Investigating fatty acid composition in the diet to determine biomarkers indicative of obesity-related disease.

A dissertation submitted by

Sharyn Carnahan, B.BioSc

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Abstract

This research investigated the role that dietary fatty acids have in the detection of obesity-related diseases. While studies have focused on whether fatty acid composition changes due to various obesity-related diseases, *e.g.* Cardiovascular, metabolic syndrome, very few have investigated whether fatty acids can be used as biomarkers to detect the predilection towards obesity. In response this project investigated a range of animal and plant oils, as well as liver and adipose tissue samples to determine whether the composition of fatty acids is similar between a low fat diet and a high carbohydrate high fat diet, simulating the unhealthy diet eaten by many. Through the use of fatty acid methyl esters (FAME) which were derivitized using two different methods using either H_2SO_4 or BF_3 as catalysts, samples were analysed by GC-MS. While both methods were viable, BF_3 proved to be the more reliable method. Previous research completed by this laboratory was extended using tissue samples not previously examined. Results indicated that supplementation of the diet by EPA (C20:5) possibly attenuates the impact of obesity-related inflammation ($p<0.05$). Increased levels of n-3 PUFA in tissues from a diet supplemented with EPA indicates that preferential metabolism of anti-inflammatory eicosanoids may occur. The outcome of this research indicates that the composition of fatty acids produced by varying diets does differ and therefore may have an impact on obesity-related diseases. This research has contributed to current knowledge by extending previous findings (Poudyal *et al.*, 2012b) from other tissues and provides further indication that study should continue in examining what role eicosanoids play in attenuating obesity-related diseases.

Declaration

I certify that the work reported in this thesis is entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for assessment in any other course of study at any other university.

Signature of candidate: Date:

Sharyn Maree Carnahan

Endorsement

Supervisor: Dr Mark Lynch

Signature: Date:

Dr. Mark Lynch

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I would like to dedicate this project to my father, who passed away during the year. It was your battles with health that led me to take up this field of study.

In Memory of

ROY COLIN FRANCE

(1946 – 2013)

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List of Abbreviations

AA	Arachidonic acid
amu/sec	atomic mass unit/second
ALA	α-Linolenic acid
□C	Degrees Celsius
$C_4H_{10}O$	Diethyl Ether
$CHCl_3$	Chloroform
cm	Centimetre
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic Acid
C18:2	Linoleic acid
C18:3	α-Linolenic acid
C20:3	Dihomo-γ-Linolenic Acid
C20:4	Arachidonic acid
C20:5	Eicosapentanoic acid
C22:6	Docosahexanoic acid
DGLA	Dihomo-γ-Linolenic Acid
DHA	Docosahexanoic acid
EPA	Eicosapentanoic acid
et al	*et alia,* and others
FA	Fatty acid
FAME	Fatty acid methyl esters
GC-MS	Gas Chromatography-Mass Spectrometry

g	grams
h	hours
H_2SO_4	Sulphuric acid
kg	kilograms
kPa	kilopascal
LA	Linoleic Acid
LT	leukotriene
m	metre
MeOH	methanol
mL	millilitre
mm	millimetre
NMR	Nuclear Magnetic Resonance
µL	microlitre
µm	micrometre
min	minute
MUFA	monounsaturated fatty acid
PG	prostaglandin
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
SCD-1	stearoyl-CoA-desaturase 1
TAG	triacylglycerides
TFA	*trans*- fatty acids
TX	thromboxane
UFA	unsaturated fatty acid
UPLC-MS	Ultra Performance Liquid Chromatography – Mass Spectrometry

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Obesity poses one of the greatest challenges to public health in the 21st century with over 500 million adults afflicted (World Health Organisation, 2008) and is the Western world's leading preventable cause of death. It can be defined as excess body fat that has accumulated to the extent that it may have a detrimental effect on health leading to a reduced life expectancy and/or increased health problems (World Health Organisation, 2008). Obesity is caused by a positive energy balance (energy intake exceeding energy expenditure) which results in an expansion of adipose tissue stores and metabolism of fatty acids in cells which may have maladaptive responses to lipids (Poirier, 2007). Obesity has a significant impact on both the physical and psychological health of those who are affected as it leads to an increased risk of morbidity and mortality (Poirier, 2007).

Obesity is associated with numerous metabolic disorders, such as dyslipidaemia, Type 2 diabetes and long term cardiovascular complications (Haslam and James, 2005). Dietary fat and carbohydrates have both been implicated in obesity but on quality rather than quantity *e.g*. *trans* fatty acids (TFA) are created through the transformation of unsaturated fatty acids from their natural *cis*- form to the *trans*- form, and are abundant in the 'western' diet. They take on similar properties to saturated fatty acids (SFA) and appear to be more atherogenic and promote insulin resistance (Odegaard and Pereira, 2006). Saturated fatty acids and *trans*- fatty acids are generally accepted as being detrimetnal to health as studies have shown a

correlation between levels of these lipids and disease (Muoio, 2010, Quehenberger and Dennis, 2011). Both TFA and SFA have been found to be associated with systemic inflammation (Mozaffarian *et al.*, 2004).

Research has indicated that monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids found in dietary fat could possibly attenuate symptoms and risk factors of obesity in rats fed high carbohydrate, high fat diets (Poudyal and Brown, 2013).

1.2 Fatty Acids

Fatty acids are biomolecules that are present in all organisms and play a physiologically important role as building blocks in biomembranes, as signalling molecules and as energy storage. Fatty acids are carboxylic acids with long aliphatic tails that are either saturated (no double bonds) or unsaturated (one or more double bonds). Naturally occurring fatty acids have straight chains of an even number of carbon atoms between 4 and 28 carbons in length.

A tissue's fatty acid content is dependent on two major contributors. Firstly, fatty acids can be synthesised *de novo* through lipogenesis to produce short chain fatty acids (SCFA) (C2 to C6). These SCFA are elongated through medium chain fatty acids (C8 to C12) to form palmitic acid (C16:0) and with further elongation to stearic acid (C18:0). Enzymatic desaturation can add a double bond to produce palmitoleic (C16:1) and oleic (C18:1) acids respectively. Palmitic, stearic, palmitoleic and oleic acids can be derived from the diet as well. In mammals, essential fatty acids are not made in the body and must be derived from the diet. Linoleic acid (LA; C18:2) and α-Linolenic acid (ALA) (C18:3) are the only two essential fatty acids. LA is elongated and desaturated to form dihomo-γ-linolenic acid (DGLA; C20:3), arachidonic acid (AA; C20:4) and docosapentaenoic acid (DPA; C24:5). ALA is elongated and

desaturated to form eicosapentaenoic acid (EPA; C20:5) through to docosahexaenoic acid (DHA; C20:5) (Poudyal and Brown, 2013). Arachidonic acid (C20:4) and EPA (C20:5) are the precursors of the eicosanoids, such as prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) and as such are physiologically and metabolically essential.

Dietary fat sources are generally found to be the 18-carbon fatty acid series: linoleic (LA)(C18:2), α-linolenic (ALA) (C18:3), oleic (C18:1) and stearic (C18:0) (Poudyal *et al.*, 2013) with the modern Western diet generally containing more linoleic rather than α-linolenic acid (Rett and Whelan, 2011). Excess fatty acids are stored as TAG's in adipose tissue. Triacylglyerides have twice the energy per gram of carbohydrates and proteins.

Fatty acids can either be beneficial or detrimental to the human body. Studies have found that n-3 (ALA, EPA, DHA) PUFA are beneficial, with anti-inflammatory properties through their conversion into eicosanoids (PG, TX, LT) while n-6 (LA, DGLA, AA, DPA) PUFA tend to elicit a pro-inflammatory response from their eicosanoids (Poudyal *et al.*, 2013). The predominant n-6 PUFA is arachidonic acid (C20:4) which converts to series-2 PG, series-2 TX, series-4 LT and other lipooxygenase and cyclooxygenase products (Arita *et al.*, 2005, Serhan *et al.*, 2007) which are regulators of cell function with inflammatory effects. EPA, the key intermediary n-3 PUFA, and DHA derived eicosanoids (series-3 PG, series-3 TX, series-5 LT, resolvins, docosatrienes and neuroprotectins) (Arita *et al.*, 2005, Serhan *et al.*, 2007) appear to antagonize the pro-inflammatory effects of n-6.

Prostaglandins, Thromboxanes and Leukotrienes are assigned to either the 1,2,3,4, or 5 series based on the number of double bonds contained in the fatty acid.The n-3

eicosanoids down regulate inflammatory genes and lipid synthesis and stimulate fatty acid degradation (Schmitz and Ecker, 2008).

The same series of desaturase and elongase enzymes are utilised by both n-3 and n-6 fatty acids in conversion to eicosanoids which leads to preferential metabolism towards the excess PUFA causing significant decreases in conversion of the other (Figure 1). Therefore, increase consumption of n-3 PUFA leads to reduced synthesis of inflammatory eicosanoids from n-6 arachidonic acid and elevated production of the anti-inflammatory n-3 converted eicosanoids.

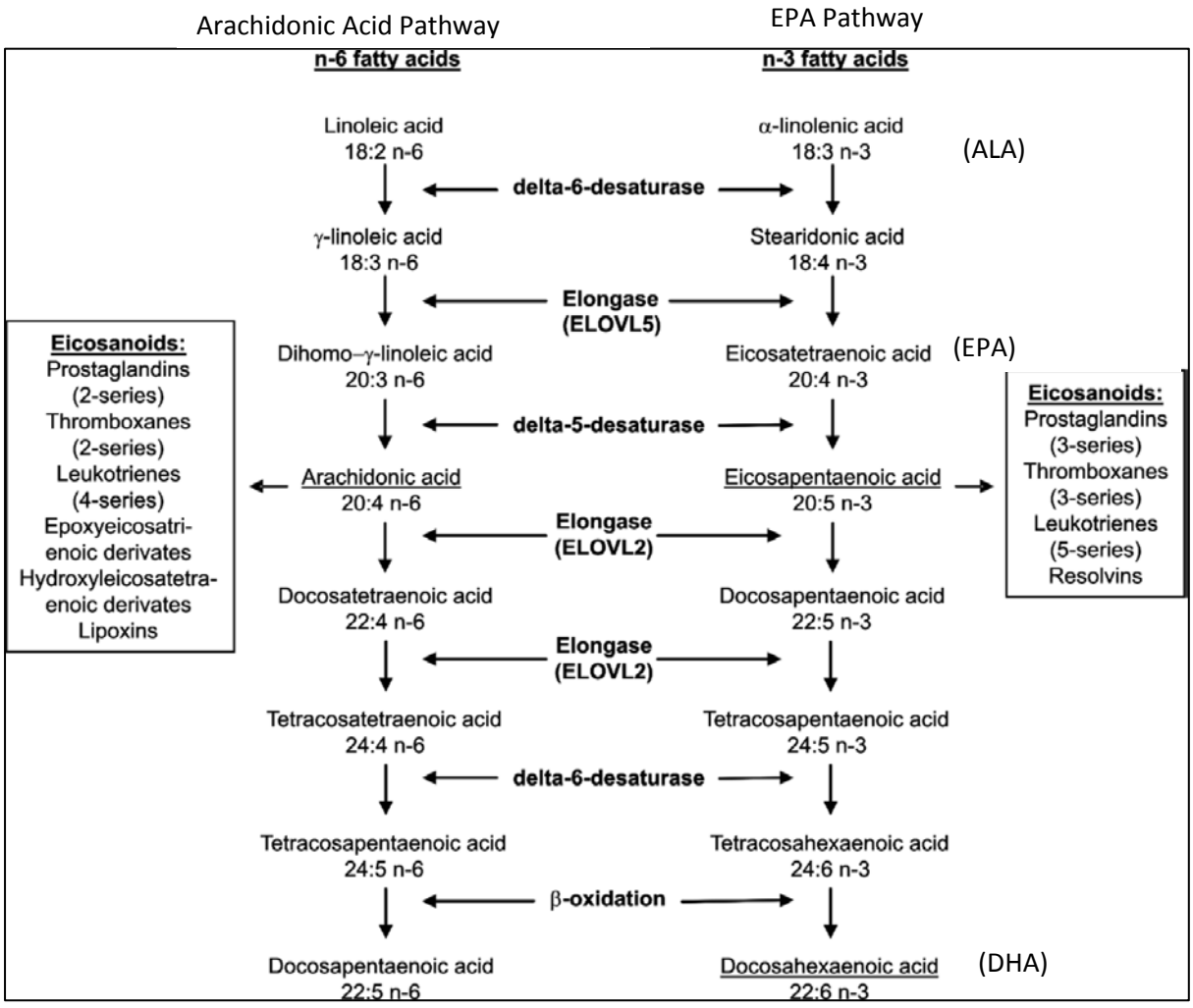


Figure 1. n-3 and n-6 fatty acid metabolism indicating the same series of desaturase and elongase enzymes needed for metabolism. Eicosanoids metabolised by arachidonic acid (n-6) or eicosapentaenoic acid (EPA) (n-3). (Schmitz and Ecker, 2008)

Until recently, it had been thought that due to the similar chemical structures of fatty acids they would produce similar biological actions. Earlier studies from our laboratory investigating ALA, EPA and DHA and effects on rats fed a high-carbohydrate, high-fat diet, found that the biological response to EPA and DHA were similar. ALA-rich supplementation increased the concentration of DHA in various tissues, indicating that ALA was also involved in the production of DHA (Poudyal *et al.*, 2012b). The C18 unsaturated fatty acids have shown a tendency for partitioning lipids away from the abdomen (Poudyal *et al.*, 2012b) suggesting that it is the proportion of these fatty acids in the dietary lipid pool and not just the diet that plays a significant role in response to long chain fatty acid synthesis (Poudyal *et al.*, 2012b).

Abdominal and visceral fat have been linked to metabolic disturbances, and, an increased risk in cardiovascular disease and Type 2 diabetes. Therefore these fat deposits are more detrimental than peripheral fat storage (Rosito *et al.*, 2008). Hence, PUFA and MUFA are beneficial as the decrease in visceral fat ameliorates the risk factors associated with obesity. EPA and DHA have been shown to cause a decrease in total body fat which will also attenuate the risk factors of insulin resistance, dyslipidaemia and hypertension (Poudyal *et al.*, 2012a).

Arachidonic acid (C20:4) is a product of linoleic acid metabolism, and, as the key intermediate to pro-inflammatory eicosanoids (TX, PG, LT), is a key regulator in inflammation (Poudyal *et al.*, 2013). While too much arachidonic acid appears detrimental to health, it is proposed that the control mechanisms for fatty acid homeostasis shunt dietary arachidonic acid into specific metabolic pathways, such as the β-oxidation and the citric acid cycle ensuring utilization rather than accumulation in adipose tissue (Nelson *et al.*, 1997). Recent research has found that

unchanged proportions of arachidonic acid in liver and skeletal muscle suggest that eicosapentanoic acid (EPA) is preferentially metabolised over arachidonic acid to form anti-inflammatory and anti-hypertensive eicosanoids in these highly metabolically active adipose tissues (Poudyal *et al.*, 2012b).

Studies using genetically obese Zucker rats, which share several traits with obese humans, such as obesity and hyperinsulinaemia (Escoubet *et al.*, 1987), and Sprague-Dawley rats (Zhang *et al.*, 2009) have shown abnormal fatty acid composition in plasma tissues. Due to these similar traits rats are a useful alternative in studies on obesity.

These rat models have shown higher linoleate intake increases arachidonic acid which in turn increases prostacyclin production, stimulating signalling pathways leading to adipogenesis (Ailhaud *et al.*, 2008) .

These pathways possibly include phospholipase and/or cyclo-oxygenase activation, and, may be linked to low n-3 PUFA intake as well as excessive dietary linoleate. (Ailhaud *et al.*, 2008) also discussed the likelihood that excess dietary linoleate may play a role in adipose tissue development due to a demand for a higher intake of 'essential fatty acids'. This high n-6 PUFA intake and a very high ratio of n-6:n-3 PUFA in the diet have also been implicated in the advancement of many obesity related diseases, including CVD, cancer, inflammatory and autoimmune diseases (Robinson *et al.*, 2007).

Human studies (Novgorodtseva *et al.*, 2011) have shown that disturbances in the composition of fatty acids within plasma, red blood cells and eicosanoid synthesis, play important roles in Metabolic Syndrome with decreased amounts of stearic acid (C18:0), arachidonic acid (C20:4), and docosapentaenoic acid (C22:5) seen, while the amount of linoleic acid (C18:2), the precursor of arachidonic acid (C20:4) was

doubled (Ailhaud *et al.*, 2008, Escoubet *et al.*, 1987, Novgorodtseva *et al.*, 2011). There is also increasing evidence that obesity and Type 2 diabetes may be associated with a morphological defect in the mitochondria that leads to the inability of skeletal muscle to oxidise lipids, with the intracellular environment partitioning lipids towards storage instead (Consitt *et al.*, 2009). Additional research is needed into the relationship between obesity and the accumulation of lipid intermediates and the impact that lipids have on insulin action (Consitt *et al.*, 2009).

1.3 Markers indicative of disease state

1.3.1 Biomarkers

Zhang *et al.* (2009) discussed the necessity of assessing the essential nutrient status of individuals to provide a more comprehensive systemic metabolic response to dietary, lifestyle and environmental influences. By increasing our understanding of the metabolic processes involved in the pathogenesis of obesity, a comprehensive understanding of the disease can be developed.

Biomarkers are indicators of normal biological and pathogenic processes or responses to therapeutic interventions that can be objectively measured. They are molecules found in blood or other body fluids or tissues that could be used as an indication of disease and could be used for routine screening tests, monitoring therapy or, prediction of therapeutic responses (Zhang *et al.*, 2009). Previous studies have used amino acids such as tyrosine and glutamine, proteins and cytokines (Cao *et al.*, 2008, He *et al.*, 2012, Kim *et al.*, 2010), as biomarkers to identify molecules that discriminate healthy lean from healthy obese individuals. The analysis of fatty acid profiles in blood, plasma and tissue is becoming an important tool in clinical studies as an endpoint for detecting biomarkers of disease *e.g.* cardiovascular and other chronic diseases, and drug compliance (Masood and

Salem, 2008). Previous research into obesity generally relied on subjective data, such as questionnaires. Difficulties in this form of research arise as fat intake is rarely observed directly and there can be a recall bias and measurement errors as well as changes in diet while filling out questionnaires (King *et al.*, 2006). From this point of view a biological marker would be useful especially if it could be obtained using a blood sample which would provide a cheap, simple and a less time-consuming process to determine fat intake. With advancements in technology in the last three decades, detection of biomarkers with GC-MS has become a more time-effective, less costly and less invasive way of measurement. A number of physio-chemical properties of fatty acids, such as chain length, degrees of saturation and various other structural features could be used in the detection of biomarkers. For example, total fat, fatty acids, essential fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and eicosanoids could all potentially be utilised as biomarkers, and have already been identified as useful markers of cardiovascular disease (Masood and Salem, 2008).

Studies have shown conflicting results when it comes to biomarkers representing total fat while red blood cell fatty acid concentrations of MUFA, PUFA and SFA do not appear to be adequate. EPA, DHA and oleic acid may provide short-term indicators of relative intake rather than total fat intake (Poppitt *et al.*, 2005). Previously, *trans*-fatty acids were a good biomarker of dietary fatty acids, as they are generally not naturally found but have been manufactured during the processing of food. However, with the decreased prevalence in the diet they are becoming less helpful (Baylin *et al.*, 2002). By comparing fatty acids, a biomarker profile may be created to indicate total fat intake. By using samples of red blood cells, plasma phospholipids and cholesterol esters, King *et al.* (2006) measured fatty acid status

and produced predictive models that were specific and sensitive in discriminating between low fat and high fat intakes.

Studies by Baylin *et al.* (2002) Fuhrman *et al.* (2006), Thiébaut *et al.* (2009) examined biomarkers for relative fatty acid intakes and have produced outcomes where C18:2 and C18:3 showed strong correlations with dietary intake when measured in adipose tissue (Baylin *et al.*, 2002). n-6 and n-3 PUFA in phospholipids have been noted to be long-term biomarkers of relative intake by Fuhrman *et al.* (2006). The study also reported that oleic acid (C18:1) in plasma and red blood cells is a valid long-term biomarker of total MUFA intake (Fuhrman *et al.*, 2006). The red blood cell MUFA findings were corroborated by Thiébaut *et al.* (2009) while finding that red blood cell saturated fatty acids do not appear to be a suitable biomarker.

EPA and DHA have been found to attenuate disease states in a number of studies (Masood and Salem, 2008, Poudyal *et al.*, 2012b). They have been found to be antagonistic to C20:4, and, the eicosanoids that are subsequently produced by it. In the past, EPA and DHA were obtained mainly through the consumption of fish. However, with the availability of EPA/DHA fortified foods and supplements due to the benefits of these fatty acids, the biomarker can indicate not only the dietary intake of fish but also EPA/DHA intake (Harris and von Schacky, 2004). Further research to determine the time-period of intake of plasma EPA and DHA may show that they are useful dietary biomarkers. Studies indicate that the Omega-3 Index, which is the sum of EPA and DHA in red blood cell membranes as a percentage of the total fatty acids, may also be a good biomarker of n-3 intake (Harris and von Schacky, 2004). Significant correlations between the Omega-3 Index, plasma phospholipid and whole blood EPA and DHA have been found (Harris and von Schacky, 2004).

The presence of these fatty acids in plasma can act as important biomarkers in cardiovascular disease risk (Masood and Salem, 2008) and other related diseases. However, it must be noted that some fatty acids are also endogenous *e.g.* palmitic acid (C16:0) and oleic acid (C18:1), and therefore show up in testing regardless of diet. It is likely that successful biomarkers of disease need to come from exogenous sources, such as LA (C18:2) and ALA (C18:3). Baylin *et al.* (2002) found high diet - adipose tissue correlations for both of these acids. However, there was no correlation between dietary PUFA and adipose tissue C20:4. These findings suggest that there is little endogenous conversion of C18:2 to C20:4. Further research is needed to ascertain the usefulness of fatty acids as biomarkers.

1.4 Methods of Analysis

1.4.1 Transesterification methods

With the nutritional and health benefits of fatty acids becoming evident, their analysis is of growing importance. Most fatty acids in biological samples are either triacylglycerols or phospholipids. The derivatization of fatty acids from biological samples are generally transesterifications (Carrapiso and Garcia, 2000). In order to analyse lipid samples, extraction is commonly done prior to esterification. Gas chromatographic analysis of fatty acids esters, such as methyl esters (FAME), rather than of the free fatty acids, is the preferred method as their analysis is more accurate and selective. FAME are prepared more often than other esters due to their greater volatility, therefore improving peak shape and resolution of the more common fatty acids (C14:0 – C22:0) (Carrapiso and Garcia, 2000).

The most commonly used lipid extraction method is the Folch method (Folch *et al.*, 1957). This rapid and simple method of extraction is similar to the Bligh and Dyer (1959) method (Bligh and Dyer, 1959). Many current methods are based on these

two methods, as they both require the same solvents (chloroform and methanol) and, similar procedures. Both methods are efficient, easily reproduced and safe to perform.

Transesterification is a catalysed substitution reaction where TAGs are reacted with an alcohol (*e.g.* methanol, ethanol) to form glycerol and three equivalents of FAME. In the acid catalysed reaction, the carbonyl carbon and oxygen from the methanol hydroxyl group interact. As this bond is formed, electroneutrality is restored by deprotonation. Cleavage of the glycerol group and subsequent protonation gives the three FAME's and glycerol products. The same products are generated when BF_3 is used as the catalyst.

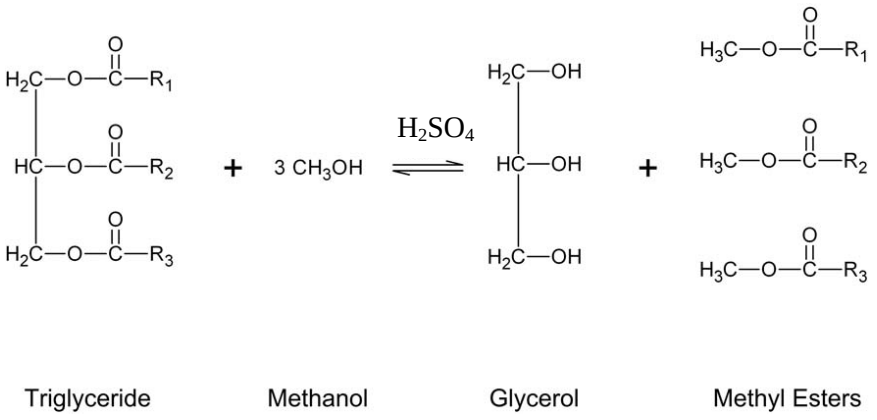


Figure 2. Transesterification reaction with methanol and a sulphuric acid (H_2SO_4) catalyst.

(Diagram: ww2.mackblackwell.org)

A number of studies have utilised a similar approach to the current study being undertaken using a combination of sulphuric acid (H_2SO_4) and methanol to transesterify lipids (Antolin *et al.*, 2008, Wei and Zeng, 2011). Methods utilising boron trifluoride (BF_3) are also used in lipid derivatization (Araujo *et al.*, 2008, Park *et al.*, 2010). The current study will attempt to validate the results of adipose

tissue/liver samples previously transesterified under a BF_3 method (Poudyal *et al.*, 2012b) by comparison with a H_2SO_4/methanol procedure.

1.4.2 Analysis methods

There are two major high throughput tools that are used in metabolomics to identify metabolites – Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS). MS is the most frequently used method as it has a high level of sensitivity and is a powerful tool not only to detect and quantify metabolites but also to investigate the molecular structure (Zhang *et al.*, 2012). When combined with gas chromatography (GC) distinct metabolite classes can be detected reducing the complexity of the mass spectra.

Due to the complexity of the human metabolome, a multi-platform approach to analysis provides a more comprehensive understanding of alterations to metabolism. Combining approaches allows for complementary analytical outcomes, which can broaden the metabolic variations identified (Zhang *et al.*, 2012). A study undertaken by Williams *et al.* (2006) investigated the metabolic regulatory mechanisms present in diabetes. The metabolic profile was obtained from normal Wistar-derived (n=5) and Zucker (*fa/fa*) (n=6) obese rats using multiple analytical platforms, including NMR, UPLC-MS and GC-MS. The study was able to detect differences in the profiles of the two strains of rats. For example, a number of biomarkers including cholesterol, arachidonic acid (C20:4), oleic acid (C18:1), palmitic acid (C16:0), monooleoylglycerol and low density and very low density lipoproteins were found to be higher in the Zucker variety. A follow up study using only Zucker rats (n=18) detected over 200 compounds in each plasma sample. It found that lean (n=6) and lean/*fa* (n=6) genotypes had similar compounds while the *fa/fa* (n=6) genotype was

distinct from the others. Evidence obtained from GC-MS analysis, indicated that differences in plasma were due to disease states rather than the strain of rat, when related to the previous Wistar –Zucker rat study (Major *et al.*, 2006).

Both of the studies' samples were analysed using the same conditions. The samples were analysed using NMR, GC-MS and UPLC techniques (Major *et al.*, 2006, Williams *et al.*, 2006). By performing two complementary studies using the same conditions and evidence correlated between both studies it showed that the GC-MS is a valuable tool that is reliable and robust in performing research. Also, by using similar samples with different techniques they were able to distinguish overlaps in metabolites between the GC-MS and NMR but not with the UPLC (Major *et al.*, 2006, Williams *et al.*, 2006). This indicates that by using a multiplatform analytical study a wider range of unique markers can be identified.

While most methods using GC-MS have analysis times of over 30 minutes (Araujo *et al.*, 2008, Baylin *et al.*, 2002), Ecker *et al.* (2012) developed a rapid method for quantification of positional and geometric isomers of FAMEs using a highly polar column (10m x 0.10mm, 0.20µm) which runs for 17.2 minutes. This method has been shown to be robust and reliable for routine analysis of plasma and tissue extractions and has been used in various studies (Ecker *et al.*, 2010a, Ecker *et al.*, 2010b). Fast, reliable methods are useful in processing large numbers of samples with a relatively quick turnaround.

1.5 Conclusion

Obesity is a worldwide public health issue with numerous risk factors and co-morbidities. It appears that fatty acids play a role in the development of many of these disease states, but the how and why are still to be elucidated. Research has

shown that dietary supplementation with fatty acids, particularly n-3 PUFA can attenuate some of the negative impacts of obesity but how they work is still not well understood. Currently there are few studies addressing the differences in metabolism between healthy and obese individuals. This study will describe how fatty acids could be suitable biomarkers of the pathophysiology of obesity. The study will utilise liver, and adipose tissue samples from rats fed a high-carbohydrate, high-fat diet by the Brown Research Group at USQ.

1.6 Research Project

1.6.1 Research Hypothesis

That fatty acid composition of the liver and adipose (retroperitoneal fat) tissue is altered by a diet that is high in carbohydrates and fats, and, these differences can be quantified.

1.6.2 Research Objectives

The object of this project is to investigate whether the fatty acid composition of liver and adipose (retroperitoneal fat) tissue in rats is altered dependant on the diet that they are fed. Results from previous research (Poudyal *et al.*, 2012b) will be validated by using an alternative method of derivitization.

The objectives of this project are:

- Establish a methodology for the analysis of fatty acids by comparing results from BF_3 method to H_2SO_4 method.
- Extend previous research by analysing liver and adipose tissue samples

- Evaluate if fatty acid composition is altered based on the diet fed to rats on the HCHF rat model of obesity
- Determine if dietary supplementation with EPA alters fatty acid composition.

CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1 Chemical Preparations

Chloroform, methanol, heptadecanoic acid (C17), sodium hydroxide, 2-propanol, sodium chloride and 14% boron trifluoride in methanol were obtained from Sigma-Aldrich. HPLC grade methanol was obtained from Lab Scan Analytical Sciences. Concentrated sulphuric acid (H_2SO_4) was obtained from Proanalys. Milli-Q high purity water was used. All other reagents and solvents were of analytical grade or better and were used with no further purification, unless otherwise noted.

Two multi-acid standard mixtures were used to check performance of the gas chromatograph/mass spectrometer :Fatty Acid Methyl Ester Mix (C14:0 to C22:0) was obtained from Sigma Aldrich and for identification purposes: GLC-462 Reference Standard, containing a mixture of 28 fatty acid methyl esters obtained from Nu-check Prep Inc.

Oils that were used in the large scale H_2SO_4 method development included plant oils (chia, flax, olive, avocado, macadamia, canola and safflower) and animal oils (beef tallow, EPA and DHA). These were kindly supplied by Dr. Hemant Poudyal.

2.2 Chromatographic conditions

Fatty acid methyl esters were identified using gas-chromatography-mass spectrometry. A Shimadzu GC-2010 gas chromatograph with an AOC-20i autoinjector sampled from an AOC-20s autosampler using an RTX-5MS capillary column (30m x 0.25mm x0.25µm) (serial number 801339). A Shimadzu GCMS-

QP2010 Plus mass spectrometer detector using an electron ionisation ion source was used to produce the mass spectra.

Oven conditions for this method were developed using a standard of FAMEs (C14:0 to C22:0) (Sigma Aldrich) and esterified C17. An initial oven temperature of 150°C was held for 5 minutes, the temperature was increased to 180°C at a rate of 19°C.min^{-1}. Upon reaching 180°C the oven was held at this temperature for 16 minutes. The temperature was increased to 200°C at a rate of 8°C.min^{-1} and held for a further 5 minutes, then increased again to 240°C at 8°C.min^{-1} and held for a further 20 minutes. The method required a total run time of 55.08 minutes.

A volume of 1.0 µL was injected for each sample with an injection temperature of 250°C utilising a 10:1 split ratio using ultra-pure helium carrier gas at a linear velocity of 25.0 cm.s^{-1} at a pressure of 26.1 kPa. The column flow rate was 0.43 mL.min^{-1}. A purge flow of 3.0 mL.min^{-1} was used, with a total flow rate of 7.8 mL.min^{-1}.

The mass spectrometer conditions used an ion source temperature of 285°C and interface temperature of 250°C with a MS scan mode of 40 - 400 m/z with an initial solvent cut time of 4 minutes. Events were measured every 0.5 seconds at a scan speed of 759 amu/sec.

2.3 Preparation of Tissue samples

Liver and adipose (retroperitoneal fat) tissue samples were kindly supplied by Dr Hemant Poudyal and were collected as part of studies described previously (Poudyal *et al.*, 2012b) .

Experiments for this project were conducted on liver and adipose (retroperitoneal fat) tissue samples. For each method (H_2SO_4 and BF_3) samples were prepared with corn starch diet (CS) (n=3) as the healthy control, high carbohydrate high fat (HCHF)

(n=3) as the unhealthy control, CS with EPA supplementation (CEPA) (n=3) and HCHF with EPA supplementation (HEPA) (n=3) as the treatment groups.
Using forceps and scissors approximately 1 – 5 g of tissue was cut from the sample and added to a pre-weighed 15 mL polypropylene centrifuge tube. Tubes were then re-weighed and the mass of the sample accurately determined.

2.4 Lipid Extraction

The extraction of lipids from liver and adipose tissue samples was undertaken using a manual solvent extraction method using a 2:1 chloroform/methanol mixture.
To a tissue sample prepared as above, 10 mL of chloroform/methanol solvent was added. With the cap firmly on this was mixed on a mechanical shaker for 30 minutes. The sample was then centrifuged at 4000 rpm for 5 minutes. The solvent (lower layer) was aspirated from the tube and placed into a 50 mL polypropylene centrifuge tube. This extraction process was repeated a further two times and all of the extracted solvent was combined.
5 mL of 5% NaCl solution was added to the chloroform extract and the mixture was centrifuged at 4000 rpm for 5 minutes to separate the different phases. The aqueous phase was removed and discarded. The sample was mechanically shaken for a further 30 minutes and centrifuged for 5 minutes at 4000 rpm. Any remaining water was removed.
Samples were transferred to pre-weighed glass vials and the solvent was evaporated under a gentle stream of compressed air on a hot plate set at 50°C.
Dried samples were stored under nitrogen for future fatty acid analysis.

2.5 Transesterfication using H_2SO_4 Method

2.5.1 Bulk Oils

This method was developed using a variety of plant oils (chia, safflower, canola, macadamia, olive, avocado and flax) and animal oils (beef tallow, EPA and DHA). The procedure for transesterfication was a modified version of that detailed in Folch *et al.* (1957). A sample of 1.0 g of oil was combined with 25 mL methanol and 1 mL of H_2SO_4 in a round bottomed flask with a magnetic stirrer. This was placed in a glycerol bath, ensuring the liquid was 2 - 3 mm below the level of the glycerol. The mixture was heated at reflux for two hours. After two hours, heating was discontinued, the flask was removed from the glycerol and allowed to cool.

To the mixture 25 mL water was added and this was transferred to a 250 mL separatory funnel. The mixture was extracted with 20 – 30 mL of diethyl ether. The ether layer was collected and the aqueous phase was further extracted with 20 – 30 mL of diethyl ether. This process was repeated. The combined ether extracts were washed three times with portions of 20 – 30 mL of water.

The diethyl ether extract was transferred to a clean beaker. Excess ether was evaporated off under a stream of compressed air. Prior to total evaporation samples were transferred to glass storage vials where evaporation was completed. Samples were re-suspended with HPLC grade methanol for analysis by GC-MS.

2.5.2 Lipids Derived from Tissue Samples

Samples collected from lipid extraction were transesterfied to produce fatty acid methyl esters (FAME). The procedure for transesterfication was a modified version of that conducted at large scale (see section 2.5.1).

Sulphuric Acid Method

To 0.02 g of the lipid extracts 5.5 mL methanol, 0.5 mL of H_2SO_4 and a few anti-bumping granules were added. The test tubes were placed in a bath of glycerol and heated at 65°C for two hours. After two hours, the heating was discontinued, the tubes were lifted out of the glycerol and allowed to cool.

A volume of 6 mL water was added to the sample and the mixture was transferred to a 50 mL separatory funnel. Samples were extracted with 10 mL of ether for 30 seconds with the pressure released periodically. The aqueous phase was removed and the ether layer collected. The aqueous phase was extracted with further 10 mL portions of ether twice more. The combined ether extracts were washed three times with 10 mL portions of water. The ether phase was transferred to a glass vial and the ether was evaporated under a stream of compressed air. Samples were re-suspended with HPLC grade methanol for GC-MS analysis.

2.6 Transesterfication using BF_3 Method

2.6.1 Internal standard solution

A mass of 100 mg of heptadecanoic acid (C17) was dissolved in isopropanol and was made up to 10.0 mL in a volumetric flask.

2.6.2 Reagent preparation

Reagents prepared for use were 0.5 M methanolic NaOH. This was prepared fresh prior to each set of experiments, by dissolving 1 g of NaOH in 50 mL CH_3OH. Boron Trifluoride-Methanol (BF_3-CH_3OH) 14% was used as supplied by Sigma Aldrich. NaCl was saturated by dissolving 500 g in 500 mL of water.

2.6.3 Transesterification of extracted fatty materials

All 12 HEPA and CEPA liver and retroperitoneal fat samples were prepared at once and transesterified together.

All 12 control HCHF and CS liver and retroperitoneal fat tissue samples were prepared and transesterified at the same time.

About 15 - 30 mg of prepared sample was placed in a test tube with 100 µL of the internal standard solution and 0.5 mL of 0.5 M methanolic NaOH. The tube was then flushed with N_2 and a vented stopper was placed loosely in the top.

Test tubes were heated in a 95°C water bath for 3 - 5 minutes to saponify the fats. Tubes were cooled and then 2.5 mL BF_3-methanol was added using a syringe through the vented stopper. Tubes were then placed in the water bath for 5 minutes to esterify the fatty acids before being cooled to room temperature. Heptane (2.0 mL) was added with a syringe. The tubes were mixed using a vortex mixer. A volume of 5 mL of saturated NaCl was added and the tubes were again mixed.

Once the phases had separated, the heptane phase was collected and placed in a 2 mL autosampler vial with a clean Pasteur pipette. The tubes were capped and were subsequently analysed by GC-MS.

2.6.4 Calibration Standard

For calibration with the C17 internal standard, three working standards were prepared with 50, 100 and 150 µL of the C17 internal standard present in each test tube (Figure 3).

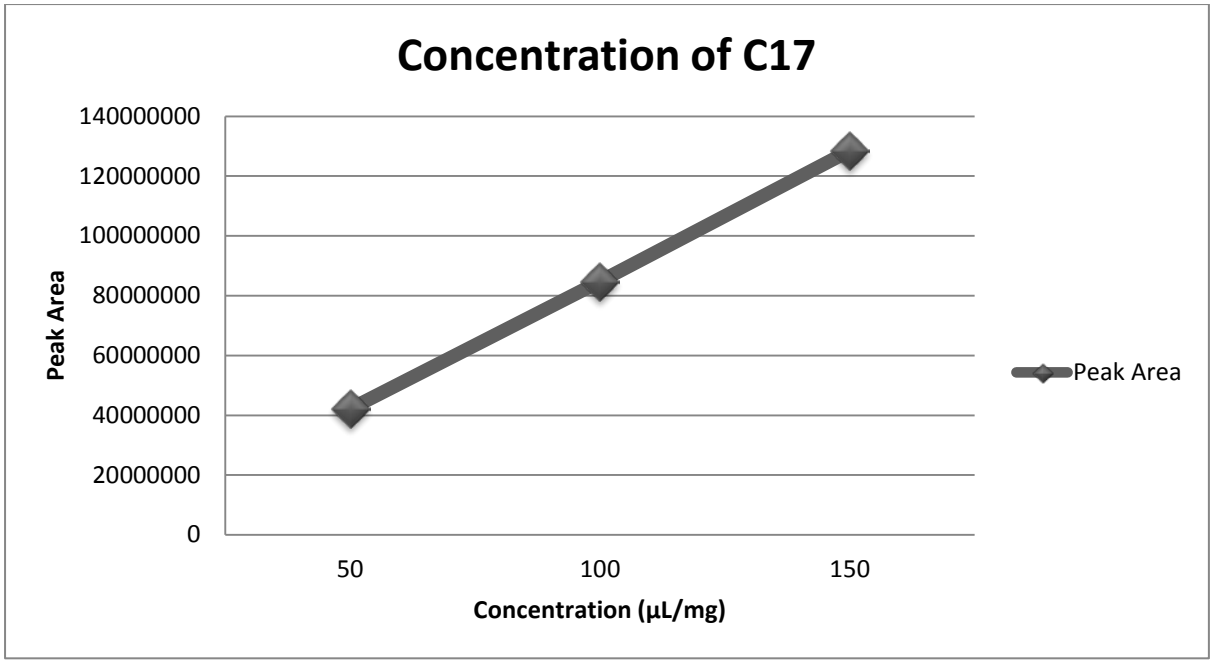


Figure 3. Concentration vs. Peak Area of C17 Standard.

2.6.5 Method validation

For method validation purposes four blinded standards of saturated fatty acids (Lauric, Myristic, Palmitic and Stearic) were prepared by Dr Hemant Poudyal as per the BF_3 derivitization method previously described, these were analysed using the same GC-MS program for quality control purposes.

2.7 Statistical Analysis

All data are presented as mean±SEM. Data from animal oil, plant oil, liver and adipose tissue samples were tested by one way analysis of variance (ANOVA). When interaction and/or main effects were significant, means were compared using Newman-Keuls multiple comparison *post hoc* test. A *P* value of <0.05 was considered as statistically significant. All statistical analyses were performed using GraphPad Prism version 6.0 for Windows (San Diego, CA, USA).

CHAPTER 3

RESULTS

3.1 Method Validation

Table 1 and Figures 4-7 present the validation results of the four blinded standards. Each of the samples are indicated in Table 2 with above 80% purity, except sample 2 which is at 69.56%.The retention times of the samples are indicated in Figures 3-7. Sample 1 has a retention time of 7.368 which correlates to lauric acid (C12:0), sample 2 at 11.348 is myristic acid (C14:0), sample 3 or palmitic acid (C16:0) at 18.479 and sample 4 at 28.974 correlates with stearic acid (C18:0). The internal standard of C17 has a retention time of 24.066 (Table 2).

Table 1. Fatty acid profile of blinded standards used as method validation.

Fatty Acid (g/100 of total)	**Sample 1 (n=3)**	**Sample 2 (n=3)**	**Sample 3 (n=3)**	**Sample 4 (n=3)**
C10:0	2.25±1.12	0.00±0.00	0.00±0.00	0.00±0.00
C12:0	86.00±10.63[b]	0.00±0.00[a]	4.38±4.38[a]	0.00±0.00[a]
C12:1	11.75±11.75	0.00±0.00	0.45±0.45	0.00±0.00
C14:0	0.00±0.00[b]	69.56±1.51[a]	6.98±3.49[b]	0.00±0.00[b]
C14:1n-5	0.00±0.00[b]	30.44±1.51[a]	0.00±0.00[b]	0.00±0.00[b]
C16:0	0.00±0.00[b]	0.00±0.00[b]	88.20±1.34[a]	1.08±1.08[b]
C18:0	0.00±0.00[b]	0.00±0.00[b]	0.00±0.00[b]	80.95±7.90[a]
C18:2n-6	0.00±0.00	0.00±0.00	0.00±0.00	15.14±7.57
C18:3n-3	0.00±0.00	0.00±0.00	0.00±0.00	2.83±1.41
Total Saturated	88.25±11.75	69.56±1.51	99.55±0.45	82.03±8.98
Total Unsaturated	11.75±11.75	30.44±1.51	0.45±0.45	17.97±8.98
Total Monounsaturated	11.75±11.75	30.44±1.51	0.45±0.45	0.00±0.00
Total Polyunsaturated	0.00±0.00	0.00±0.00	0.00±0.00	17.97±8.98

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. $P < 0.05$. No superscript within a row indicates no significant difference between groups.

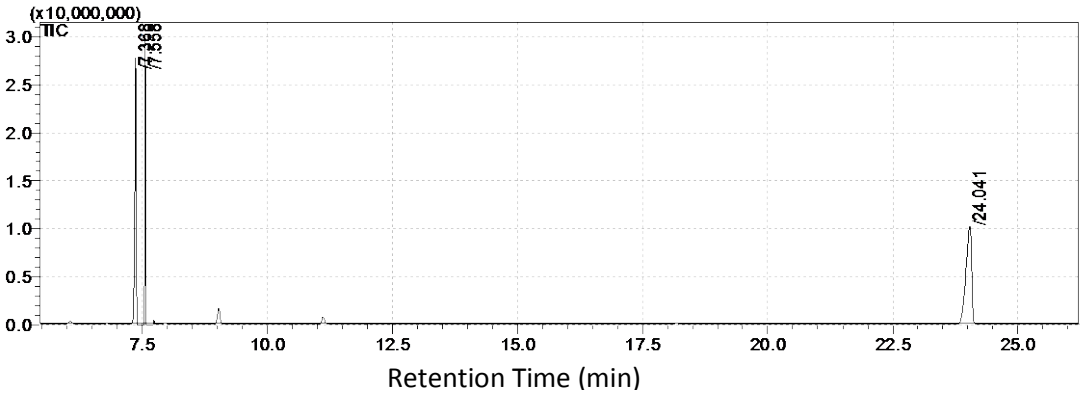


Figure 4. Chromatogram indicating retention time for sample 1.

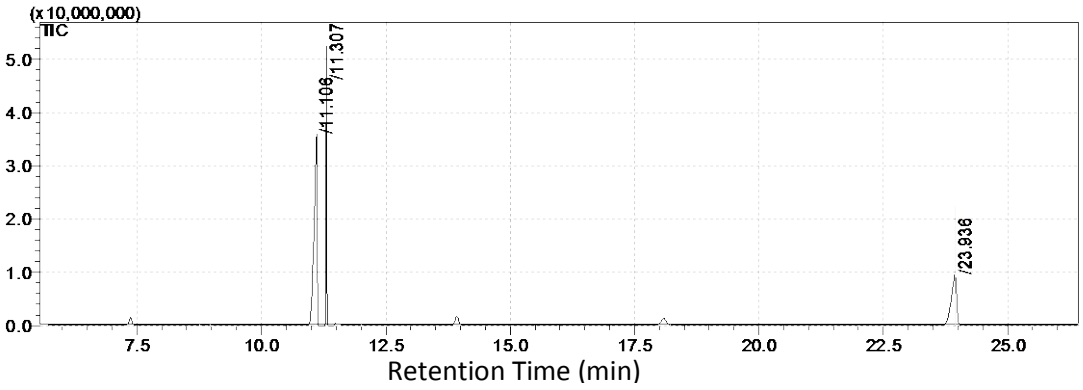


Figure 5. Chromatogram indicating retention time for sample 2.

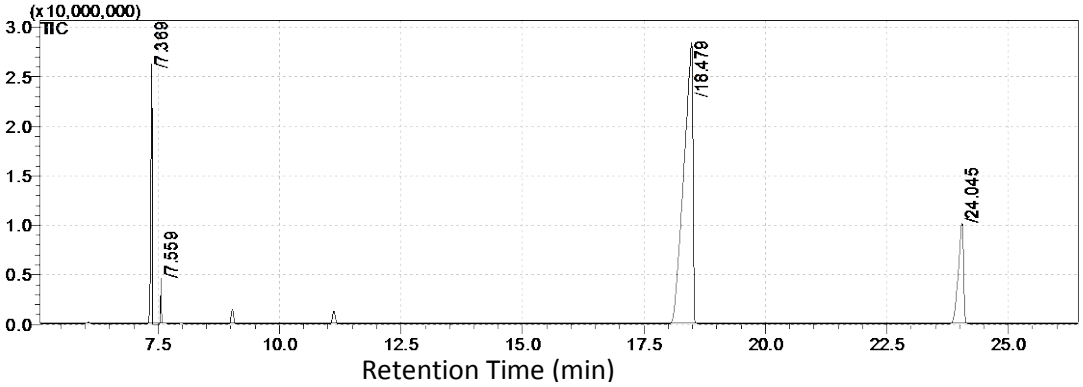


Figure 6. Chromatogram indicating retention time for sample 3.

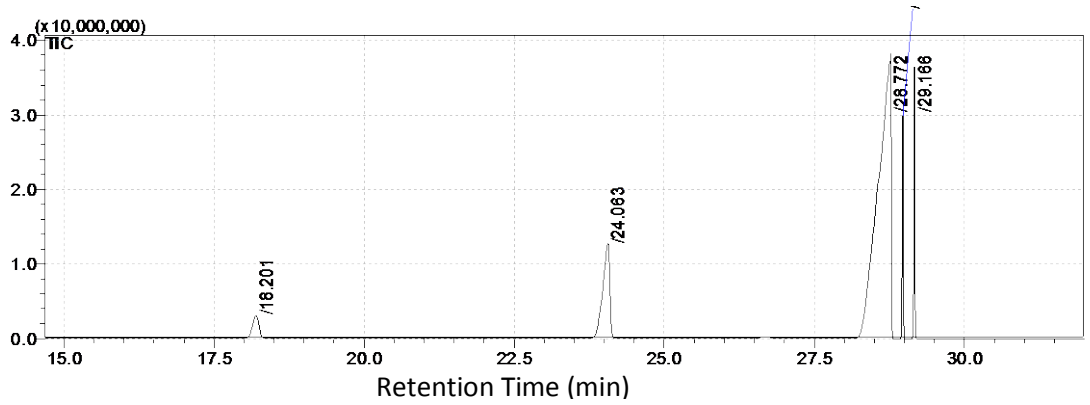
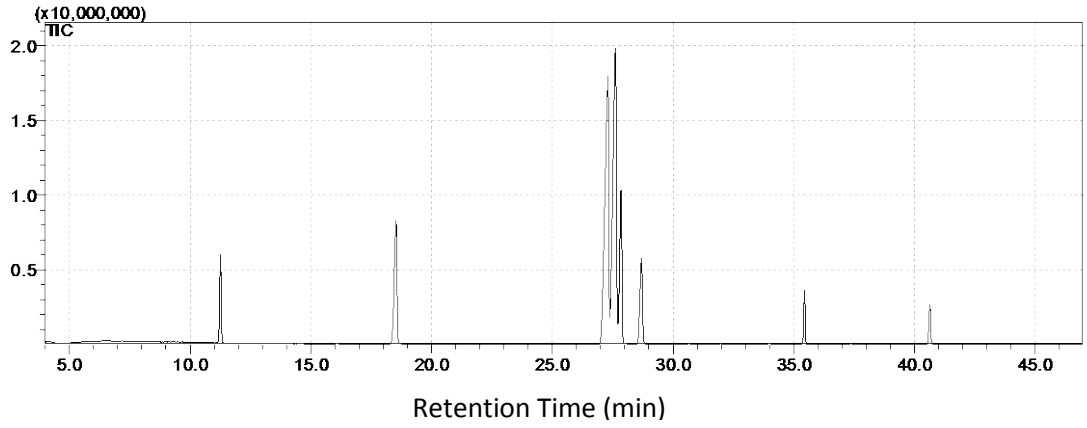


Figure 7. Chromatogram indicating retention time for sample 4.

Table 2. Retention Time of Saturated Fatty Acid Samples and C17 Standard.

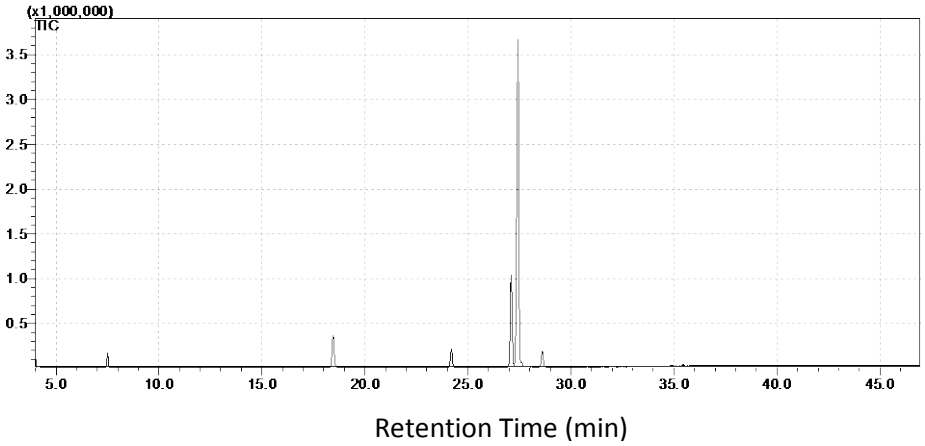
Fatty Acid	***Retention Time (min)***
C12:0	7.368
C14:0	11.348
C16:0	18.479
C17:0	24.066
C18:0	28.974

Oven conditions for the GC-MS analysis were developed using a standard of FAMEs (C14:0 to C22:0) (Sigma Aldrich) and esterified C17 (Figures 8 and 9).



Peak No	Peak name	Ret. Time. (min)	Area	Area %
1	C14:0	21.340	20572069	3.86
2	C16:0	30.639	55277415	10.38
3	C18:2	38.239	173835459	32.66
4	C18:1	38.571	172108330	10.28
5	C18:1cis-9	38.831	54700119	32.33
6	C18:0	39.761	34520835	6.48
7	C20:0	51.005	10588956	1.99
8	C22:0	60.617	10746239	2.02

Figure 8. Chromatogram and retention time of FAME standard mix.



Peak No	Peak name	Ret. Time. (min)	Area	Area %
1	BHT	7.505	595150	1.58
2	C16:0	18.455	2492701	6.61
3	C17:0	24.198	1265193	3.36
4	C18:2	27.096	6726191	17.84
5	C18:3	27.428	25250010	66.95
6	C18:1	27.642	168138	0.45
7	C18:0	28.604	1210405	3.21

Figure 9. Chromatogram and retention time of esterified C17 and adipose tissue.

The retention time of C10:0 to C24:1 indicates that the fatty acids were eluting from the column in order of chain length. However, the unsaturated fatty acids were eluting from the column earlier than the saturated fatty acids as indicated on Figure 10 and Table 3. For example, the C18 series of fatty acids has the order of C18:3 (RT = 26.221), C18:2 (RT= 26.925), C18:1n-9 (RT=27.277), C18:1n-7 (RT = 27.497) and C18:0 (RT=28.447).

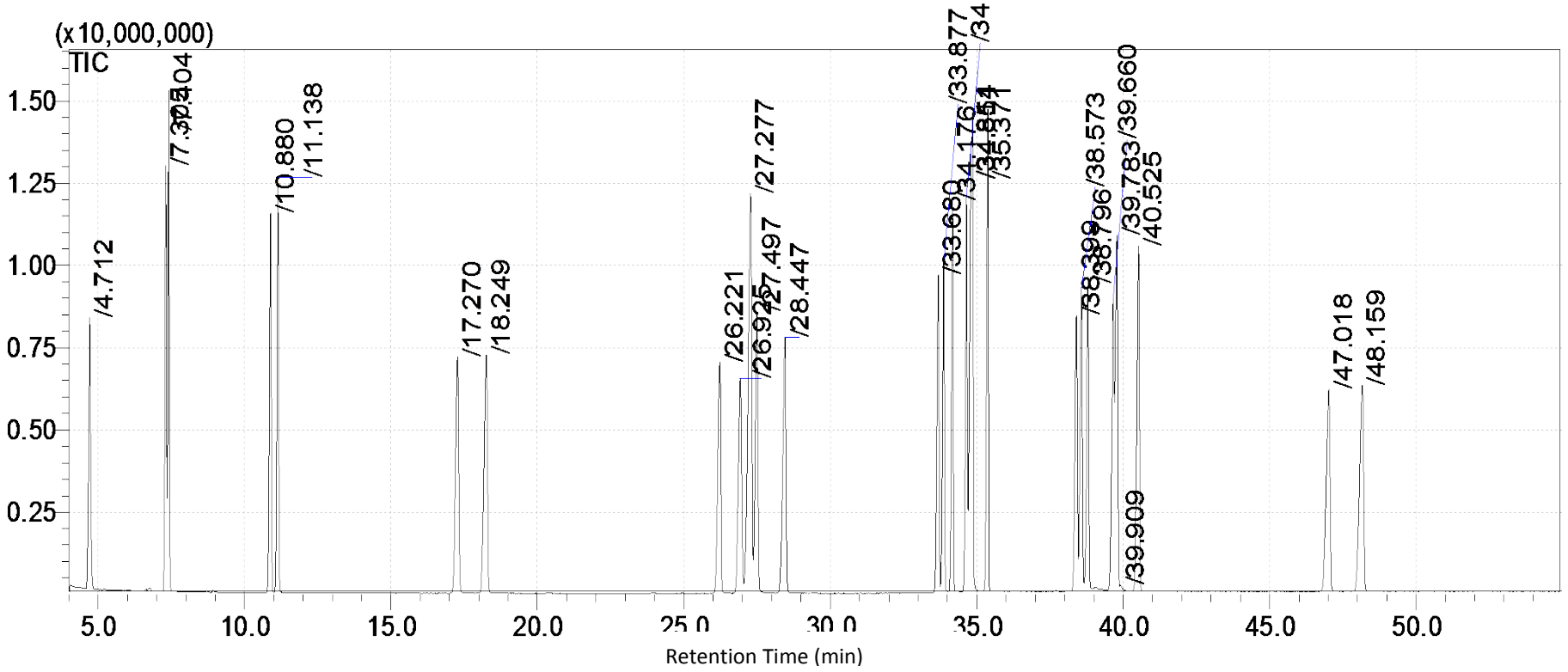


Figure 10. Chromatogram indicating retention time of fatty acid standards (C10:0 to C24:0).

Table 3. Retention time of Standard fatty acids (C10:0 to C24:0).

Fatty Acid	**Retention Time (min)**
C10:0	4.712
C12:1	7.305
C12:0	7.404
C14:1	10.88
C14:0	11.138
C16:1	17.27
C16:0	18.249
C18:3	26.221
C18:2	26.925
C18:1	27.277
C18:1	27.497
C18:0	28.447
C20:4	33.68
C20:5	33.877
C20:3	34.176
C20:2	34.65
C20:1	34.811
??	34.854
C20:0	35.371
C22:6	38.399
??	38.573
??	38.796
??	39.66
C22:1	39.783
??	39.909
C22:0	40.525
C24:1	47.018
C24:0	48.159

?? Indicate unidentified fatty acids.

3.2 Analysis of Dietary Components

Various dietary components from both plant and animal sources were analysed to determine their fatty acid profiles ($p<0.05$). Samples from rat liver and adipose (retroperitoneal fat) tissues were then investigated to identify the fatty acid profiles as a comparison to the fatty acids profiles ($p<0.05$) of the diet that the animals were fed.

3.2.1 Analysis of Plant Oils

The fatty acid profiles of the various plant oils that are consumed as dietary components indicate that there is a high percentage of unsaturated fatty acids in macadamia (97.50±0.02), canola (94.28±0.09) and chia (93.52±2.80) while safflower (73.69±3.22) has the lowest amount (Table 4). Safflower has the highest amount of saturated fatty acid with 26.31±3.22 while macadamia has the lowest levels with 2.50±0.02 (Table 4).

There are higher levels of C16:0 in olive (15.16±2.48) and avocado (15.22±0.01) compared to the other samples, while C16:1 are higher in the avocado (5.68±0.36), chia (2.67±2.67) and macadamia (4.15±0.01) samples (Table 4). There is no C16:1 in safflower, flax and canola and only a minimal amount (0.14±0.14) in olive oil (Table 4).

In the C18 series, safflower (18.02±4.09) has the highest proportion of C18:0 while avocado has none (Table 4). Chia (90.58±2.58), flax (89.11±0.59) and safflower (73.11±3.19) have high polyunsaturated (C18:2; C18:3) fatty acids while macadamia (91.17±0.01), canola (76.97±1.33), avocado (76.35±0.01) and olive (75.48±1.07) are highest in monounsaturated (C18:1) fatty acids with flax having no monounsaturated fatty acids and chia (2.94±2.73) and safflower (0.58±0.04) only having low levels (Table 4). Safflower (73.11±3.19) consists mainly of C18:2, while macadamia (2.62±0.12) has the lowest level. C18:2 is the fatty acid that varies the most across the various samples (Table 4). Chia and flax have high levels of

C18:3 (69.16±2.00 and73.30±0.45 respectively), while there is none present in safflower, olive, avocado and canola, with only a minimal amount (3.70±0.15) found in macadamia (Table 4).There was only low levels of C20:4 found in canola (0.62±0.01) and chia (0.08±0.08) with none in the other samples (Table 4).

Table 4. Fatty acid composition of various plant oils used as dietary components.

Fatty Acid (g/100 of total)	CHIA (n=3)	SAFFLOWER (n=3)	FLAX (n=3)	OLIVE (n=3)	MACADAMIA (n=3)	AVOCADO (n=3)	CANOLA (n=3)
C16:0	2.89±2.89^{b}	8.29±1.45^{b}	4.93±0.64^{b}	15.16±2.48^{a}	1.88±0.01^{b}	15.22±0.01^{a}	4.06±0.06^{b}
C16:1n-7	2.67±2.67^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.14±0.14^{a}	4.15±0.01^{ac}	5.68±0.36^{bc}	0.00±0.00^{a}
C18:0	3.59±0.13^{a}	18.02±4.09^{b}	5.97±0.58^{a}	0.61±0.61^{a}	0.62±0.02^{a}	0.00±0.00^{a}	1.66±0.03^{a}
C18:1n-9	0.27±0.13^{e}	0.58±0.04^{e}	0.00±0.00^{e}	75.34±1.89^{c}	87.02±0.01^{a}	70.68±0.36^{d}	76.97±1.33^{b}
C18:2n-6	21.34±0.55^{b}	73.11±3.19^{a}	15.81±0.58^{c}	8.75±1.54^{d}	2.62±0.12^{e}	8.42±0.01^{d}	16.69±1.43^{c}
C18:3n-3	69.16±2.00^{b}	0.00±0.00^{d}	73.30±0.45^{a}	0.00±0.00^{d}	3.70±0.15^{c}	0.00±0.00^{d}	0.00±0.00^{d}
C20:4n-6	0.08±0.08^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.62±0.01^{a}
Total Saturated	6.48±2.80^{be}	26.31±3.22^{d}	10.89±0.59^{ce}	15.77±2.38^{ac}	2.50±0.02^{b}	15.22±0.01^{ce}	5.72±0.09^{bc}
Total Unsaturated	93.52±2.80^{ae}	73.69±3.22^{d}	89.11±0.59^{ce}	84.23±2.38^{bc}	97.50±0.02^{a}	84.78±0.01^{bc}	94.28±0.09^{ac}
Total Monounsaturated	2.94±2.73^{c}	0.58±0.04^{c}	0.00±0.00^{c}	75.48±1.07^{b}	91.17±0.01^{a}	76.35±0.01^{b}	76.97±1.33^{b}
Total Polyunsaturated	90.58±2.58^{a}	73.11±3.19^{b}	89.11±0.59^{a}	8.75±1.54^{d}	6.33±0.03^{d}	8.42±0.01^{d}	17.31±1.42^{c}

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. *P* <0.05. No superscript indicates no significant difference between groups.

3.2.2 Analysis of Animal Oils

The profiles of the animal oils tested indicate that beef tallow consists of a high percentage (64.84±0.68) of saturated fatty acids (C14:0 – 4.62±0.08; C16:0 - 32.24±0.31; C18:0 – 27.98±0.28) with lower levels of unsaturated fatty acids, mainly monounsaturated C18:1 (32.23±0.31) and a small amount of polyunsaturated C18:2 (1.11±0.02) (Table 5). EPA and DHA samples are 100% polyunsaturated fatty acids. EPA (91.86±1.49) consists mainly of C20:5 and DHA (90.26±0.25) of C22:6 which is consistent with their chemical nature (Table 5). The composition of EPA (2.73±0.66) and DHA (3.90±2.39) indicate that low levels of C20:4 are present. DHA (5.84±2.50) also contains C20:5 (Table 5).

Table 5. Fatty acid composition of Animal oils.

Fatty Acid (g/100 of total)	**BEEF TALLOW (n=3)**	**EPA (n=3)**	**DHA (n=3)**
C14:0	4.62±0.08	0.00±0.00[a]	0.00±0.00[a]
C16:0	32.24±0.31	0.00±0.00[a]	0.00±0.00[a]
C16:1n-7	1.36±0.01	0.00±0.00[a]	0.00±0.00[a]
C18:0	27.98±0.28	0.00±0.00[a]	0.00±0.00
C18:1n-9	32.23±0.31	0.00±0.00[a]	0.00±0.00[a]
C18:2n-6	1.11±0.02	0.00±0.00[a]	0.00±0.00[a]
C20:4n-6	0.00±0.00	2.73±0.66	3.90±2.39
C20:5n-3	0.00±0.00[a]	91.86±1.49[b]	5.84±2.50[c]
C22:6n-3	0.00±0.00[a]	5.41±0.83[b]	90.26±0.25[c]
Total Saturated	64.84±0.68	0.00±0.00[a]	0.00±0.00[a]
Total Unsaturated	35.16±0.68	100.00±0.00[a]	100.00±0.00[a]
Total Monounsaturated	34.04±0.66	0.00±0.00[a]	0.00±0.00[a]
Total Polyunsaturated	1.11±0.02	100.00±0.00[a]	100.00±0.00[a]

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. *P* <0.05. No superscript indicates no significant difference between groups.

3.3 Analysis of Tissue Samples

3.3.1 Analysis of Liver samples

Fatty acid profiles of liver from rats fed with corn-starch (CS) or high carbohydrate high fat (HCHF) diets and with EPA supplementation (CEPA and HEPA) were analysed using differing methods of derivitization. Each group was compared against each other group.

The H_2SO_4 method indicates a number of differences ($p<0.05$): Palmitoleic acid (C16:1n-7) in CS (2.60±0.01) treatment is higher than in the HCHF (0.35±0.35) samples ($p<0.05$) with none in the CEPA and HEPA samples, stearic acid (C18:0) in the HCHF (8.56±1.75) is lower than in the HEPA fed groups (32.34±8.34) ($p<0.05$) while it is also different between the CS (14.16±0.47) and HCHF ($p<0.05$) (Table 6). Oleic acid (C18:1n-9) in the HCHF (55.54±2.90) group is higher than in the CS (33.18±2.0), CEPA (24.08±2.13) and HEPA (15.86±8.29) groups ($p<0.05$) (Table 6). The total monounsaturated fatty acid composition of the CS fed group (39.87±2.95) differs from the HEPA group (16.98±8.87)($p<0.05$) (Table 6). In the HCHF group (57.58±4.14) the monounsaturated fatty acid composition is higher than to the CS (39.87±2.95), CEPA (24.67±2.94) and HEPA (16.98±8.87) groups ($p<0.05$) (Table 6).

Table 6. Liver fatty acid composition in groups fed CS, HCHF, CEPA and HEPA diets using H_2SO_4 derivitization method.

Fatty Acid (g/100 of total)	CS (n=3)	HCHF (n=3)	CEPA (n=3)	HEPA (n=3)
C14:0	0.49±0.49	0.99±0.50	0.00±0.00	0.00±0.00
C16:0	31.38±0.97	27.09±3.90	38.27±5.80	38.64±7.10
C16:1n-7	2.60±0.01	0.35±0.35[a]	0.00±0.00[a]	0.00±0.00[a]
C18:0	14.16±0.47[ab]	8.56±1.75[b]	25.71±2.92[ab]	32.34±8.34[a]
C18:1n-9	33.18±2.50[b]	55.54±2.90[a]	24.08±2.13[b]	15.86±8.29[b]
C18:2n-6	4.21±1.23	2.70±0.05	5.08±2.66	5.08±2.55
C20:4n-6	8.57±1.54	1.89±0.95	3.22±1.74	4.69±2.40
C22:6n-3	1.37±0.70	2.09±1.33	3.05±1.55	2.27±2.27
Total Saturated	45.98±0.82	36.64±5.02	63.97±8.70	70.98±15.44
Total Unsaturated	54.02±0.82	63.36±5.02	36.03±8.70	29.02±15.43
Total Monounsaturated	39.87±2.95[b]	57.58±4.14[a]	24.67±2.94[bc]	16.98±8.87[c]
Total Polyunsaturated	14.15±3.39	5.78±0.88	11.36±5.92	12.04±6.60

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. *P* <0.05. No superscript within a row indicates no significant difference between groups.

Using the BF_3 derivatization method DHA differs between CS (3.94±1.55) and HCHF (4.01±0.96) as well as HCHF (4.01±0.96) and HEPA (6.53±0.10) (Table 7). Total polyunsaturated fatty acids are also differing between CS (20.21±4.90) and CEPA (15.30±1.22) as well as HCHF (14.84±2.29) and HEPA (27.41±0.87) (Table 7). Low levels of Dihomo-γ-linolenic acid (C20:3n-6) were found in CS (0.28±0.28) and HEPA (0.36±0.36) with none in HCHF or CEPA (Table 7).

Table 7. Liver fatty acid composition in groups fed CS, HCHF, CEPA and HEPA diets using BF3 derivatization method.

Fatty Acid (g/100 of total)	CS (n=3)	HCHF (n=3)	CEPA (n=3)	HEPA (n=3)
C14:0	0.36±0.36	0.73±0.43	0.00±0.00	0.00±0.00
C16:0	30.67±2.80	28.48±1.65	36.65±1.66	28.71±0.32
C16:1n-7	1.37±1.37	1.00±0.65	0.00±0.00	0.00±0.00
C18:0	22.78±4.62	18.15±4.60	25.98±1.22	25.67±2.16
C18:1n-9	24.61±7.65	34.33±9.35	22.06±0.67	18.21±3.25
C18:2n-6	6.95±1.64	6.94±1.79	7.97±0.91	9.47±0.51
C20:4n-6	8.18±2.41	6.37±1.61	4.32±0.25	10.62±1.23
C20:3n-6	0.28±0.28	0.00±0.00	0.00±0.00	0.36±0.36
C20:5n-3	0.87±0.47	0.00±0.00	1.83±0.95	0.43±0.43
C22:6n-3	$3.94±1.55^{bc}$	$4.01±0.96^{ab}$	$1.18±1.18^{b}$	$6.53±0.10^{c}$
Total Saturated	53.81±6.02	48.32±6.07	62.64±2.27	54.38±2.43
Total Unsaturated	46.19±6.02	51.68±6.07	37.36±2.27	45.62±2.43
Total Monounsaturated	25.98±8.98	36.83±8.09	22.06±2.10	18.21±3.25
Total Polyunsaturated	$20.21±4.90^{abc}$	$14.84±2.29^{a}$	$15.30±1.22^{ac}$	$27.41±0.87^{b}$

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. *P* <0.05. No superscript within a row indicates no significant difference between groups.

3.3.2 Analysis of Adipose (retroperitoneal fat) Samples

Tables 8 and 9 show the fatty acid profile of adipose (retroperitoneal fat) tissue from rats fed a corn-starch (CS) or high carbohydrate high fat (HCHF) diets and with EPA supplementation (CEPA and HEPA) using differing methods of derivitization. Each group was compared to each other group.

Using the H_2SO_4 method, palmitic acid (C16:0) is lower in the CEPA samples (25.94±10.92) than HCHF (59.31±1.13), HEPA (58.12±0.85) and CS (46.52±1.46) (Table 9). Arachidonic acid (C20:4n-6) is found in low levels in CEPA (3.87±3.87) and HCHF (0.18±0.18) but not in CS or HEPA (Table 9). Total saturated fatty acids are highest in the CEPA fed samples (47.74±1.28), followed by HEPA (38.22±3.02),

CS (38.11±1.75) and HCHF (32.89±1.14) (Table 8). Total unsaturated fatty acids are highest in HCHF (67.11±1.14) with CEPA (52.27±1.29) having the lowest amount (Table 9). Of these amounts, 64.14±0.49 (HCHF) and 43.86±7.82 (CEPA) are monounsaturated fatty acids (Table 8).

Differences in fatty acid composition between the CS group and the treatment group (CEPA) ($p<0.05$) also occurred. However, there was no difference found between the HCHF and HEPA groups. For example, oleic acid (C18:1) decreased between CS (46.52±1.46) and CEPA (25.94±10.92) ($p<0.05$), but were similar in HCHF (59.31±1.13) and HEPA (58.12±0.85) (Table 8). Total saturated fatty acid increased from the CS (38.11±1.75) to the CEPA (47.74±1.28) groups, while total unsaturated fatty acid levels decreased with CS (57.6±1.75) to CEPA (52.27±1.29), this mainly being a decrease in monounsaturated fatty acid levels of 52.22±2.28 (CS) to 43.86±7.82 (CEPA) (Table 8).

In the HCHF and HEPA groups, total saturated fatty acids increased from 32.89±1.14 (HCHF) to 38.22±3.02 (HEPA), total unsaturated fatty acids decreased from 67.11±1.14 (HCHF) to 61.78±3.02 (HEPA) with a corresponding decrease in MUFA of 64.14±0.49 (HCHF) to 59.41±1.95 (HEPA) (Table 8).

Table 8. Adipose (retroperitoneal fat) fatty acid composition in groups fed CS, HCHF, CEPA and HEPA diets using H_2SO_4 derivatization method.

Fatty Acid (g/100 of total)	CS (n=3)	HCHF (n=3)	CEPA (n=3)	HEPA (n=3)
C14:0	2.10±0.10^{ab}	3.62±0.54^{b}	1.37±0.72^{a}	3.10±0.15^{ab}
C16:0	32.85±1.12	23.90±0.72	33.90±4.25	26.51±2.67
C16:1n-7	5.70±0.82	1.79±0.94	14.65±12.11	0.00±0.00
C18:0	3.16±0.60	5.37±0.93	12.47±6.11	8.61±0.97
C18:1n-9	46.52±1.46^{a}	59.31±1.13^{a}	25.94±10.92^{b}	58.12±0.85^{a}
C18:2n-6	5.38±1.54	2.78±0.60	3.49±1.92	2.37±1.19
C20:4n-6	0.00±0.00	0.18±0.18	3.87±3.87	0.00±0.00
C22:6n-3	0.00±0.00	0.00±0.00	1.05±1.04	0.00±0.00
Total Saturated	38.11±1.75^{bc}	32.89±1.14^{bc}	47.74±1.28^{a}	38.22±3.02^{b}
Total Unsaturated	57.6±1.75^{ab}	67.11±1.14^{ab}	52.27±1.29^{c}	61.78±3.02^{a}
Total Monounsaturated	52.22±2.28^{ab}	64.14±0.49^{b}	43.86±7.82^{a}	59.41±1.95^{ab}
Total Polyunsaturated	5.38±1.54	2.97±0.71	8.41±6.57	2.37±1.19

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. *P* <0.05. No superscripts within a row indicate no significant differences between groups.

There was no difference ($p<0.05$) between any of the fatty acids in the adipose tissue despite the diet given when using the BF_3 derivatization method. Oleic acid (C18:1n-9) was the most abundant fatty acid with (61.90±10.52) and HEPA (61.06±0.48) levels higher than in the CS (38.74±10.69) and CEPA (40.76±2.58) (Table 9). Palmitic acid (C16:0) was the next highest with HCHF (23.02±4.88) and HEPA (22.98±0.22) levels lower than in the CS (32.11±4.32) and CEPA (6.88±0.50) (Table 9). Both CS (2.98±2.97) and CEPA (0.17±0.17) had low levels of arachidonic acid (C20:4n-6) while the HCHF and HEPA groups had none (Table 9). EPA (C20:5n-3) was found in the CEPA (0.44±0.44) group but no others (Table 9). DHA (C22:6n-3) was in CS (2.44±2.00), HCHF (0.19±0.18) and CEPA (0.40±0.40) (Table 9). Total saturated fatty acids were higher in the CS (44.62±5.77) and CEPA

(42.44±5.41), while the total unsaturated fatty acids were higher in the HCHF (69.72±5.41) and HEPA (64.54±0.49) groups (Table 9). Monounsaturated fatty acids were higher across all four groups compared to polyunsaturated fatty acids (Table 9).

Table 8. Adipose (retroperitoneal fat) fatty acid composition in groups fed CS, HCHF, CEPA and HEPA diets using BF_3 derivatization method.

Fatty Acid (g/100 of total)	**CS (n=3)**	**HCHF (n=3)**	**CEPA (n=3)**	**HEPA (n=3)**
C14:0	1.17±0.59	3.39±1.01	2.03±0.13	2.55±0.20
C16:0	32.11±4.32	23.02±4.88	35.94±3.35	22.98±0.22
C16:1n-7	4.01±2.14	2.89±1.94	6.88±0.50	1.15±0.58
C18:0	11.27±5.42	3.89±1.95	4.46±2.37	9.38±0.86
C18:1n-9	38.74±10.69	61.90±10.52	40.76±2.58	61.06±0.48
C18:2n-6	6.06±3.05	3.57±2.56	5.98±3.24	2.32±0.48
C20:0	0.06±0.06	0.00±0.00	0.00±0.00	0.56±0.28
C20:4n-6	2.98±2.97	0.00±0.00	0.17±0.17	0.00±0.00
C20:5n-3	0.00±0.00	0.00±0.00	0.44±0.44	0.00±0.00
C22:6n-6	2.44±2.00	0.19±0.18	0.40±0.40	0.00±0.00
Total Saturated	44.62±5.77	30.30±5.40	42.44±5.41	35.46±0.49
Total Unsaturated	55.38±5.77	69.72±5.41	57.56±5.41	64.54±0.49
Total Monounsaturated	43.90±12.16	65.94±8.05	50.57±2.98	62.22±0.96
Total Polyunsaturated	11.48±7.32	3.76±2.73	6.99±3.40	2.32±0.48

Each value is a mean±SEM. Number of repetitive experiments indicated within parenthesis. Means within a row with unlike superscript are significantly different. P <0.05. No superscripts within a row indicate no significant differences between groups.

CHAPTER 4

DISCUSSION

4.1 Analysis of Method

The generation of fatty acid methyl esters (FAMEs) is necessary for the analysis of long chain fatty acids (>C12) by gas chromatography. The separation of the carboxylic acids is complicated by their relatively high polarity, so relatively nonpolar derivatives, such as FAMEs, which are more volatile are produced. FAME can be produced using acidic or alkaline conditions (Ruiz-Rodriguez *et al.*, 2010), this project utilized the acidic conditions produced by heating fats with an excess of methanol in the presence of either H_2SO_4 or BF_3 catalysts. While both methods produced FAME there were a number of noteworthy differences.

4.1.1 Sulphuric Acid (H_2SO_4) method

This method is a very simple method to use as only two chemicals (H_2SO_4 and Methanol) are involved in the derivatization process and it only uses diethyl ether and distilled water in the separation and extraction process. It is neither expensive nor dangerous and has good GC response (Antolin *et al.*, 2008). Another advantage is that sufficient FAME is produced to enable multiple analyses. However, a number of limitations were also found to exist with this method. While the method was suitable for large scale samples, when it was scaled down to test tube amounts the sample regularly discoloured. This may have been due to the methanol solvent evaporating too quickly and the residual H_2SO_4 charring the fatty acid and is problematic in achieving optimum results as FAMEs may not be produced. The hot plate used in this procedure was a limitation as it did not heat accurately, with temperatures varying as it could not be set specifically at 65°C to enable the reaction

to take place at the optimum temperature. These issues could be solved by using a water bath that is set at a consistent temperature.

Another limitation is the length of time that the derivatization process takes, as each sample takes at least 2 hours followed by the extraction and separation process this limits the number of samples that can be prepared at any one time. However, previous research has indicated that the process can take as little as 10 minutes with the addition of further chemicals (*n*-hexane, 1 M sodium hydroxide), higher temperature and occasional shaking (Antolin *et al.*, 2008).

A large volume of chemicals are need for this method, particularly the separation and extraction processes. Even in the small scale experiments 30 mL of diethyl ether and 40 mL of water was required.

During the separation and extraction phase of the method, there is the possibility of losing sample through spillage, or remaining in the glassware used.

4.1.2 BF_3 Method

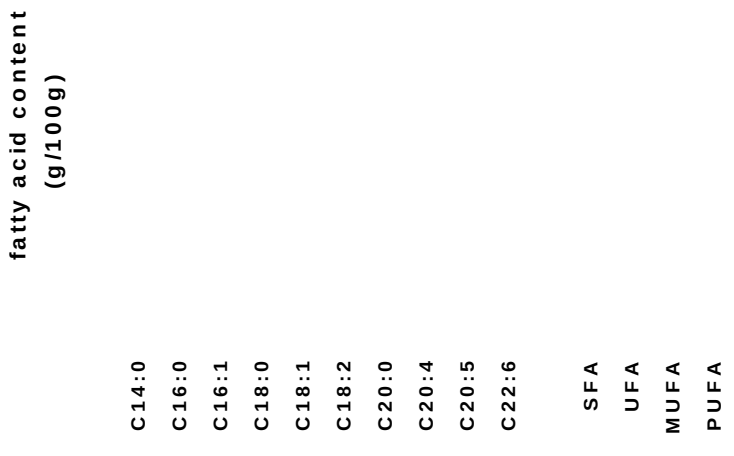
The BF_3 method allows for fast and effective methylation (Antolin *et al.*, 2008, Ruiz-Rodriguez *et al.*, 2010), as multiple samples were able to be prepared simultaneously enabling analysis by GC-MS to occur sooner. Temperature was controlled at a steady 95°C using a water bath, which controlled solvent evaporation. As BF_3 is volatile the need for a more complicated work up procedure is removed. However, limitations exist as the reagent is expensive and doesn't have a long shelf life even when refrigerated (Antolin *et al.*, 2008). BF_3 has greater toxicity, consequently greater care needed to be taken during the procedure. Also only sufficient FAME was produced for a single GC-MS auto sampler vial to be prepared. There have also been reports that BF_3 leads to irreversible damage of the GC

column (Ruiz-Rodriguez *et al.*, 2010), which is another consideration to make when using this method.

4.1.3 Comparison of results

The results obtained confirm that both BF_3-methanol and H_2SO_4-methanol derivatization methods are suitable to use for the analysis of fatty acids. The results achieved from both liver and adipose tissue samples indicate that there was no significant difference ($p>0.05$) between the methods (Figure 11) therefore no definitive conclusion, can be made as to the method better suited to the derivatization process. Therefore in considering that one of the objectives of this project is to validate previous results (Poudyal *et al.*, 2012b), comparisons will be made using the BF_3 methodology results as this is the more accepted method of derivatization in prior literature. Comparisons will be made between the two methods where appropriate.

a.



c.

	fatty acid content (g/100g)
C14:0	
C16:0	
C16:1	
C18:0	
C18:1	
C18:2	
C20:0	
C20:4	
C20:5	
C22:6	
SFA	
UFA	
MUFA	
PUFA	

b.

	fatty acid content (g/100g)
C14:0	
C16:0	
C16:1	
C18:0	
C18:1	
C18:2	
C20:0	
C20:4	
C20:5	
C22:6	
SFA	
UFA	
MUFA	
PUFA	

e.

	fatty acid content (g/100g)
C14:0	
C16:0	
C16:1	
C18:0	
C18:1	
C18:2	
C20:4	
C20:3	
C20:5	
C22:6	
SFA	
UFA	
MUFA	
PUFA	

d.

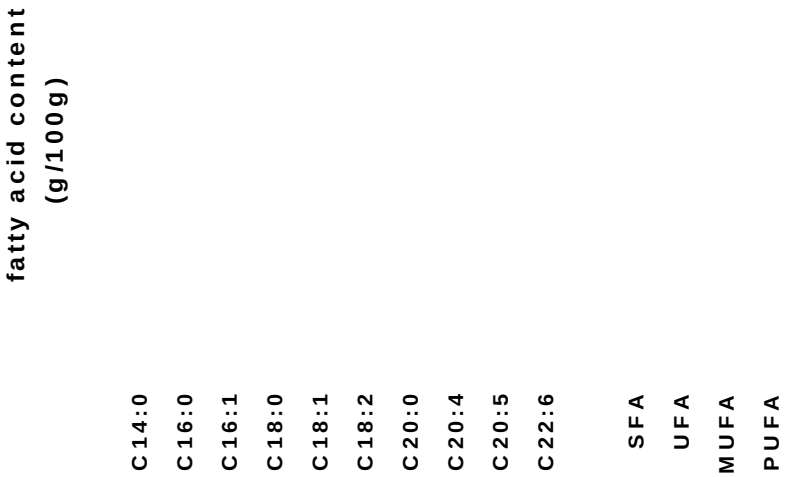
	fatty acid content (g/100g)
C14:0	
C16:0	
C16:1	
C18:0	
C18:1	
C18:2	
C20:0	
C20:4	
C20:5	
C22:6	
SFA	
UFA	
MUFA	
PUFA	

f.

	fatty acid content (g/100g)
C14:0	
C16:0	
C16:1	
C18:0	
C18:1	
C18:2	
C20:4	
C20:3	
C20:5	
C22:6	
SFA	
UFA	
MUFA	
PUFA	

g.

	fatty acid content (g/100g)
C14:0	
C16:0	
C16:1	
C18:0	
C18:1	
C18:2	
C20:0	
C20:4	
C20:5	
C22:6	
SFA	
UFA	
MUFA	
PUFA	



h.

Figure 11. Derivatization Method Comparison (BF_3 vs. H_2SO_4) in various feeding conditions (CS, HCHF, CEPA, HEPA) in adipose (a-d) and liver (e-h) tissue.

4.2 Analysis of Treatments

Plant and animal oils were tested for method validation purposes as well to identify fatty acid compounds that were present. As samples were of relative purity they could be compared to other literature and used to validate the methodology of this project. These oils were analysed using the H_2SO_4 method due to ease of use in bulk samples and cost considerations.

4.2.1 Plant Oils

Plant oils consist mainly of unsaturated fatty acids. Chia, flax and safflower oils have high levels of polyunsaturated fatty acids (PUFA) (Figure 13). Olive, macadamia, avocado and canola oils consist mainly of monounsaturated fatty acids (MUFA) (Figure 13). Safflower, olive and avocado oils have the highest levels of saturated fatty acids (SFA) (Figure 13) amongst the plant oils that were tested. Oleic acid and linoleic acid were found in all samples. Oleic acid is the predominant dietary MUFA (Asif, 2011) found in plants, results indicated it was highest in macadamia oil, followed by canola, olive and avocado, with low levels found in chia and safflower (Figure 12). Linoleic acid was highest in the safflower sample with varying levels in all other samples (Figure 12). α-linolenic acid was found in flax, chia and a low level in macadamia (Figure 12). The typical 'western' diet contains much more linoleic acid than α-linolenic acid (Gillingham *et al.*, 2011). Both linoleic and α-linolenic acids are eicosanoid precursors with linoleic acid ones tending to be pro-inflammatory and α-linolenic acid ones anti-inflammatory (Poudyal *et al.*, 2011) indicating possible implications in the development of obesity.

A very small amount of arachidonic acid was found in chia and canola (Figure 12) indicating elongation from linoleic acid. (Individual plant oil graphs see Appendix 1).

Figure 12. Individual fatty acid composition of plant oils.

Figure 13. Total fatty acid composition of plant oils.

Results are comparable to those found in literature (Asif, 2011, Poudyal *et al.*, 2012a, Poudyal *et al.*, 2012b) indicating that the methodology used was appropriate. However, it must be noted that while types and proportions of fatty acids were similar, the overall values were higher possibly due to the extracted samples being used being a better quality than metabolised samples as in the literature values which were measured after dietary supplementation. It is also possible that values are slightly different due to different GC columns being used to analyse the samples or to natural variation between the samples themselves.

4.2.2 Animal Oils

Animal oils differ in fatty acid composition. The samples of EPA and DHA that were examined were essentially 'pure'. Most studies utilize a mix of EPA/DHA through fish oil supplements, capsules or an oily fish diet therefore it is unclear if they have independent effects or if the result is due to the mixture.

Results indicated that the samples of EPA and DHA were both 100% unsaturated fatty acids, as expected. EPA consisted mainly of n-3 fatty acids with EPA and a small amount of its derivative DHA and also a small amount of C20:4 (Figure 14). DHA was found to consist of DHA with low levels of its precursor EPA and C20:4 (Figure 14). It was not clear from our analysis whether the C20:4 was the pro-inflammatory arachidonic acid (C20:4n-6), or the anti-inflammatory eicosatetranoic acid (C20:4n-3). However, previous studies on EPA have found it to be involved in the production of anti-inflammatory eicosanoids (Bagga *et al.*, 2003, Duda *et al.*, 2009, Feller and Gawrisch, 2005, Poudyal *et al.*, 2012b, Schmitz and Ecker, 2008, Yashodhara *et al.*, 2009), therefore it may be postulated that it is C20:4n-3 as it is a precursor to EPA.

The beef tallow that was tested was found to contain mostly SFA, particularly palmitic (C16:0) and stearic (C18:0) acids. A lower amount of UFA, was found with oleic (C18:1) being the main fatty acid and a low level of LA (C18:2n-6) (Figure 15).

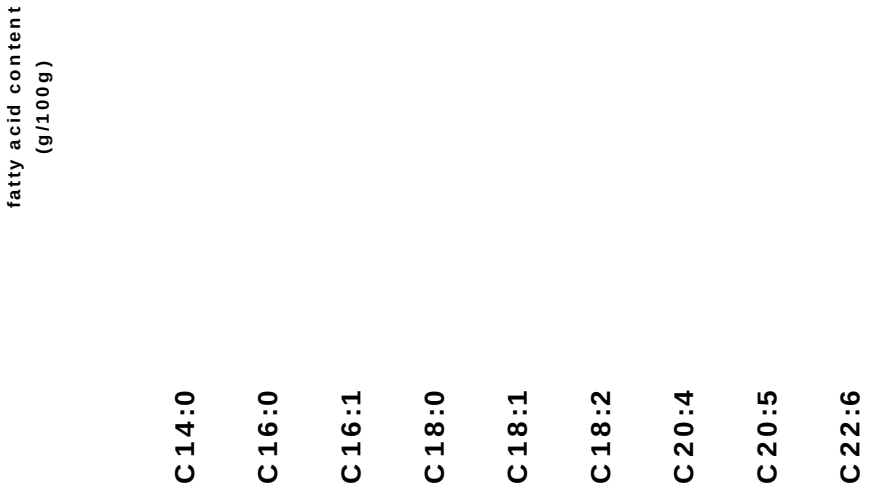


Figure 14. Individual fatty acids composing animal oils (EPA, DHA and beef tallow).

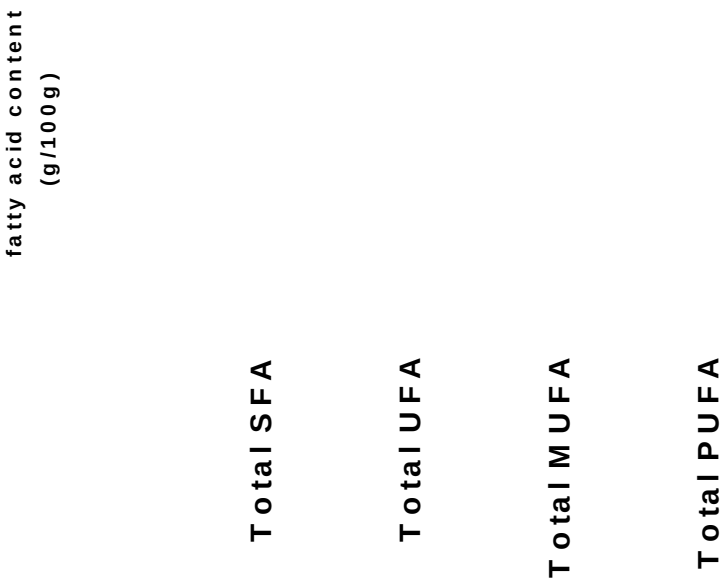


Figure 15. Total fatty acid composition of animal oils (EPA, DHA and beef tallow).

Animals are unable to produce fatty acids over than C18:1 due to a lack of necessary enzymes, therefore cannot form linoleic acid (C18:2n-6), α-linolenic acid (C18:3n-3) or longer fatty acids *de novo.* However, animals can further extend linoleic (C18:2n-6) and α-linolenic acids (C18:3n-3), so they are essential to metabolism and are only gained from external sources, such as plants (Poudyal *et al.*, 2012a).

Plant and animal oil results are similar to other research undertaken (Poudyal *et al.*, 2012a) indicating that the H_2SO_4 method is viable, particularly when done on a large scale to provide sufficient fatty acids to be converted to FAME for GC-MS analysis.

4.3 Analysis of Tissue Samples

4.3.1 Tissue Samples

Most biological tissues are involved in fatty acid metabolism with the liver and adipose tissue the most important (Frayn *et al.*, 2006). Adipose fatty acids are released to circulate to other tissues, while liver fatty acids act as a substrate for re-esterification within the endoplasmic reticulum to make triacylglycerol and to be secreted as very low density lipoprotein (VLDL) (Frayn *et al.*, 2006). Evidence from previous studies suggests that individual fatty acids may have selective and potentially independent effects on cardiovascular health, (Li *et al.*, 2008, Poudyal *et al.*, 2011) whether this extends to attenuation of obesity-related symptoms is still relatively unknown.

Liver (n=3) and adipose/retroperitoneal fat (n=3) tissue samples from the HCHF rat model of obesity were examined for differences in fatty acid composition between diet and also with supplementation of ‘pure’ EPA.

4.3.1.1 Liver Samples

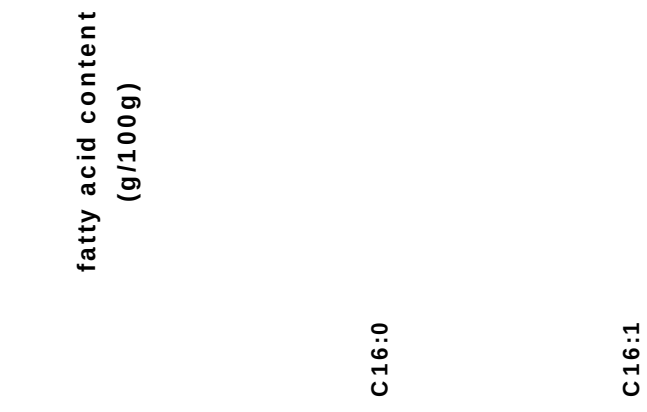
Studies indicate that dietary EPA partially replaces the n-6 PUFA’s from various cells including hepatocytes by inhibiting stearoyl-CoA-desaturase-1 (SCD-1) (Simopoulos, 2002). Of the four SCD genes that have been discovered SCD-1 is the only desaturase expressed by the liver (Li *et al.*, 2008). The conversion of n-3 and n-6 fatty acids share the same series of enzymes and competition exists for metabolism with an excess of one causing a significant decrease in the conversion of the other.

(Schmitz and Ecker, 2008). If n-3 fatty acids can be detected at higher levels than n-6 fatty acids, then the conversion to anti-inflammatory eicosanoids is possible. There was a slight increase in palmitic acid (C16:0) in the CEPA group compared to CS, while there was a low level of palmitoleic acid (C16:1) in the supplementation groups, (Figure 16) indicating the possible inhibition of SCD-1, as this enzyme induces as *cis*-double bond in the Δ^9 position, and is the rate limiting factor in the biosynthesis of MUFA (Li *et al.*, 2008). Similarly oleic acid (C18:1) was lower in the CEPA and HEPA fed liver sample (Figure 16).

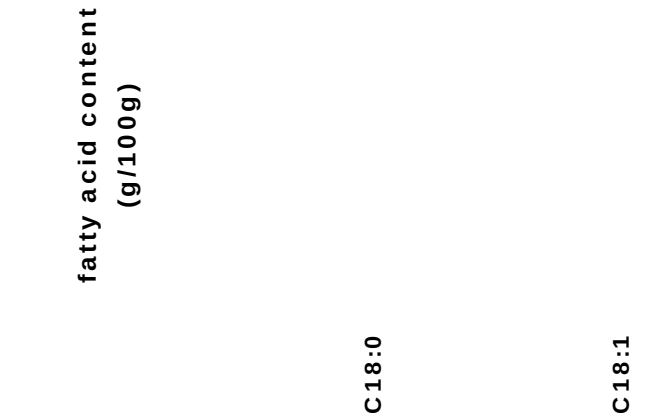
DGLA (C20:3n-6) was found in low levels in CS and CEPA groups (Figure 17). DGLA is an intermediary to arachidonic acid (C20:4n-6) which is the key intermediate in the production of pro-inflammatory eicosanoid metabolites (TX_2, PG_2, LT_4) (Serhan *et al.*, 2007). DHA (C22:6) was found to be higher in HEPA than HCHF and EPA (C20:5) was higher in CEPA than CS (Figure 17), both these results are as expected with the supplementation of EPA into the diet.

Total SFA increased between the diet and supplementation groups, while the total UFA decreased (Figure 17), in line with SCD-1 being inhibited. PUFA decreased from the CS to the CEPA groups and increased from the HCHF to the HEPA group (Figure 17). Studies have found that high fish oil feeding decreases liver fatty acid synthase (FAS) and SCD-1 expression (Sun *et al.*, 2011) for example Inuit and Japanese diets are high in fish-derived EPA and DHA (Greene *et al.*, 2013, Li *et al.*, 2008). However, the incidence of obesity is low, suggesting there may be a beneficial effect of n-3 PUFA enriched fish oil in preventing and alleviating obesity. Engler *et al.* (2000) found that in spontaneously hypertensive rats that a DHA enriched diet for 6 weeks decreased the Δ^9 desaturase in hepatic microsomes, and was accompanied by an increase in palmitic acid and decrease in palmitoleic acid.

As MUFA make up a large portion of cellular lipids the decrease in their biosynthesis via SCD inhibition may account for PUFA fat lowering effects.



a.



b.

Figure 16. Comparison of (a) C 16:0 and C16:1 (b) C18:0 and C18:1 levels in liver under CS, HCHF, CEPA and HEPA feeding conditions.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

a.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

b.

c.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

d.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

e.

fatty acid content
(g/100g)
CS
ICHF
CEPA
HEPA

f.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

g.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

h.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

i.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

j.

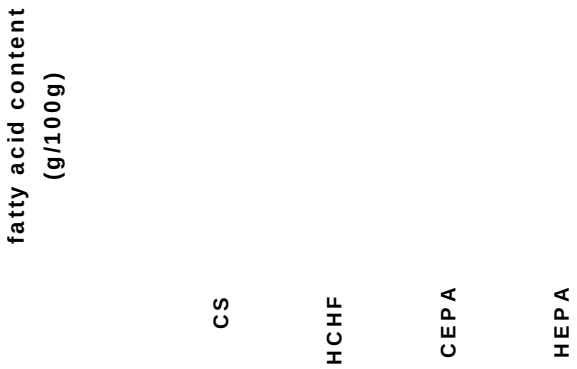
fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

k.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

l.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA



m.

Figure 17. Fatty acid content of liver under CS, HCHF, CEPA and HEPA feeding conditions. (a) C16:0 (b) C16:1 (c) C18:0 (d) C18:1 (e) C18:2 (f) C20:3 (g) C20:4 (h) C20:5 (i) C22:6 (j) Total SFA (k) Total UFA (l) total MUFA (m) Total PUFA

4.3.1.2 Adipose Samples

Dietary fatty acids are an important source of adipose tissue fatty acids and not just the amount but also the composition plays a significant role in adipose tissue metabolism. (Fernández-Quintela *et al.*, 2007). Adipose tissue is seen as a reliable biomarker for long term dietary intake due to its ability to store fatty acids for up to 2 years (Iggman *et al.*, 2010), especially for the essential fatty acids: linoleic and α-linolenic acids, as well as EPA and DHA. However, it is still unclear whether it is an appropriate biomarker to indicate the tendency towards obesity-related diseases.

Although experiments yielded no significant differences ($P>0.05$), there were a number of trends emerging, that correlate with previous research (Poudyal *et al.*, 2012b). Palmitic (C16:0) and oleic (C18:1n-3) acids had the highest levels with slight

increases between the CS to CEPA groups and decreases from HCHF to HEPA groups (Figure 18), as with the liver samples this could possibly be due to EPA inhibiting the enzymatic activity of SCD-1. Stearic acid (C18:0) decreased in the CEPA group compared to the CS group, while there was an increase in the HEPA group compared to the HCHF group (Figure 18).

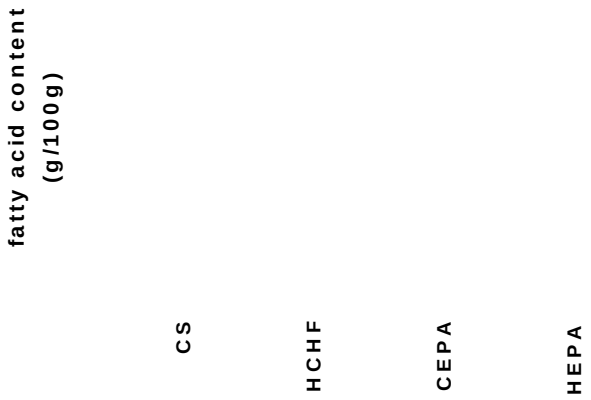
An increased amount of EPA was found in CEPA compared to the dietary CS group, as expected with dietary supplementation. A minimal amount appears to have been converted to DHA in the CEPA supplementation group (Figure 18). A larger difference was found between EPA and DHA in the HEPA supplementation group, suggesting that increased conversion occurred (Figure 18). As there is no α-linolenic acid present in any of the groups examined, it is assumed that the EPA and DHA comes from the diet. There is also the assumption that the linoleic acid that appears is the key precursor to the arachidonic acid found in all groups (Figure 18), which may lead to preferential metabolism of pro-inflammatory eicosanoids.

Previous research (Poudyal *et al.*, 2012b) using rats on the same diet, indicated α-linolenic acid was present, this is contradictory to current results, possibly due to differences in the GC-MS columns used. Changes in the methodology are warranted to specifically quantify α-linolenic acid particularly the use of a fatty acid specific column such as the Agilent DB-23.

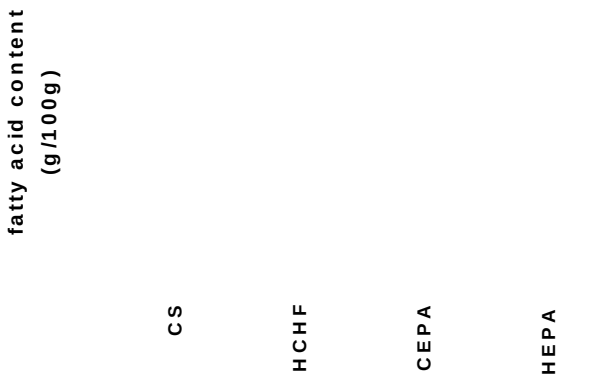
Total SFA marginally decreased between CS and CEPA, and increased between HCHF and HEPA (Figure 18). A corresponding change in unsaturated fatty acid composition appeared with slight increases between CS and CEPA and decreases between HCHF and HEPA (Figure 18). MUFA increased in CEPA and decreased in HEPA, while PUFA decreased in both supplementation groups. Overall UFA, and MUFA, was higher in the HCHF and HEPA groups while PUFA and SFA were higher

in the CS and CEPA groups (Figure 18). Studies have found that fish oil enriched diets prevent abdominal fat accumulation compared to other dietary oils, suggesting that n-3 PUFA supplements play an important role in preventing weight gain and improving weight loss (Micallef *et al.*, 2009).

a.



b.



fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

c.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

d.

e.

fatty acid content (g/100g)

CS

HCHF

CEPA

HEPA

f.

fatty acid content (g/100g)

CS

HCHF

CEPA

HEPA

g.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

h.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

i.

fatty acid content (g/100g)

CS

HCHF

CEPA

HEPA

j.

fatty acid content (g/100g)

CS

HCHF

CEPA

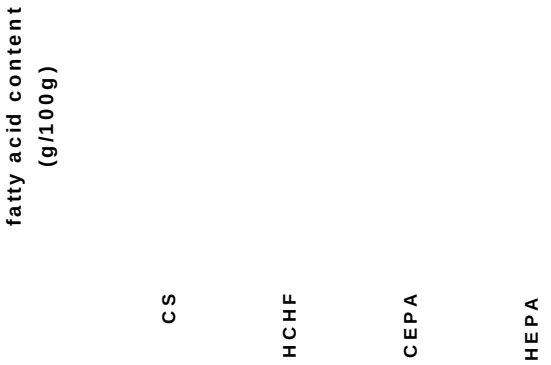
HEPA

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

k.

fatty acid content
(g/100g)
CS
HCHF
CEPA
HEPA

l.



m.

Figure 18. Fatty acid content of adipose tissue under various feeding conditions. (a) C16:0 (b) C16:1 (c) C18:0 (d) C18:1 (e) C18:2 (f) C20:0 (g) C20:4 (h) C20:5 (i) C22:6 (j) Total SFA (k) Total unsaturated fatty acid (l) Total MUFA (m) Total PUFA

Studies show that EPA and DHA ameliorate obesity-induced inflammation (Poudyal *et al.*, 2012b, Schmitz and Ecker, 2008) and with increased levels of EPA and DHA in the liver samples between the diet (CS) and supplementation (CEPA) groups, and decreased levels between the HCHF and HEPA groups in the adipose tissue there is an indication that it is being utilized by the body to reduce the obesity-related inflammation.

Results from the liver and adipose tissue samples indicate that very little EPA is being deposited in the liver while more was found in the adipose tissue, this may indicate that it is being desaturated and elongated into DHA or it could also be metabolized into energy.

There is the possibility that supplementation of the diet with EPA is inhibiting the enzymatic activity of SCD-1 to elongate and desaturate linoleic acid to arachidonic acid. This could provide some evidence supporting the beneficial effects of DHA and EPA on improving obesity.

4.4 Limitations

A number of limitations exist with this research project. Firstly, the sample size was limited to n=3 per oil and tissue type, for more conclusive results a larger sample size is necessary. Secondly, the GC column that was used was not specifically made for fatty acid analysis, therefore issues arose with separation of fatty acids *e.g.* C18 series *cis*- and *trans*- isomers of C18:1 eluted together. A more suitable column to use would be the DB23 High-polarity column produced by Agilent, as it is specifically designed for the analysis of fatty acid methyl esters (FAMEs) and provides excellent resolution for *cis*- and *trans*- isomers with very little overlap of FAMEs of the same chain length (Agilent, 2013).

4.5 Future Directions

There are a number of different directions that future research could take including the following:

- Further research could be undertaken using individual oils, particularly avocado, macadamia and canola to determine their capacity to affect obesity as these have yet to be fully explored. This would complement previous studies done on the effects of chia seed oil (Poudyal *et al.*, 2012a), EPA and

DHA (Poudyal *et al.*, 2012b) where the focus of the research was on cardiovascular health.

- Further research needs to be undertaken in this area to determine which fatty acids could be utilized as biomarkers to suggest that a person is at risk of developing serious diseases as a consequence of being obese. However, to do this it would possibly be more beneficial to use plasma samples, particularly in human trials as they are more readily accessible. If plasma samples were to be used then further studies using the rat model of obesity may be necessary first to ensure that the most appropriate biomarkers are studied. Arachidonic acid could possibly be used as a biomarker in liver and adipose samples although this would be more difficult to achieve in humans.
- The pro-inflammatory eicosanoids, for which arachidonic acid is a precursor could possibly be used as a biomarker for obesity related diseases. It is also possible that the anti-inflammatory eicosanoids could be investigated, focusing on EPA as the key intermediate. Research into the two essential fatty acids and their relationship to obesity could possibly be an area of interest with an increase of linoleic acid tending towards pro-inflammatory eicosanoids or increases in α-linolenic acid towards the anti-inflammatory eicosanoids. Investigations of the n-6:n-3 ratio - LA:ALA and also AA:EPA
- Future studies could also continue to investigate the fatty acid profiles of other tissues, such as plasma, whole blood, and heart, as well as investigating faecal and urine samples to gain an understanding of the entire ‘journey’ of fatty acid from food through metabolism to excretion. This could be extended to describe how the profiles differ for each fatty acid at each stage of the journey.

CHAPTER 5

CONCLUSION

Results achieved by this project are consistent with previous plasma and cardiovascular fatty acid composition studies (Poudyal *et al.*, 2012b). This research goes someway to filling the gap by utilizing liver and adipose (retroperitoneal fat) tissue to elucidate the fatty acid composition further by using rats on the same diet and supplementation of EPA. Previous studies have indicated that EPA and DHA ameliorate obesity-related inflammation (Poudyal *et al.*, 2012b, Schmitz and Ecker, 2008). Current results indicate that EPA levels increased in liver samples between the diet (CS and HCHF) groups and the supplementation (CEPA and HEPA) groups, while having a corresponding decrease in adipose tissue, possibly indicating that the EPA is being utilized by the body to reduce obesity related inflammation. There is the possibility that by supplementing the diet with EPA it is inhibiting the enzymatic activity of SCD-1 to elongate and desaturate linoleic acid to arachidonic acid, as indicated by decreased levels of arachidonic acid in the CEPA groups of both the liver and adipose tissue samples, providing some evidence supporting the beneficial effects of DHA and EPA on improving obesity.

In conclusion, results appear to confirm that while the quantity of fat that is consumed leads to obesity it is more likely that it is the quality of fat that is the final determining factor in whether obesity-related diseases become apparent.

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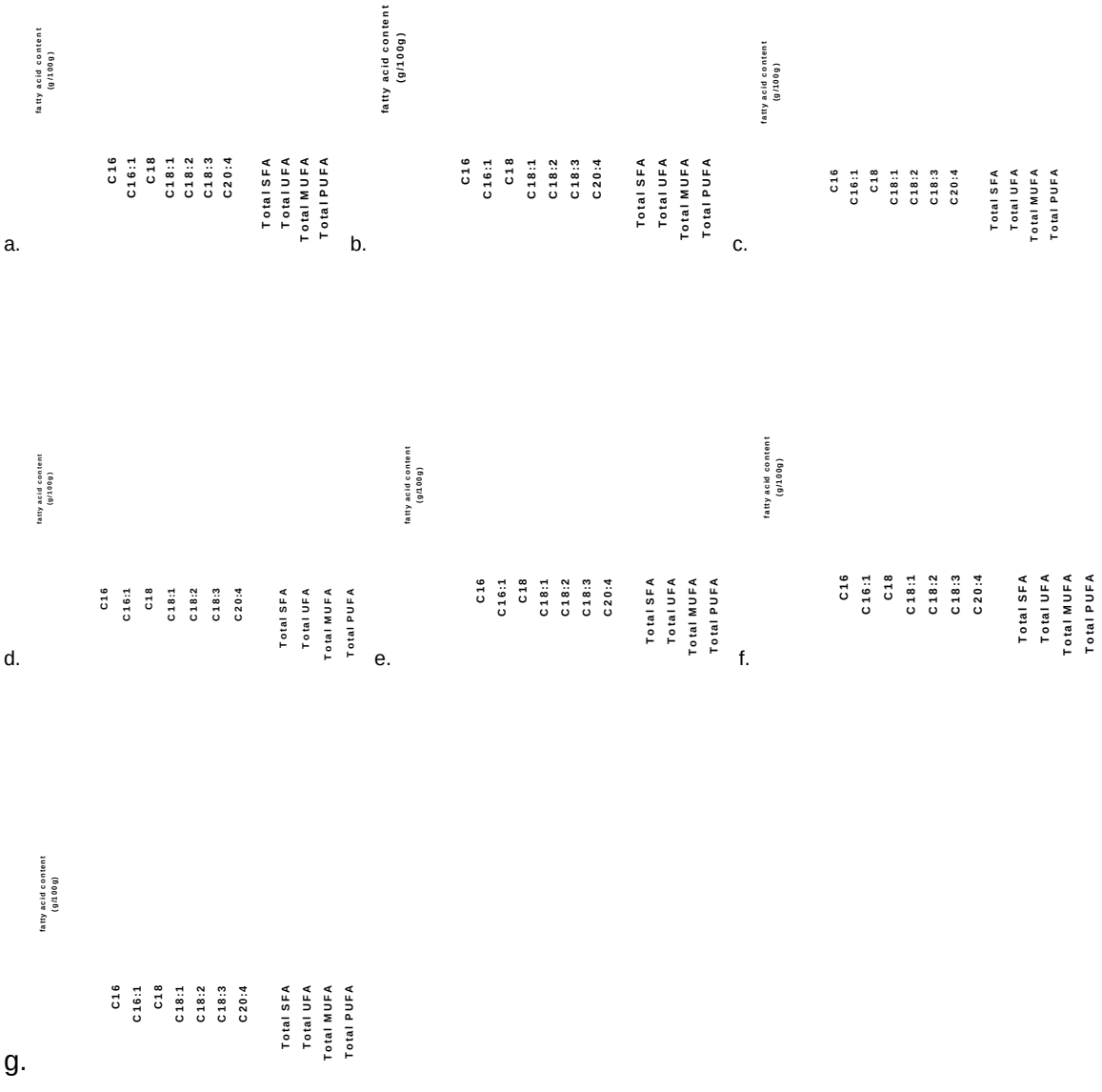
APPENDIX ONE

Graphs of Fatty Acid Content

Plant Oils



a. b. c. d. e. f. g.

Fatty acid composition of a variety of plant oils (a) chia (b) safflower (c) flax (d) olive (e) macadamia (f) avocado (g) canola

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

a.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

b.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

c.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

d.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

e.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

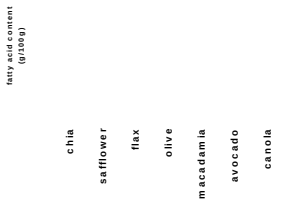
f.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

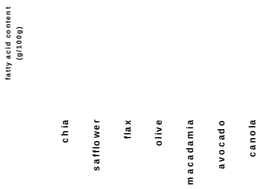
g.

fatty acid content (g/100g)
chia
safflower
flax
olive
macadamia
avocado
canola

h.



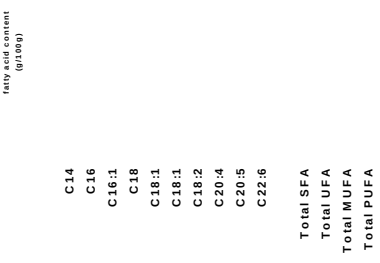
i.



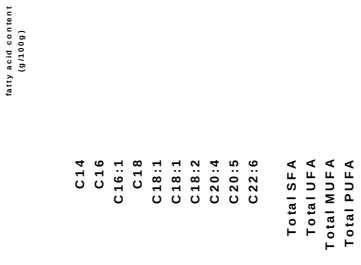
j.

Individual fatty acid content of a variety of plant oils (a) C16:0 (b) C16:1 (c) C18:0 (d) C18:1 (e) C18:2 (f) C18:3 (g) C20:4 (h) total SFA (i) total MUFA (j) Total PUFA

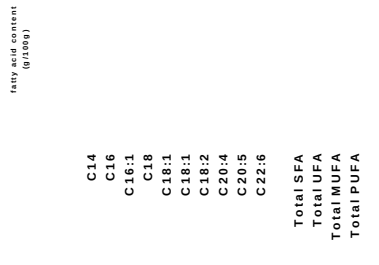
Animal Oils



a.



b.

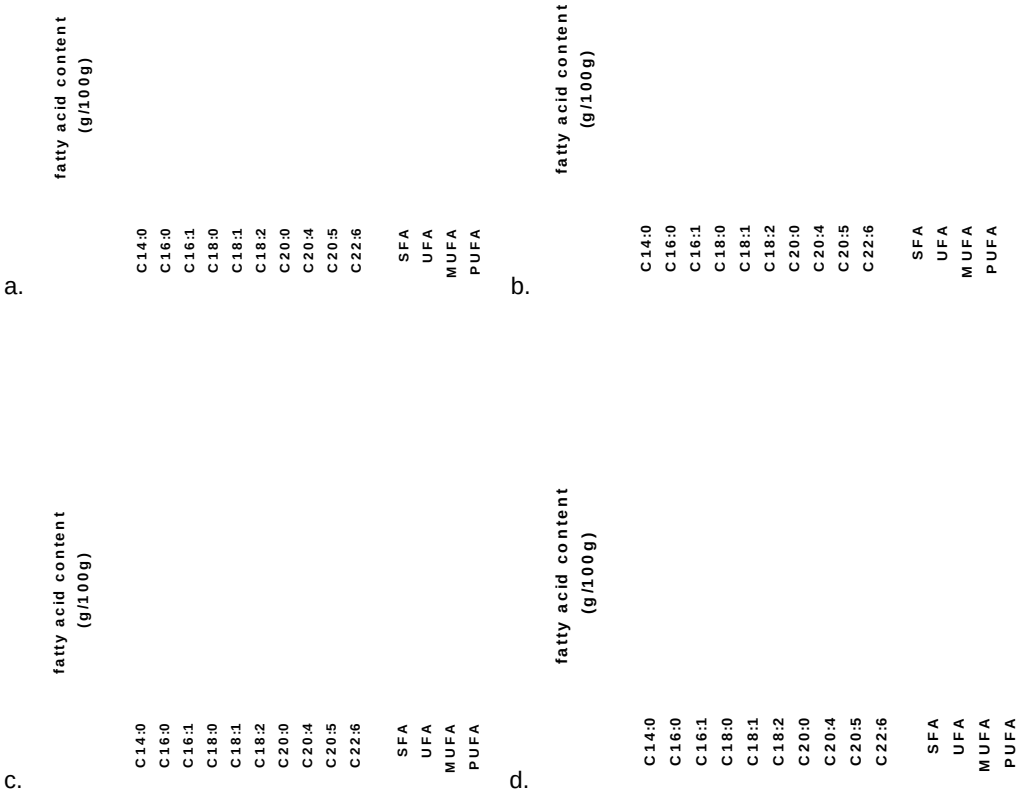


c.

Fatty acid composition of a variety of animal oils (a) EPA (b) DHA (c) Beef tallow

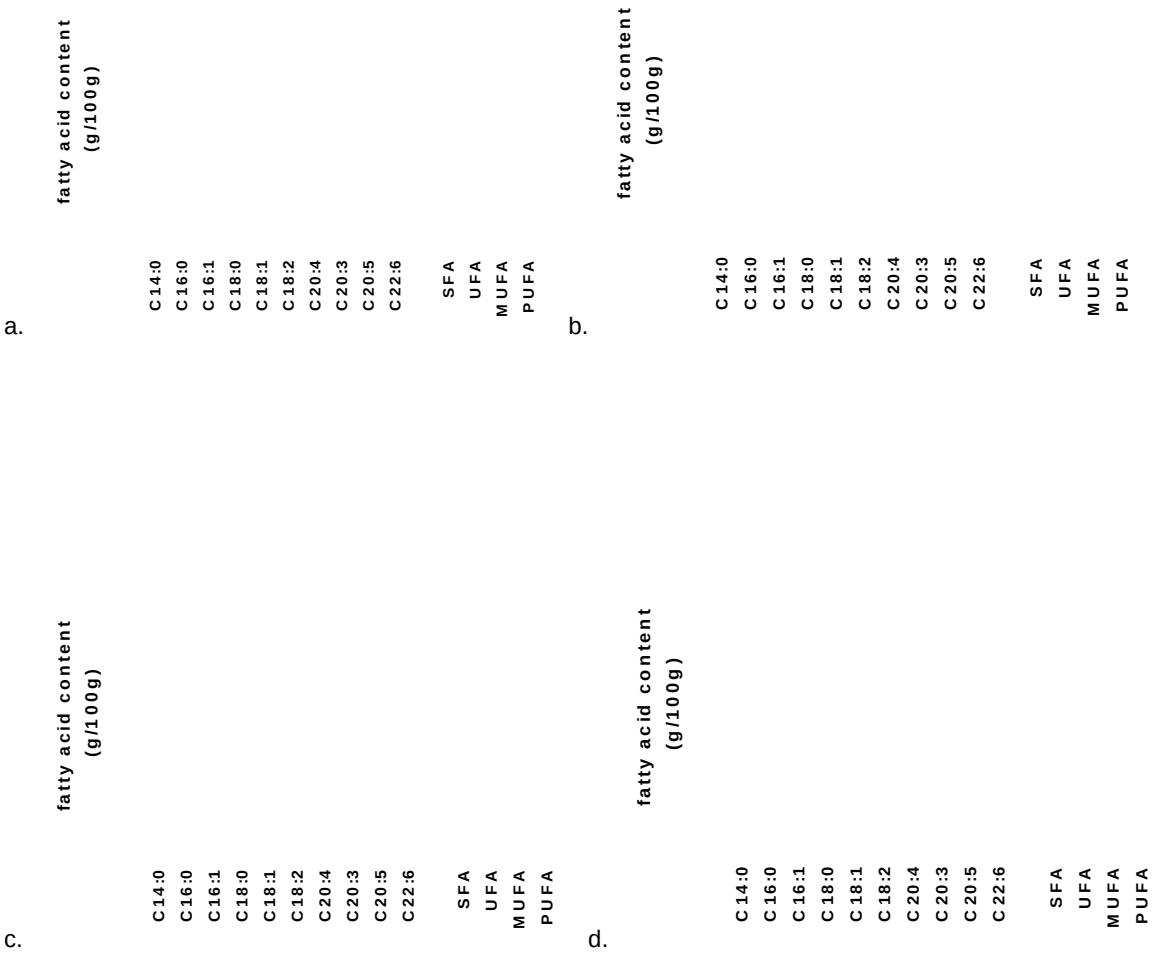
Individual fatty acid content of a variety of animal oils (a) C16:0 (b) C18:0 (c) C18:1 (d) C18:2 (e) C20:4 (f) C20:5 (g) C22:6 (h) total SFA (i) total UFA (j) Total MUFA (k) Total PUFA

Adipose Tissue



Fatty acid composition of Adipose Tissue in a variety of dietary conditions (a) CS (b) HCHF (c) CEPA (d) HEPA

Liver Tissue



Fatty acid composition of Liver Tissue in a variety of dietary conditions (a) CS (b) HCHF (c) CEPA (d) HEPA