998). This rationale is also chemically sensible as long chain PUFAs have a greater propensity to undergo oxidation due to the greater number of activated bisallylic carbon centres on the acyl chain (Halliwell & Gutteridge, 1999). This may result in the removal of these peroxidised FAs by phospholipase A_2 as it has a greater affinity for peroxidised than normal phospholipids (Farooqui *et al.*, 2000). While it is possible that this alone may explain the reduction in the relative abundance of long chain PUFAs and increase in less-unsaturated FAs, the fact that the increase in the latter is occurring predominantly in PA species suggests that there may be a specific replacement with shorter-chain less-unsaturated PUFAs as a protective action against further free radical attack.

Reports that long-chain PUFAs are reduced in rodent skeletal muscle with exercise (Kriketos *et al.*, 1995; Helge *et al.*, 1999) tend to contradict the increases observed in human muscle (Andersson *et al.*, 1998; Helge *et al.*, 2001) and would suggest a deleterious effect on insulin sensitivity (Borkman *et al.*, 1993; Helge *et al.*, 1998b; Helge & Dela, 2003). While reductions in phospholipids containing long-chain PUFAs were also observed in the current study, another interesting finding is the different effect of exercise training on the phospholipid profile of glycolytic and oxidative muscle fibres. Whereas only a small difference in speciation between the white and red vastus lateralis was observed in sedentary animals, it was far greater following 8 weeks of exercise training, predominantly due to the change in glycolytic muscle fibres. These fibres have been shown to have very little insulin stimulated glucose uptake (James *et al.*, 1985), and as a result the observed reductions in long chain PUFAs would not likely have a major impact upon insulin sensitivity. It should also be highlighted that the relative abundance of a number of phospholipid molecular species containing long-chain PUFAs, *e.g.* PC(18:1,20:4) and PI(18:0,22:6), were not reduced after exercise and it may be that specific phospholipid molecular species are a more explicit determinant of alterations in insulin sensitivity than total phospholipid FA composition. Of note was that the abundance of PE(18:0,22:6) was significantly higher in the insulin sensitive oxidative muscle than in the glycolytic muscle. Furthermore, this level did not decrease after exercise training in the oxidative as it did in the glycolytic muscle. As high levels of membrane DHA (22:6, n-3) content have been linked to increases in insulin sensitivity (Sohal *et al.*, 1992; Clandinin *et al.*, 1993) it poses the question as to whether this molecule is selectively spared in the insulin sensitive tissue.

In conclusion, this study has demonstrated that rat skeletal muscle membrane phospholipids undergo a significant rearrangement under the influence of exercise training, which is in contrast to the minor changes in FA profile previously observed in these rodents (Turner *et al.*, 2004). Furthermore, this rearrangement is more prominent in glycolytic than oxidative fibres. In spite of this, information regarding the role of

particular phospholipid molecular species in physiological regulation is limited and therefore additional work is required to determine the functional significance of the observed changes. Further research in this area will prove to be highly informative as to the role of biological membranes in metabolism and their adaptation to various factors, *e.g.,* exercise and diet and their impact upon disease states such as insulin resistance and the metabolic syndrome.

Chapter 5

The Effect of Exercise on the Skeletal Muscle Phospholipid Molecular Species Profile of Rats Fed a Carbohydrate-Free High Saturated Fat Diet

5.1 Introduction

The data presented in the previous chapter provides the first direct evidence that exercise is associated with a large and significant remodelling of rat skeletal muscle membranes. Furthermore, this remodelling is observed at the level of phospholipid molecular species and is not seen in the total phospholipid FA profile as determined by GC. There is also existing evidence, as discussed in Chapter 1, that diet has a large influence upon the skeletal muscle phospholipid FA composition of rats (Pan & Storlien, 1993; Ayre & Hulbert, 1996; Turner *et al.*, 2004) and humans (Andersson *et al.*, 2002). Additionally, data presented in Chapter 3 (section 3.3.6) illustrates that diet also has a large effect at the level of phospholipid molecular species. In this chapter the effect of exercise on the same rodent muscles, *i.e.* red and white vastus lateralis will be examined when rats are fed a high saturated fat diet. This diet is also deficient in n-3 PUFAs, effectively altering the supply of essential FAs and so it is reasonable to expect some difference in the results obtained from the high-fat fed rats described in this chapter and the high-carbohydrate fed rats discussed in Chapter 4. Possible differences between diet groups include (i) an alteration in the skeletal muscle phospholipid molecular species profile of sedentary animals and consequently (ii) a difference in the effect of exercise.

A number of studies have reported that a short-term high-fat diet can elicit adaptive responses in skeletal muscle that increase exercise endurance capacity in rats (Miller *et al.*, 1984; Simi *et al.*, 1991; Lapachet *et al.*, 1996). Indeed, the rats used in this study demonstrated an increase in running endurance capacity when compared to the rats on the high carbohydrate laboratory chow diet discussed in the previous chapter (Lee *et al.*, 2001). An increase in the percentage contribution of fat oxidation to total energy supply during exercise may be partially responsible for the observed increase in endurance following a high-fat diet (Helge *et al.*, 1998a). A recent study on endurance-trained cyclists suggests that increased muscle triacylglycerol content may be at least partly responsible for this shift in substrate utilisation (Zderic *et al.*, 2004). Nevertheless, work

by Ayre and Hulbert (1997) found that the ratio of n-3 to n-6 PUFAs in the diet is a more crucial factor than total fat content in determining endurance capacity. They found that untrained-rats fed a diet high in n-6 PUFAs had a running endurance capacity 44% greater than those on a high n-3 diet (n = 4, $P < 0.02$). A significant decrease in the ratio of n-3 to n-6 FA content of soleus and extensor digitorum longus (EDL) phospholipids, 0.1 vs 1.2 and 0.2 vs 1.2 respectively ($P < 0.05$) was also apparent. Membrane lipid composition is known to influence the activity of membrane bound proteins (Wu *et al.*, 2004) and it is possible that changes in energy metabolism may be partially due to these influences. If this is the case, diet-induced alterations in muscle phospholipid composition may play a role in determining endurance capacity and based on the work described above the oxidative muscle (RVL) would be expected to exhibit a greater proportion of molecular species containing n-6 PUFAs than those described in Chapter 4.

In spite of the possible benefits to endurance performance, high-fat diets are known to induce insulin resistance in rat skeletal muscle (Kraegen *et al.*, 1986; Hansen *et al.*, 1998; Wilkes *et al.*, 1998; Kim *et al.*, 2000b; Axen *et al.*, 2003). It again appears that the types of fat present in the diet are important factors in the facilitation of this metabolic impairment. When Storlien *et al.* (1991) fed rats isocaloric diets that differed only in FA profile, insulin resistance was observed in animals fed diets high in saturated, monounsaturated or n-6 PUFAs but not in the rats fed high n-3 PUFAs. While the exact mechanism for this reduction in insulin action is not known, high-fat diets have been associated with reduced GLUT-4 expression (Kahn & Pedersen, 1993) and impaired insulin stimulation of both glycogen synthase activity (Oakes *et al.*, 1997) and glucose transport in skeletal muscle (Han *et al.*, 1997; Zierath *et al.*, 1997; Hansen *et al.*, 1998). Recent work by Han and co-workers has established that a large number of insulin-sensitive changes in phospholipid molecular species occur in diabetic rat myocardium (Han *et al.*, 2000). Clore *et al* (1998) have also reported correlations between the PUFA content of PC (but not PE) and glucose disposal during a hyperinsulinemic-euglycemic clamp in vastus lateralis of healthy humans (r = 0. 58, $P < 0.01$, n = 27). These studies

suggest a possible link between diet-induced alterations in muscle membrane lipid content and impaired insulin action (see also Chapter 1, section 1.2.1). There is also evidence that this impairment in glucose metabolism following a high-fat diet can be at least partially reversed by exercise training (Kraegen *et al.*, 1989; Kim *et al.*, 2000a). A recent study by Helge and Dela (2003) suggests that this too may be linked to muscle phospholipid FA content.

In Chapter 4, data were presented demonstrating an increase in the abundance of PE(18:0,22:6) and a decrease in the abundance of Plasmenyl PE(16:0,20:4) ions in oxidative compared to glycolytic muscle. In the current study it is expected that the magnitude of this difference will be blunted as the only PUFA present in the diet was 18:2 n-6. The previous study also indicated that glycolytic muscle has a greater level of remodelling than the oxidative muscle under the influence of exercise training. Accordingly, both glycolytic (WVL) and oxidative (RVL) muscles have again been analysed to see if the FA content of the diet influences this finding.

Although no difference in phospholipid molecular species profile was observed between low and high exercise training intensity in the previous chapter, the type of FAs available may have an impact on phospholipid molecular species profile as demand for β-oxidation increases with exercise intensity and energy requirements. This would be particularly pertinent in these rats that have depleted glycogen stores in both liver and RVL (Lee *et al.*, 2001). Consequently, both the high and low intensity training regimes have again been analysed. This will also allow a direct comparison to the results obtained in Chapter 4.

5.2 Methods

As mentioned in section 4.2 all muscle tissue for this study was supplied by Professor John Hawley and his exercise metabolism group at RMIT University, Melbourne, Victoria, Australia.

The methods used in this study were identical to those described in Chapter 4 (see section 4.2) with the notable exception of the diet composition. In this study, rats were fed a high fat diet that was devoid of carbohydrates (0 E% carbohydrate, 21.9 E% protein and 78.1 E% fat). While such a diet is extreme, and not attainable in free-living humans, the diet and training interventions were designed and performed by the exercise metabolism group at RMIT University to test the effect of a high fat diet and exercise training on running endurance (Lee *et al.*, 2001). Even so, this diet was chosen as studies investigating the effect of high-fat diets on endurance have traditionally used such extreme diets (Miller *et al.*, 1984; Conlee *et al.*, 1990; Lapachet *et al.*, 1996). Furthermore, this allows the examination of exercise-induced changes in phospholipid molecular species profile under the influence of two vastly different diets. The FA composition of the diet was determined using gas chromatography as previously described (Pan & Storlien, 1993) and is presented in Table 5-1 (for a more detailed explanation see Chapter 3, section 3.2.4). All experimental procedures were approved by the Animal Experimentation Ethics Committee of RMIT University.

Table 5-1: ***Fatty acid composition of the diet***

Fatty acid	% Total
16:0	15.0
16:1 (n-7)	17.5
17:0	16.5
18:0	16:1
18:1(n-9)	29.4
18:2 (n-6)	5.4

Values are expressed in mol % and also appear in Table 3-1.

5.3 RESULTS

5.3.1 Compliance with training programs and anthropometric adaptations

All animals in the exercise training groups completed each of the prescribed training sessions (see section 4.2.1). There were no significant differences in the body mass of animals between the sedentary (SED), low intensity training (LOW) and high intensity training (HIGH) groups at the end of the 8 wk experimental period (272 ± 10 g). The effect of exercise training intensity on endurance running capacity and mitochondrial enzyme activity for these rats has been reported previously (Lee *et al.*, 2001). In brief, the trained rats from both the LOW and HIGH groups demonstrated an improved endurance capacity compared to the untrained rats ($P < 0.05$), as did the high intensity trained rats compared to the low intensity trained rats ($P < 0.05$). In the analysis of enzyme activities (Lee *et al.*, 2001) a small but significant increase in the activity of β-hydroxy-acyl-CoA dehydrogenase was also observed in the red vastus of the rats in the HIGH compared to the LOW group (8.9 ± 0.5 vs 6.2 ± 0.9; $P < 0.05$). Changes in the activities of other

mitochondrial enzymes, *i.e.,* citrate synthase and carnitine palmitoyl transferase, however, were minimal (Lee *et al.*, 2001).

5.3.2 Phospholipid molecular species composition

A comparison between exercise training and the observed phospholipid molecular species profile of the high-fat fed rats is presented in Tables 5.2 and 5.3 (neutral and acidic phospholipids, respectively). A comparison of significant training-induced changes in phospholipid profile between the glycolytic and oxidative fibres is displayed in Figure 1.

5.3.2.a Training comparisons

No novel molecular species were observed in either glycolytic or oxidative muscles after exercise training. Nevertheless, a number of overall significant changes in the profile of both neutral (Table 5-2, column T) and acidic (Table 5-3, column T) phospholipids were observed following exercise training. Increases in the abundance of PA(18:1,18:2) (P<0.05), plasmenyl PE(16:0,18:2) (P<0.01), PC(18:0,20:4) (P<0.005) and PC(16:0,18:2) (P<0.005) ions were all observed with training. Training was also associated with decreases in the abundance of PE(18:0,22:6) (P<0.05), PC(16:0,20:4) (P<0.05), PI(18:0,22:6) (P<0.005) and PC(16:0,22:6) (P<0.0005) ions as well as the isobaric ions at *m/z* 747 [PA(18:0,22:6), PA(18:1,22:5) and PG(16:0,18:1)] (P<0.05) and 742 [PE(18:0,18:2) and PE(18:1,18:1)] (P<0.0005).

Table 5-2: *Neutral phospholipid molecular species*

Molecular species	*m/z*	SED		LOW		HIGH		P Value		
	$[M+Li]^+$	WVL	RVL	WVL	RVL	WVL	RVL	T	F	TxF
PC(16:0,18:1)	766	12.5±0.3	15.4±0.5†	12.3±0.6	14.4±0.3†	12.4±0.3	14.3±0.4†	NS	<0.0001	NS
PC(16:0,18:2)	764	10.4±0.4	12.6±0.3	12.8±0.9	16.1±1.0*†	11.4±0.6	15.2±0.8†	<0.005	<0.05	NS
PC(16:0,20:4)	788	43.4±0.7	36.0±1.0†	42.6±0.9	36.0±0.4†	41.3±0.9	34.3±0.5†	<0.05	<0.0001	NS
PC(16:0,22:6)	812	8.3±0.4	8.8±0.5	7.2±0.3	6.8±0.3*	7.6±0.3	7.1±0.3*	<0.0005	NS	NS
PC(18:0,18:2) PC(18:1,18:1)	792	6.0±0.5	8.0±0.3	7.6±1.8	8.5±0.3	7.3±01.1	9.5±0.2*	NS	NS	NS
PC(18:0,20:4)	816	9.4±0.4	10.0±0.3	8.4±0.8	10.1±0.2	10.2±0.2	11.1±0.4	<0.005	NS	NS
PC(18:1,20:4)	814	10.1±0.3	9.1±0.1	9.2±0.7	8.2±0.3	9.8±0.3	8.6±0.3	NS	NS	NS

m/z, mass-to-charge ratio; M, molecular mass; Li, lithium; T, training; SED, sedentary; LOW, low intensity training (8 m min^{-1}); HIGH, maximal voluntary running intensity (28 m min^{-1}); F, fibre; WVL, white vastus lateralis; RVL, red vastus lateralis; NS, not significant. *Significantly different from SED ($P<0.05$). †Significantly different from WVL ($P<0.05$). Values are expressed as mean normalised ion intensities ± S.E.M. after correction for isotope effects (n=6 per group).

Table 5-3: *Acidic phospholipid molecular species*

Molecular species	*m/z*	SED		LOW		HIGH		*P* Value		
	$[M-H]^-$	WVL	RVL	WVL	RVL	WVL	RVL	T	F	TxF
PA(16:0,18:2)	671	1.1±0.1	1.2±0.1	1.2±0.1	1.6±0.2	1.4±0.1*	1.4±0.2	NS	NS	NS
PA(16:0,20:4) PA(18:2,18:2)	695	2.6±0.3	2.8±0.3	2.5±0.2	2.6±0.2	3.0±0.2	2.6±0.2	NS	NS	NS
PA(18:0,20:4) Cardiolipin$(18:2)_4$	723	0.5±0.1	0.9±0.3	0.4±0.1	0.9±0.1	0.3±0.1	1.2±0.1†	NS	<0.05	NS
PA(18:0,22:6) PA(18:1,22:5) PG(16:0,18:1)	747	6.6±0.3	6.3±0.3	7.0±0.3	6.2±0.2	5.8±0.1‡	6.0±0.2	<0.05	NS	NS
PA(18:1,18:1) PA(18:0,18:2)	699	2.0±0.1	2.6±0.2	1.9±0.1	2.5±0.1	2.4±0.1	2.7±0.2	NS	<0.05	NS
PA(18:1,18:2)	697	1.5±0.1	2.2±0.2	1.9±0.2	2.8±0.3	1.9±0.2	2.8±0.3	<0.05	<0.05	NS
Plasmenyl PE(16:0,18:2)	698	1.4±0.1	1.9±0.2	1.8±0.2	2.6±0.3	1.9±0.2	2.8±0.3	<0.01	NS	NS
Plasmenyl PE(16:0,20:4)	722	9.3±0.5	9.3±0.5	10.0±1.0	9.7±1.0	10.8±0.6	9.3±0.9	NS	NS	NS
PE(16:0,22:6) PS(16:0,18:0)	762	2.6±0.2	2.5±0.2	2.5±0.1	2.3±0.2	2.7±0.1	2.5±0.2	NS	NS	NS
PE(18:0,18:2) PE(18:1,18:1)	742	4.0±0.2	5.5±0.2	4.9±0.3	7.7±0.6*†	5.5±0.4	7.3±0.3*†	<0.0005	<0.05	NS
PE(18:0,20:4)	766	23.4±1.0	25.2±1.3	24.6±0.5	27.3±0.5	25.0±0.6	24.5±0.4	NS	NS	NS
PE(18:0,22:6)	790	10.3±0.5	13.6±0.9†	8.9±0.2	12.1±0.5†	10.4±0.3	12.8±0.4†	<0.05	<0.0001	NS
PS(18:0,22:6)	834	3.6±0.4	2.4±0.5	2.8±0.5	1.9±0.6	3.9±0.2	2.9±0.2	NS	<0.05	NS
PI(18:0,20:4)	885	29.7±1.0	22.0±1.1†	28.2±1.5	18.5±2.2†	23.7±1.7	20.3±1.7	NS	<0.005	NS
PI(18:0,22:5)	911	1.5±0.1	1.5±0.2	1.3±0.1	1.2±0.1	1.4±0.1	1.3±0.1	NS	NS	NS
PI(18:0,22:6)	909	1.6±0.1	1.7±0.2	1.4±0.1	1.3±0.1	1.3±0.1	1.2±0.0	<0.005	NS	NS

m/z, mass-to-charge ratio; M, molecular mass, H, hydrogen; T, training; SED, sedentary; LOW, low intensity training (8 m min^{-1}); HIGH, maximal voluntary running intensity (28 m min^{-1}); F, fibre; WVL, white vastus lateralis; RVL, red vastus lateralis; NS, not significant. *Significantly different from SED (P<0.05). ‡ Significantly different from LOW (P<0.05). †Significantly different from WVL (P<0.05). Values are expressed as mean normalised ion intensities ± S.E.M. after correction for isotope effects (n=6 per group).

The number of significant training effects within fibre type, however, was far less than the overall effects of training (Figure 1). The largest effect of exercise was found to be significant increases in PC(16:0,18:2) ions (P<0.05) in the red vastus lateralis (Figure 5.1). This increase only reached significance, however, in the low intensity training group when compared to the sedentary animals (Table 5-3). Increases in the abundance of PC(18:0,20:4) ions (P<0.05) in the high intensity trained rats and isobaric PE(18:0,18:2) and PE(18:1,18:1) ions (P<0.05) in rats from both training regimes compared to the sedentary rats were also apparent in RVL. Conversely, decreases were observed in the abundance of red vastus PC(16:0,22:6) ions (P<0.05) in both the low and high intensity groups.

Only two significant exercise training-induced changes in phospholipid molecular species profile were observed in WVL. The first was an increase in the level of PA(16:0,18:2) ions (P<0.05). This was detected in the high but not the low intensity training regime when compared to the sedentary animals. The second was a decrease in the abundance of isobaric ions at *m/z* 747 (P<0.05), corresponding to PA(18:0,22:6), PA(18:1,22:5) and PG(16:0,18:1) ions. These ions were of lower abundance in the rats from the high intensity training group than those in the low intensity training group.

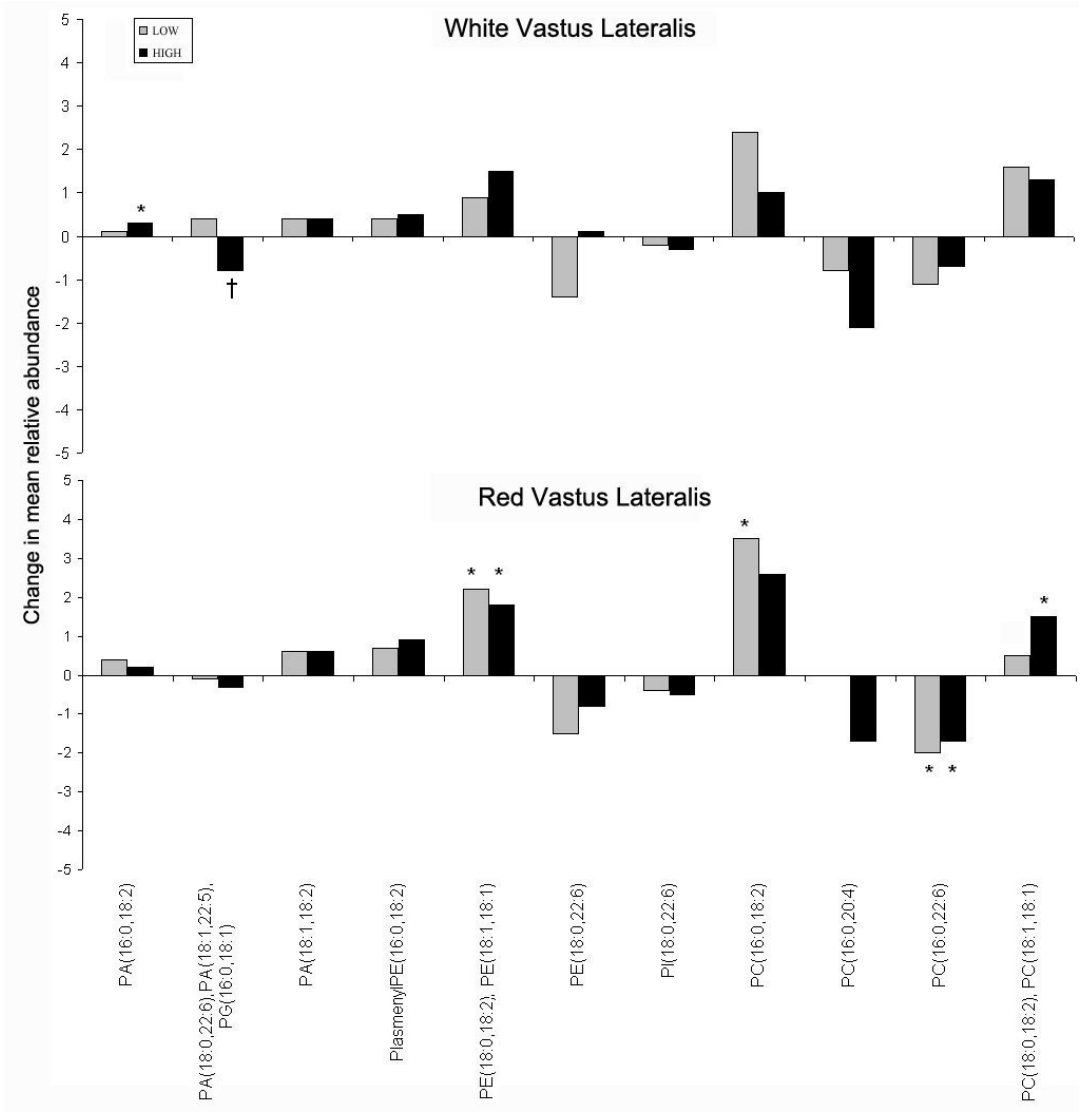


Figure 5-1: ***Change in mean relative abundance of phospholipid molecular species after exercise training in White (top) and Red (bottom) vastus lateralis.***

Only species with a significant overall effect of exercise training are displayed (seeTables 5-2 and 5-3 column T). LOW, low intensity training (8 m min^{-1}); HIGH, maximal voluntary running intensity (28 m min^{-1}). PC, phosphatidylcholine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol. Values are expressed as the absolute change in mean normalised ion intensity from the SED group (n=6 per group).

**Significant change from SED group of same muscle type (P<0.05).*

†Significant change from LOW group of same muscle type (P<0.05).

5.3.2.b Comparison of oxidative and glycolytic fibres

A comparison of fibre types independent of training regime demonstrates a number differences in the phospholipid profile between the two muscles in both the acidic (Table 5-2, column F) and neutral phospholipids (Table 5-3, column F). The largest difference between the two muscles was a decrease in PC(16:0,20:4) ions and increases in PE(18:0,22:6) and PC(16:0,18:1) ions in the oxidative (RVL) compared to the glycolytic (WVL) muscle (P<0.0001 for each). The red vastus also had a higher abundance of PC(16:0,18:2) and PA(18:1,18:2) ions, as well as the isobaric ions at *m/z* 699 [PA(18:1,18:1) and PA(18:0,18:2)], *m/z* 723 [PA(18:0,20:4) and Cardiolipin$(18:2)_4$] and *m/z* 742 [PE(18:0,18:2) and PE(18:1,18:1)] (P<0.05 for each). A lower abundance of PI(18:0,20:4) (P<0.005) and PS(18:0,22:6) ions (P<0.05) was also seen in the red vastus compared to the white vastus.

Only four significant differences in phospholipid speciation between white and red vastus lateralis were observed in the sedentary animals, all at a significance level of P<0.05. They were a lower level of PI(18:0,20:4) and PC(16:0,20:4) ions and a higher level of PE(18:0,22:6) and PC(16:0,18:1) ions in the oxidative muscle fibres. The number of differences in phospholipid profile between the two muscles increased in the low intensity training group with the additional differences being a greater abundance of both PC(16:0,18:2) ions (P<0.05) and the isobaric PE(18:0,18:2) and PE(18:1,18:1) ions (P<0.05) in RVL. In the high intensity training group red vastus lateralis was also found to have a greater level of ions at *m/z* 723 [PA(18:0,20:4) and Cardiolipin$(18:2)_4$] (P<0.05) than white vastus lateralis. Also, the difference in abundance of PI(18:0,20:4) ions between the two muscles was no longer apparent.

5.3.2.c Interaction of training and fibre type

The interaction of training and fibre type was found to have no significant effect upon the phospholipid molecular species profile of the skeletal muscle used in this study.

5.4 DISCUSSION

The results presented in the previous chapter demonstrate that exercise has the ability to remodel skeletal muscle membranes at the level of phospholipid molecular species and that this remodelling is not revealed when analysing the total phospholipid FA profile by GC. The results presented in this chapter provide novel data describing the effect of low and high intensity exercise training on the phospholipid molecular species profile of both oxidative (RVL) and glycolytic (WVL) muscle fibres of rats fed a carbohydrate-free, high-fat diet. Differences in the phospholipid profile between these two muscle fibre types have also been characterised.

It has been previously reported that long-chain n-3 PUFAs are higher in insulin sensitive type I and type IIa fibres while long-chain n-6 PUFAs predominate in type IIb fibres (Kriketos *et al.*, 1995). This preference for n-6 PUFAs was particularly apparent for PI(18:0,20:4) and PC(16:0,20:4) with the white vastus having a significantly greater abundance of each of these phospholipids compared to the red vastus. In contrast, the data presented in Chapter 4 indicates no significant difference in the abundance of these phospholipids between the two muscle types. This may be explained by the larger percentage of 20:4 n-6 containing phospholipids observed in the animals on the high fat diet, *i.e.,* as the availability of this long-chain n-6 PUFA increases the difference between muscles in the abundance of the major phospholipids containing this FA increases. Of particular interest is the higher abundance of PE(18:0,22:6) in RVL when compared to WVL. In spite of the fact that the diet was completely deficient in n-3 PUFAs the ratio of PE(18:0,22:6) abundance in oxidative to glycolytic muscle was almost identical across both diet groups. For example, in the sedentary rats the ratio of PE(18:0,22:6) ion abundance in red vastus to white vastus was 1.35 for the carbohydrate diet group and 1.32 for the fat diet group. It is also interesting to note that despite the large difference in dietary FAs, particularly the lack of essential n-3 FAs (see Table 5.1), the phospholipid molecular species observed are the same as those reported in the previous chapter. There

is, however, a large difference in the ratio of phospholipids containing n-6 PUFAs to those containing n-3 PUFAs as shown in Figure 5-2.

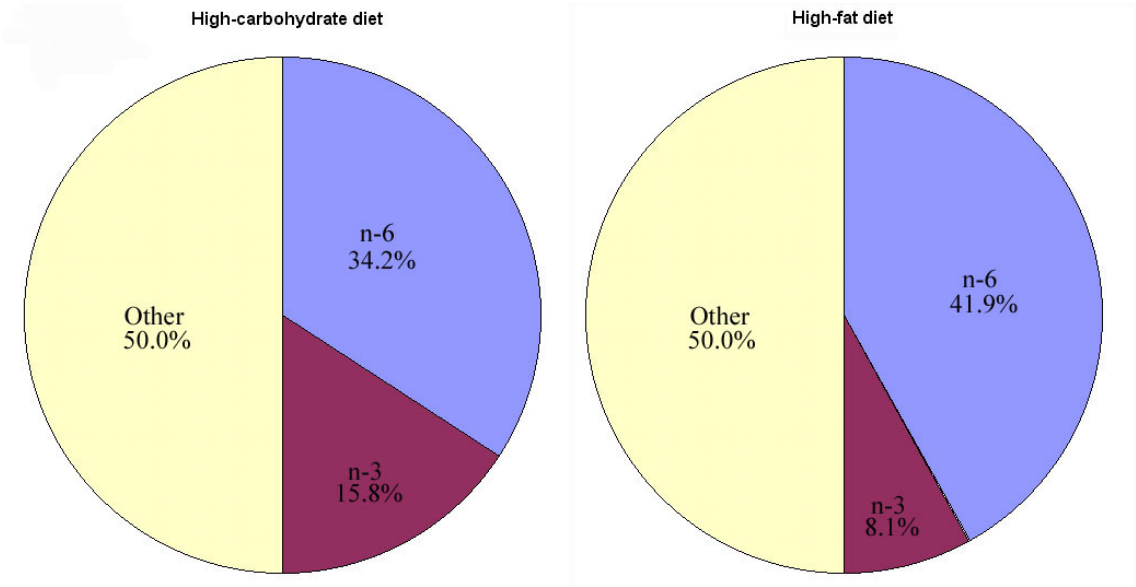


Figure 5-2: Approximate percentage of skeletal muscle phospholipids containing n-6 and n-3 PUFAs in sedentary rats from both diet groups.
Other, phospholipids not containing n-3 or n-6 PUFAs and those with isobaric species containing a mixture of both; n-3, phospholipids containing n-3 PUFAs; n-6, phospholipids containing n-6 PUFAs. Data was compiled from both red and white vastus lateralis of sedentary rats.

The abundance of a number of phospholipid molecular species was altered in association with exercise training. Exercise-induced changes appear to be facilitated through the remodelling of phospholipid species already present in the membrane and did not involve the creation of novel molecular species. This observation is consistent with the findings discussed in the previous chapter. Also in agreement with Chapter 4 is the finding that the largest changes where found to occur in PE and PC species (see Figure 5.1). This again suggests that remodelling of the membrane is occurring in both the exofacial and cytofacial leaflets. The greatest observed effect of training on an individual phospholipid molecular species was a increase in the abundance of PC(16:0,18:2) in oxidative muscle.

Unfortunately, there is little data describing the role of this phospholipid in biological processes. Although the changes in phospholipid molecular species reaching significance differed between the rats fed a high-fat, n-3 deficient diet in the current study and the high-carbohydrate, high n-3 fed rats discussed in the previous chapter, the pattern of exercise-associated changes in phospholipid molecular species was very similar between the two diet groups (*c.f.* Figure 4.2 and Figure 5.1). The most noticeable difference in this pattern for a particular phospholipid molecular species is PE(18:0,22:6). In Chapter 4, this species was found to be the phospholipid most influenced by exercise training, showing a large decrease in abundance in glycolytic muscle. In the current investigation a significant overall effect of exercise was again observed for this species, however, the reduction in abundance was much smaller and did not reach significance within either fibre type. This may be explained by the 47 % decrease in abundance of this species in the glycolytic-white vastus witnessed in the current study compared to that reported in the previous chapter and consequently a dampening of the effect of exercise. The large difference in the abundance of this phospholipid between the two diets also demonstrates the potential for change in the phospholipid molecular species profile of skeletal muscle with diet.

The exercise-induced changes in the skeletal muscle phospholipid molecular species profile described here appear far more extensive than those seen in the total phospholipid FA profile when analysed by GC in previous studies (Kriketos *et al.*, 1995; Helge *et al.*, 1999; Turner *et al.*, 2004). This finding is in agreement with the study described in Chapter 4 and is likely due to the dilution effect of combining the FAs from all phospholipid molecular species before analysis. The obvious conclusion drawn from this finding is that while the overall FA composition of the membrane is not greatly affected by exercise there is extensive remodelling occurring at the level of phospholipid molecular species. The GC analysis of these same lipid extracts has been recently reported and revealed that the phospholipid content of 18:2 (n-6) was significantly increased ($P<0.05$) in the red vastus lateralis of rats from both training regimes when compared the sedentary rats (Turner *et al.*, 2004). This is in agreement with the current

findings that a number of phospholipid molecular species containing this FA were also significantly increased in RVL, *e.g.,* PE(18:0,18:2) and PC(16:0,18:2). In fact, the largest effect of exercise on an individual phospholipid molecular species was an increase in the abundance of the latter. Again, this highlights the accuracy and specificity of the current method as tested against the well-established method of GC.

The overall trend in phospholipid molecular species profile appeared to be (i) decreases in species containing long-chain PUFAs and (ii) an increase in their shorter-chain counterparts with exercise. This is also in agreement with the findings of Chapter 4. In contrast though, is the observation that the oxidative muscle is affected to a greater extent than the glycolytic muscle in the high-fat animals whereas the glycolytic muscle was more greatly affected in the high-carbohydrate animals. Nevertheless, it is highly probable that the shift from long to short chain PUFAs is a consequence of lipid peroxidation owing to the increase in reactive oxygen species produced by the mitochondria of exercising skeletal muscle (Mataix *et al.*, 1998; see section 4.4. for further explanation).

As discussed in Chapter 4 it is possible that some of the observed changes in phospholipid profile are the result of increases in muscle mitochondrial content, changes in fibre type and/or hypertrophy. Again, however, no changes in cardiolipin content were observed between training groups indicating no significant changes in mitochondrial content. Nonetheless, an increase in the activity of the mitochondrial enzyme β-hydroxyacyl-CoA dehydrogenase in the RVL of the high intensity training group has been previously reported (Lee *et al.*, 2001). It is likely, however, that this is simply a reflection of increased FA oxidation in response to a greater energy demand required for the high intensity training regime rather than mitochondrial biogenesis. Moreover, no significant changes in citrate synthase or carnitine palmitoyl transferase activity were reported (Lee *et al.*, 2001). The observed exercise-induced changes in phospholipid profile were decreases in phospholipids commonly associated with oxidative fibres, *i.e.,* those containing n-3 PUFAs (Kriketos, *et al.,* 1995; see chapter 4). It is therefore unlikely that

fibre type changes were a large contributor to the reported changes in phospholipid profile. The synthesis of new phospholipids to accommodate muscle hypertrophy, however, cannot be disregarded as a significant contributor to these changes as lean mass was not determined. As stated in the previous chapter, however, there is no reason to suspect increases in total phospholipid quantity (to accommodate muscle hypertrophy) would have a large influence on the phospholipid molecular species profile.

Even though oxidative muscles are activated during low intensity exercise and glycolytic muscles at higher intensity levels (Gollnick *et al.*, 1974; Armstrong *et al.*, 1987; Russell *et al.*, 2003) little difference in phospholipid molecular species profile was observed between the two training protocols in either muscle type. There was, however, one difference in the glycolytic muscle with the intensity of ions with an *m/z* of 747 being significantly lower in the rats exposed to the high intensity training protocol than those in the low intensity group. Nevertheless, no significant difference between either training regime and the sedentary animals was observed for these phospholipids. Furthermore, three phospholipid molecular species, *i.e.,* PA(18:0,22:6), PA(18:1,22:5) and PG(16:0,18:1) produced the ions observed at this *m/z* complicating the interpretation of this finding. The overall lack of difference between exercise intensities is similar to the findings of Chapter 4 and would be expected as the training protocols in the two studies were identical. Consequently, it must again be acknowledged that the extra stimulus exerted by the high-intensity regime over the low-intensity regime in this study may have been too little to result in any additional effects on phospholipid profile, even when the availability of essential n-3 FAs was limited.

The FA composition of skeletal muscle membranes is strongly influenced by diet (Pan & Storlien, 1993; Ayre & Hulbert, 1996; Andersson *et al.*, 2002; Turner *et al.*, 2004) and is associated with insulin resistance (Storlien *et al.*, 1991; Borkman *et al.*, 1993; Vessby *et al.*, 1994; Helge *et al.*, 1998b) (see sections 1.3.2 and 1.2.1 respectively). High-fat diets are also associated with insulin resistance (Kraegen *et al.*, 1986; Hansen *et al.*, 1998;

Wilkes *et al.*, 1998; Kim *et al.*, 2000b; Axen *et al.*, 2003), probably due in part to their influence on skeletal muscle phospholipids, although increased intramuscular triglyceride concentration is also a factor (Pan *et al.*, 1997; Zderic *et al.*, 2004). It appears that the type of fat present in the diet rather than total fat content may be a more important determinant of insulin action with saturated fats being inhibitory and long-chain PUFAs, particularly n-3s being beneficial (Storlien *et al.*, 1991, 2000; Sohal *et al.*, 1992; Hulbert *et al.*, 2004). Furthermore, a recent study in humans found a reduction in the percentage of saturated fats in the diet is associated with improved insulin action in humans (Vessby *et al.*, 2001). A large reduction in the abundance of phospholipid molecular species containing n-3 PUFAs was apparent in the rats fed the high-fat diet as outlined in this chapter compared to the high-carbohydrate fed rats described in Chapter 4. This is more a reflection of the FA composition than the macronutrient content of the diet. There is also evidence that exercise has an ameliorating effect on diet-induced resistance to insulin (Kraegen *et al.*, 1989; Kim *et al.*, 2000a). The finding in this study that there was an exercise-induced reduction in phospholipid molecular species containing long chain PUFAs, particularly 22:6 n-3, in insulin sensitive type I and IIa fibres would tend to suggest a reduction in insulin sensitivity. A number of phospholipid molecular species containing long-chain PUFAs, however, did not demonstrate a reduction in association with exercise training and as discussed in Chapter 4, specific phospholipid molecular species may have a greater influence on insulin sensitivity than the overall phospholipid FA composition. Nonetheless, no measures of insulin sensitivity were undertaken in these rats and it is therefore pre-emptive to draw conclusions from these findings in relation to their effect on insulin action. It tends to suggest, however, that different mechanisms may be involved in both the diet-induced impairment and the exercise-induced improvement in insulin action.

In conclusion, this study has demonstrated that the exercise-induced remodelling of skeletal muscle membranes is more extensive than is indicated by the analysis of the FA content of total phospholipids and is in agreement with the results of the previous chapter. In contrast, however, under the influence of a diet high in saturated fat and deficient in

essential n-3 PUFAs exercise appears to have a greater effect on oxidative than glycolytic muscle. This may be a function of the oxidative muscle's preference for n-3 PUFAs, *i.e.,* the regulation of its membrane lipid composition may be impaired in response to an external stressor such as exercise when these FAs cannot be obtained from the diet. Nevertheless, the analysis of biological membranes at the level of phospholipid molecular species is an emerging field and further work is required to investigate some of the questions raised in this discussion.

Chapter 6

Conclusions

The aim of this thesis was to develop ESI-MS methods for analysing phospholipid molecular species in biological samples using a hybrid quadrupole time-of–flight mass spectrometer and to then use these methods to examine:

i) the phospholipid molecular species profile of oxidative (red vastus lateralis) and glycolytic (white vastus lateralis) skeletal muscle of the rat and subsequently,

ii) the effect of exercise training (at both low and high intensity),

iii) the effect of a high fat diet,

iv) the combined effect of diet (high carbohydrate vs high fat) and exercise (low vs high intensity) on these profiles.

This thesis has demonstrated that relative changes in phospholipid molecular species profile can be accurately assessed using ESI-MS and a hybrid quadrupole time-of flight mass spectrometer. The work described in Chapter 3 demonstrated how each phospholipid molecular species could be accurately identified by standard MS/MS experiments and that the MS spectra obtained from phospholipid mixtures are highly reproducible. Furthermore, results obtained by the MS methods thus developed were compared to those attained by established GC methods. The agreement between new and traditional methods validates the application of the MS technique to measure differences between treatment groups, *e.g.,* exercise vs sedentary (Chapters 4 and 5). The MS technique also has a number of advantages over traditional methods. Such as (i) the identification of whole phospholipid molecular species and, (ii) a large reduction in sample handling. In addition to the method development work, novel fragmentation mechanisms were proposed for the CID of both [plasmenyl PE–H]$^-$ and [cardiolipin-2H]$^{2-}$ ions.

The MS method does, however, present some limitations that require consideration. These include (i) isobaric interferences, (ii) quantitation and (iii) FA double bond positional isomers. Isobaric interference may be resolved using precursor ion and neutral loss scans, however, assessment of the Q-ToF 2 found the spectra produced by these scans to be under resolved making accurate identification of phospholipids extremely difficult. This method cannot be used to quantify phospholipid molecular species as differences in the ionisation efficiency of each head group mean that the observed ion count is not an accurate measure of concentration. A number of techniques have been used to compensate for these differences such as the addition of internal standards (Han *et al.*, 2001; Koivusalo *et al.*, 2001) and the calculation of a correction factor from a calibration curve (Zacarias *et al.*, 2002). Nevertheless, the best approach for the quantitative analysis of complex phospholipid mixtures requires further investigation. Double bond positional isomers in FAs present a continuing limitation in the analysis of phospholipids using ESI-MS. The CID of these FAs can be used to determine their double bond positions (Kerwin *et al.*, 1996), however, when phospholipid molecular species are analysed this requires an MS/MS/MS (MS^3) experiment. This is not possible on standard triple quadrupole and Q-ToF instruments but could be achievable using an ion trap or Fourier Transform mass spectrometer.

This thesis presents the first profile of phospholipid molecular species in both glycolytic and oxidative rat skeletal muscle. The most conspicuous difference between the two muscle types was the greater abundance of PE(18:0,22:6) in the oxidative red vastus lateralis when compared to the glycolytic white vastus lateralis. Moreover, this difference in abundance was not affected when rats were fed a diet completely lacking essential n-3 PUFAs. James *et al.* (1985) have shown that oxidative fibres are highly responsive to insulin stimulated glucose uptake while glycolytic fibres are relatively insulin resistant. It is also well established that an increased concentration of phospholipid n-3 PUFAs, particularly 22:6 n-3 is associated with increases in insulin sensitivity that is likely due to their influence on insulin receptors (see Chapter 1 for review). This suggests that

PE(18:0,22:6) may be an important molecule in the regulation of insulin action. This proposal opposes the conclusions of Clore *et al.* (1998) who recently reported a correlation between insulin stimulated glucose uptake and the FA content of PC but no correlation with the FA content of PE. It should be noted, however, that no measures of insulin action where undertaken in the current studies and therefore the correlation between PE(18:0,22:6) and insulin action has not been directly examined. Nevertheless, this theory could be tested by increasing the concentration of PE(18:0,22:6) in skeletal muscle membranes by reconstitution (Wu *et al.*, 2004) or fusion experiments (Schneider *et al.*, 1980; Hackenbrock & Chazotte, 1986; Stillwell *et al.*, 1997) and examining the effect on insulin stimulated glucose uptake.

The MS analyses in this thesis demonstrate a substantial and consistent level of membrane phospholipid rearrangement in skeletal muscle associated with exercise. This remodelling has not been observed in other studies that have examined total phospholipid FA composition using GC (Kriketos *et al.*, 1995; Helge *et al.*, 1999; Turner *et al.*, 2004). MS analysis shows that remodelling of skeletal muscle membranes with exercise does not appear to be facilitated through the creation of novel molecular species but rather through changes in abundance of molecular species present in the membrane. It should be taken into consideration, however, that only abundant phospholipid molecular species were analysed and some novel, yet minor molecular species may have been lost in the background noise. Precursor ion and neutral loss scans, using a different instrument configuration, may aid in the detection of such species.

When rats were fed a standard laboratory chow diet (see table 4-1 for FA composition), exercise training was found to have a greater effect upon the glycolytic muscle than the oxidative muscle. The opposite was observed when rats were fed a carbohydrate-free high-fat diet that was devoid of n-3 PUFAs. This may be interpreted as a differing lipid requirement of each muscle in response to an external stressor such as exercise. That is, oxidative fibres appear to prefer n-3 PUFAs and as these FAs are damaged by the

increased levels of reactive oxygen species produced by exercising muscle (Mataix *et al.*, 1998) they may be replaced from available lipid stores. When the availability of n-3 lipids is reduced (as was the case for the high-fat diet), this cannot occur and therefore a larger change in membrane composition is observed. The changes in abundance of individual phospholipid molecular species with exercise, however, were almost identical in both muscle types and diets (see Figures 4-2 and 5-1) with the largest difference between the muscles being the number of changes reaching statistical significance. It may be, therefore, that the perceived difference in response of each muscle to exercise between the two diets is merely a function of the low numbers of animals in each group (n=6) and that increasing the number of animals analysed would see a greater number of changes reaching significance in both muscles.

Even though the study described in this thesis provides a new level of understanding skeletal muscle membrane lipids it is just scratching the surface of what will be achieved by ESI-MS lipid studies in the future. Currently there is enormous interest in "lipidomics" (Han & Gross, 2003) whereby all the lipid species present in a cell or tissue are characterised and quantified. ESI-MS will play a large role in lipidomics with the NIH already providing $35 million in funding for the development of MS methods (NIH, 2003). The use of ESI-MS in the study of phospholipids and other lipid species has the capacity to revolutionise lipid biochemistry and provide a greater understanding of biological membranes and cell function.

In summary, the methods described in this thesis are able to provide more detailed information on skeletal muscle membrane lipids (and the effect exercise training has upon them) than has been previously attained by traditional methods such as HPLC, TLC and GC. It allows the analysis of membrane lipids to move from the analysis of phospholipid class or total phospholipid FA content to the next level, *i.e.,* phospholipid molecular species. As different phospholipid head groups display a characteristic asymmetric distribution across the two membrane leaflets, this provides information as to the possible

site of change. It is also possible that as further information about particular phospholipid molecular species emerges the mechanisms involved in membrane remodelling associated with various events such as exercise and the metabolic syndrome will be determined. Furthermore, it is likely that particular phospholipid molecular species have specific interactions with membrane proteins helping to regulate their activity. The use of ESI-MS will help to identify these interactions.

Chapter 7

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