DOCTOR OF PHILOSOPHY

Analysis of triglycerides using quantitative NMR techniques

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Analysis of Triglycerides using quantitative NMR techniques

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By

Rebecca Dean

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For Mum and Dad

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Abstract

Triglycerides are an important part of the human diet; they are also used as medical supplements to control the symptoms of arthritis and other medical conditions.

Analysis of triglycerides is important to check purity, and confirmation of the structure is important for authentication of high value products such as olive oil. Currently, this is carried out using enzyme reactions and the fatty acids on each position of the triglyceride are analysed separately.

Development of methods for the direct analysis of triglycerides using ^{1}H and ^{13}C NMR has been carried out as part of this research. Using ^{1}HNMR, a quick, quantitative overview of the species of fatty acids present in a triglyceride can be achieved. A method of quantitative ^{13}C NMR was developed using $Cr(acac)_3$ as a relaxation agent. This method gave rise to the identification of characteristic signals for each individual fatty acid present in triglycerides on both the alpha- and beta-positions allowing quantitation of individual components. The error present in these NMR method is high in comparison with that from chromatographic methods, but these NMR methods are designed as a relatively quick method to look at triglycerides semi-quantitatively, not for in-depth, highly quantitative analysis.

These quantitative NMR methods were applied to investigate the triglyceride composition of olive oils and to study the seasonal changes in triglyceride composition of cows' milk. The triglyceride composition of regional cheeses was also studied, and a method to analyse the lipid composition of lamb muscle and fat was developed.

Contents

1.0 Introduction

1.1 General Introduction

"Fat is a soft greasy substance occurring in organic tissue consisting of a mixture of lipids (mostly triglycerides)."

Fat is everywhere, in the food we eat, and forms many of the products we use everyday. For this reason, it is important to understand more about fat, the places it is found, its structure and the different means available to determine this structure.

Research has been carried out investigating the structure of fats, triglycerides, phospholipids, fatty acid methyl esters and butyl esters using different techniques. Throughout this work, lipids from several sources have been analysed; vegetable and olive oils, dairy products including milk, cheese, butter and yoghurt and lamb meat and fatty tissues.

Over the course of this introduction, the different types of fat found in dietary sources will be discussed. The manipulation of animal fats will be discussed with respect to the meat and milk from ruminant animals; olive and vegetable oils will be examined, and the health implications of lipid consumption explained with respect to dietary intake and the use of lipids as nutraceutical supplements. The analytical methods used throughout the course of this research will be introduced, and others which are used within the field of lipid research will be covered briefly.

1.2 Lipid Definitions

The nature of lipids is not easily defined. There are many definitions which are widely accepted, but differ depending on the context of their use. Two widely accepted definitions are:

"A heterogeneous group of substances, associated with living systems which have the common property of insolubility in water but solubility in non-polar solvents such as the hydrocarbon paraffins."

(Coultate, 2002)

"Lipids are compounds based on fatty acids or closely related compounds such as the corresponding alcohols and sphignosine base."

(Gunstone, 1992)

The definition that will be used throughout this work covers all these aspects and includes the relevant compounds:

"Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds."

(Christie, 2003)

This definition of lipids covers a wide range of substances, found naturally in a large variety of forms. The most abundant type of lipids is triacylglycerols (triglycerides, TAGs), but there are other classes such as free fatty acids (FFAs), simple lipids, phospholipids and glycolipids.

1.3 The structures of oils and fats

Dietary fat mainly comprises of fatty acids esterified to a glycerol backbone. These fatty acids can be broken down into two main groups – saturated and unsaturated. Within these two groups, there are several sub-classifications.

Saturated fatty acids contain no double bonds, and have been found in nature containing between 2 and 32 carbons. Usually, they contain an even number of carbons and are most commonly of medium chain length, typically 14, 16 or 18 carbons.

Unsaturated fatty acids contain double bonds; they can either be mono-enoic (containing one double bond) or poly-enoic (more than one double bond). The double bonds are usually of the *cis* configuration, and in poly-unsaturates, the double bonds are most commonly methylene interrupted (separated by a methylene group, CH_2).

Another member of the unsaturated family are *trans*-fats. They have usually one double bond of the *trans*- configuration. *Trans*-fats are found naturally in animal fats – particularly those from ruminant animals. Conjugated fatty acids contain *cis*- and *trans*- double bonds in a conjugated form. Conjugated fatty acids, particularly conjugated linoleic acid (CLA) are of interest because of their health properties. CLA contains one *cis*- and one *trans*- double bond.

Fatty acids with other functional groups can also be found. Castor oil contains a large amount of ricinoleic acid, which contains a hydroxyl group, lychees and longans contain dihydrosterculic acid and other fatty acids with cyclopropane moieties.

1.3.1 Lipid Classes

Triglycerides are a member of the class of glycerolipids. Glycerolipids consist of fatty acid residues bound to a glycerol molecule via ester linkages. Monoacylglycerols contain one fatty acid residue; diacylglycerols and triacylglycerols contain two and three residues respectively. Triglycerides are usually represented by a Fisher-type projection shown in Figure 1. Triglycerides are named using a numbering system based on the stereochemistry of the glycerol backbone. Positions *sn*-1 and *sn*-3 (the α-position) are stereo-isomers, and position *sn*-2 (β-position) is structurally different.

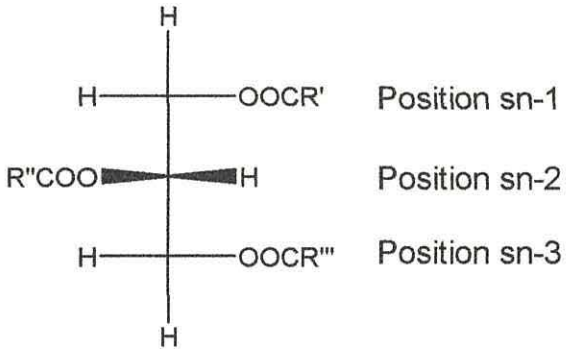


Figure 1: Stereo-specific numbering of triglycerides.

1.3.2 Other Fatty Acids

There are some fatty acids in food that are slightly less common, and arguably more interesting from a scientific standpoint. Conjugated linoleic acid refers to a family of conjugated isomers of octadecadieonoic acid (linoleic acid), which are found in some plant

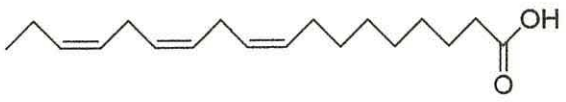
oils, in meats from ruminants such as cows, sheep and goats and dairy products. Lactobacillic acid and dihydrosterculic acid are cyclopropane fatty acids. They are found in low concentrations in dairy products (lactobacillic acid) and lychees (dihydrosterculic acid). The structure of lactobacillic acid (compound 1) is show in Figure 2.

Figure 2: Lactobacillic acid

Poly-unsaturated fats

Two groups of fatty acids of particular interest for health reasons are omega-3 (ω-3) and omega-6 (ω-6) fatty acids. These are families of polyunsaturated fatty acids with two or more skip-conjugated double bonds (bonds separated with a methylene group).

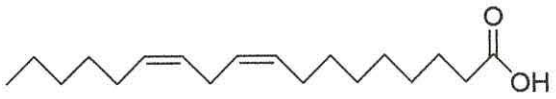
The ω-3 fatty acids are derived from linoleic acid. All ω-3 fatty acids are formed by chain elongation and desaturation of linoleic acid. Eicosapenaenoic acid (EPA) and docosahexaenoic acid (DHA) are ω-3 fatty acids. All members of the ω-3 family are essential fatty acids, meaning they cannot be synthesised in the human body but have to be taken in through dietary intake (wikepedia.org). The simplest ω-3 fatty acid is α-linolenic acid (compound 2), the structure of which is shown in Figure 3.



(2)

Figure 3: α-linolenic acid an ω-3 fatty acid .

Omega-6 fatty acids are also essential fatty acids. They are derived from linoleic acid. The structure of linoleic acid (compound 3), is shown in Figure 4.

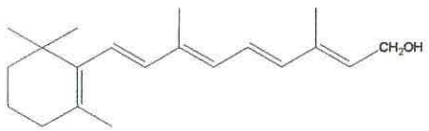


(3)

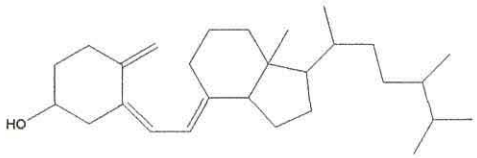
Figure 4: linoleic acid, an ω-6 fatty acid.

1.3.3 Simple Lipids

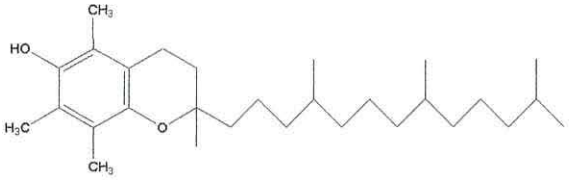
'Simple lipids' are not bound to glycerol backbones; however they are more complex in structure than free fatty acids (FFAs). Sterols such as cholesterol, leukotrienes and fat-soluble vitamins are all members of the simple lipid family. Vitamins A, D, E and K (retinol, choelcalciferol, tocopherol and menaquinone respectively) are fat soluble, unlike other vitamins which are water soluble. The structures of these simple lipids are shown in Figure 5.



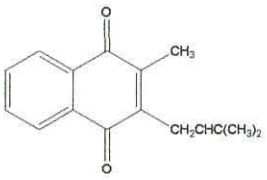
Retinol - Vitamin A (4)



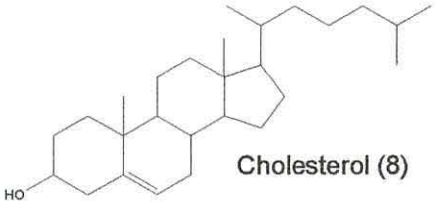
Cholecalciferol - Vitamin D (5)



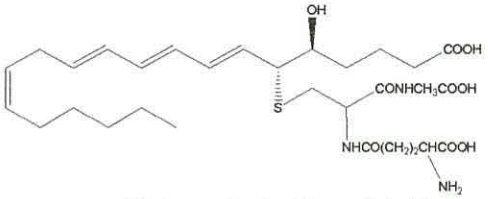
Tocopherol - Vitamin E (6)



Menaquinine - Vitamin K (7)



Cholesterol (8)



11-*trans*-leukotriene C4 (9)

Figure 5: Structures of the fat-soluble vitamins A (4), D (5), E (6) and K (7), cholesterol (8) and a leukotriene (9).

1.3.4 Phospholipids

Phospholipids consist of a glycerol backbone with fatty acids on the *sn*-1 and *sn*-2 positions. The *sn*-3 position of the glycerol is occupied by a phosphorus-containing group. Examples of common phospho-groups are: phophotidic acid; phosphotidylglycerol; diphosphotidylglycerol and phosphotidylcholine. A phospholipid is show in Figure 6.

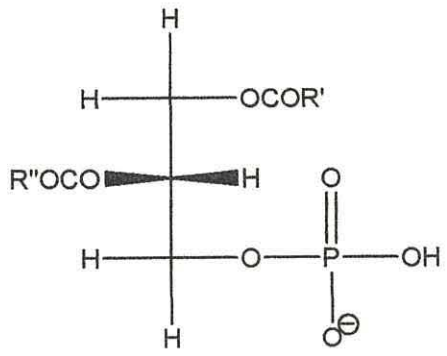


Figure 6: A phospholipid with phosphatidic acid as the phospho-group.

1.3.5 Glycolipids

Glycolipids have the general structure of a 1,2,-diacylglyceride with a glycosidic linkage to a carbohydrate group on the *sn*-3 position. The structure of a glypolipid is shown in Figure 7.

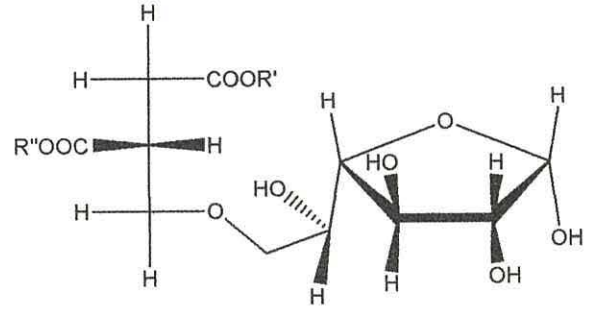


Figure 7: A glycolipid with galactose as the carbohydrate group.

1.4 The importance of lipids, oils and fats

1.4.1 Commercial importance

Natural oils and fats are used in three main industries, the food industry (80%), farming and agriculture (6%) and the oleochemical industry (14%) (Gunstone, nd).

The source of fats can be broken into two categories - those derived from animals and those derived from plants. Plant fats make up 78% of fat production worldwide.

Animal fats such as butter, lard, and tallow are usually higher in saturated fats and so are solid at room temperature. Fish oils however, are high in polyunsaturated fats and are liquid at room temperature. Vegetable oils are usually liquid at room temperature, and come from three sources; annual crops, tree crops and by-products.

Annual crops, such as rape, sunflower, groundnut and linseed are sown and harvested annually so as the demand for them changes, more or less can be planted each year. Tree crops such as oil palm, coconut and olive are slow-growing in comparison with annual crops, and the supply is not controlled by demand as it takes time for new trees to be planted and mature. Soya-bean oil, cotton oil, grapeseed oil and corn oil are created as by-products; these crops are grown and harvested for products other than oils, and their production is not driven by demand for the oil.

1.4.2 Health Importance - Diet, disease and obesity

The human diet is made up of five main components: carbohydrates; proteins; fats; water and vitamins/minerals. Each of these groups has specific functions within the diet, and each is essential to maintain a healthy body. Fat is an important part of our diet, and eating the right amount of fat is almost as important as eating the right type of fat. Eating too much fat can lead to obesity and other health problems.

According to the British Heart Foundation, in the UK, 22% of adults are clinically obese and 22% of boys and 28% of girls are overweight. This data is compounded by the fact that 88% of men and 83% of women consume too much saturated fat as part of their diet (bhf.org).

Obesity has detrimental effects on health, leading to extra pressure being placed on the heart, causing high blood pressure and strain on all major organs and joints. Too much saturated fat and cholesterol in a diet leads to atherosclerosis (the build up of plaque in coronary arteries), which in turn can cause coronary heart disease. That said, it is important to remember that fats are an important and essential part of the human diet, and it is necessary to consume them as part of a healthy diet.

1.4.3 Conjugated Linoleic Acid

In the human diet, several isomers of CLA are present; the *cis*-9, *trans*-11 isomer is present in the highest concentration, followed by *cis*-7, *trans*-9 isomer, *cis*-11, *trans*-13 isomer, *cis*-8, *trans*-10 isomer and the *trans*-10, *cis*-12 isomer, and the structures of two isomers are shown in Figure 8.

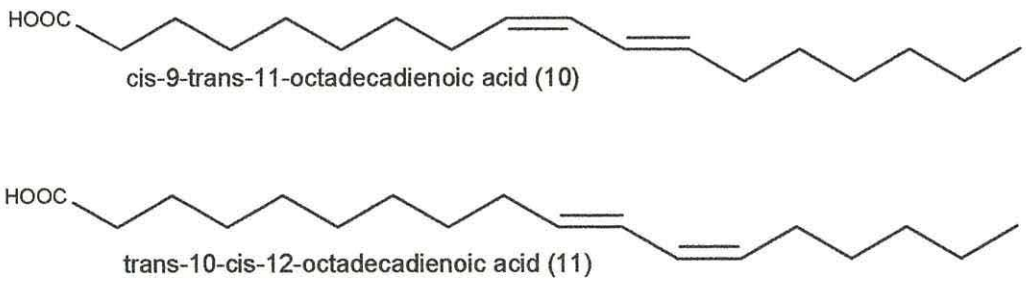


Figure 8: The structures of *cis*-9, *trans*-11 CLA (12) and *trans*-10, *cis*-12 CLA (13).

CLA comes from natural and synthetic sources in the diet; it is found in dairy products and some meats, but it is also formed during the partial hydrogenation of fats, for example in margarine production. Conjugated linoleic acid has been of growing interesting due to its biological properties, particularly its anti-carcinogenic properties and its potential to act as an anti-artherogenic.

The isomers of CLA, *cis*-9, *trans*-11 (*c9, t11*) and *trans*-10, *cis*-12 (*t10, c12*) are thought to have very different effects in the human body. *Cis*-9, *trans*-11 CLA has been reported to exhibit beneficial effects with respect to cancer and cardiovascular disease. In animal studies it has been shown to reduce mammary tumours and inhibit the growth of new tumours. Inhibitory effects on cancers of the bowel, stomach and lung have also been reported, as well as the hindrance of the systematic spread of cancer.

However, *t10,c12* CLA has very different health effects to *c9,t11* CLA (Clement *et al*, 2002). A study by P. Besnard has shown that mice fed a diet of *t10,c12* CLA develop hyperinsulinemia (over production of insulin and resistance to its metabolic effects) and fatty liver disease, a condition where >10% of the mass of the liver is fatty tissue, caused by diabetes, obesity, alcoholism and other diseases. The *c9,t11* isomer did not have these negative effects on the mice, but exhibited the usual health benefits. Although *t10,c12* CLA has been shown to be responsible for fatty tissue mass loss in mice, the detrimental side-effects are severe, so the use of *t10,c12* CLA as a tool to aid weight loss is not recommended.

The *t10,c12* isomer has been shown to have effects on the milk production of cattle, pigs and humans. A study by Baumgard showed that by supplementing the diet of dairy cows with a single isomer of CLA (the *t10,c12* isomer), at very small concentrations, the milk fat production was radically changed (Baumgard *et al*, 2001). When cows were fed *t10,c12* at a concentration of 0.05% of their dry feed intake, milk fat yield was reduced by 44%. Feed intake, milk yield and milk protein content and yield were not affected by the *t10,c12* supplementation, but the milk fat content and fat yield were. At doses of *t10,c12* CLA 0.016% of dietary dry matter, the milk fat synthesis was reduced by 25 %, but the milk fat composition was not changed markedly. At concentrations >0.016%, the proportion of *de-novo* synthesised fatty acids (medium to long chain fatty acids) decreased disproportionately when compared to preformed fatty acids. It was also shown that *t10,c12* CLA reduced the activity of Δ^9-desaturase.

1.4.4 Fats as medicines and supplements

Fats and oils are not only consumed as part of our diet, they can also be used as medicines and dietary supplements.

A combined extract of fats from olive oil and rapeseed oil, known as Lorenzo's oil, has been shown to hinder the onset of adrenoleukodystrophy, (myelin.org; Suzuki *et al*, 2001) a disease in which the fatty sheath covering nerve fibres in the brain is degraded leading to neurological disability, degeneration of the adrenal gland and eventual death (ncbi.nlm.nihgov).

The beneficial effects of cod liver oil are well known and it has recently been proven that taking cod liver oil as a nutraceutical supplement can ease the symptoms and onset of osteoarthritis and rheumatoid arthritis (Curtis *et al*, 2004).

Several studies have been carried out on the benefits of evening primrose oil as a nutraceutical supplement. It has been shown to exhibit anti-psychotic effects in schizophrenic patients (Joy, 2000). Evening primrose oil was studied as a remedy for a variety of disorders by Kang, including menopausal hot flushes, pre-menstrual syndrome, breast cysts and dermatitis (Kang, 2002). The results of this study were not conclusive however, and larger clinical trials are needed. Evening primrose oil is routinely prescribed for the treatment of mastalgia (Blommers, 2002).

1.4.5 Cell membranes

All cells are surrounded by a cellular membrane, which is essential to separate the contents of the cell from the outside world (Thomas, 2000). In eukaryotic cells, membranes are also employed within the cell to isolate organelles. The cell membrane has many functions; the main one is to maintain the integrity of the cell, but membranes are highly important for regulating the transport of substances in and out of the cell. The exterior membrane is also involved in the generation and receipt of chemical and electrical signals, and is important for cell adhesion and tissue formation. Internal cell membranes have similar functions, but are often highly involved in organelle function. The structure of a cell membrane is described as a phospholipid bi-layer. This has a fluid like structure, and hence the name "fluid mosaic model". Membranes are not made entirely of phospholipids; they also contain proteins,

glycolipids, glycoproteins, cholesterol and steroids. A schematic of a phospholipids bi-layer is shown in Figure 9.

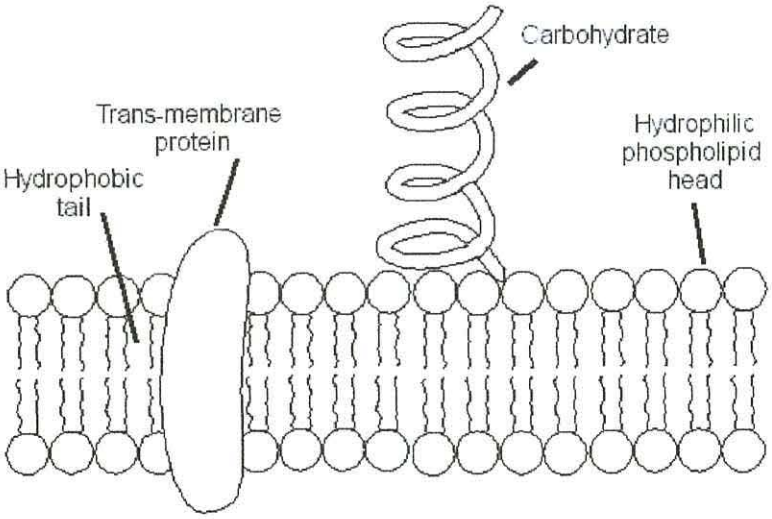


Figure 9: The structure of a phospholipid bi-layer (biochem.Arizona.edu).

1.5 Dietary Fats

The fats that make up the human diet come from three main sources, meat, dairy products and vegetable fats and oils. Dairy products and vegetable oils will be discussed in further detail below.

1.5.1 Dairy products

Dairy products are an integral part of the human diet. They supply calcium, vitamin D and important fats and proteins. Calcium and vitamin D are especially important for the formation of healthy teeth and bones and the prevention of rickets (Fraser, 2004).

Many studies have been carried out on the composition of dairy products, looking predominantly at the fat and protein content and how these are affected by diet and other factors including the breeding/lactation cycle of the animal, breed of the animal, feeding regimen as well as diet, age, season and the altitude at which it grazes.

As dairy products are not man-made, the composition of the milk can only be altered by understanding the processes that occur in the production of milk within the animal. Changes in the milk fat composition of monogastric animals are more pronounced with diet manipulation.

Cows milk contains 14 fatty acids which are present at greater than one percent, with trace fatty acids making up approximately 7 % of the total fat (w/w%) (Creamer, 1996). A study by Creamer showed the typical fatty acid content of milk fat from New Zealand cows. The data from this study is presented Table 1.

Fatty Acid		Content (w/w %)	Fatty Acid		Content (w/w %)
4:0	Butanoic	3.9	16:1	Hexadecenoic	1.5
6:0	Hexanoic	2.5	18:0	Octadecanoic	12.2
8:0	Octanoic	1.5	18:1	Octadecenoic	21.1
10:0	Decanoic	3.2	18:2	Octadecadienoic	1.4
12:0	Dodecanoic	3.6	18:2c	Conjugated Linoleic	1.1
14:0	Tetradecanoic	11.1	18:3	Octadecatrienoic	1.0
15:0	Pentadecanoic	1.2			
16:0	Hexadecanoic	27.9	Minor Acids		6.8

Table 1: Typical fatty acid content of New Zealand milk fat.

Milk fats by their very nature are complex, and the number of positional isomers on the glycerol framework which may be present is vast. Taking into account only the 14 fatty acids which are present at concentrations greater than 1 %, and the possibility that each triglyceride

may have multiple individual fatty acids present i.e. AAA, AAB or ABC, then there is the possibility of 2,744 different positional triglyceride isomers.

Altering the diet of dairy cows in attempts to produce milk containing a higher proportion of *n*-3 fatty acids has been successful. A study by Gulati showed that supplementing the diet of Friesian cows with oil mixes coated with a substance to prevent them from being absorbed in the rumen (protected seeds), changed the fatty acid composition of their milk (Gulati *et al*, 2002). Feeding cows a supplement of canola/soya bean oil (70:30, w/w) and soya bean oil/linseed oil (70:30, w/w) increased the proportion of octadecatrienoic acid (linolenic acid, C18:3) from 0.8% to 2.49% and 0.64% to 8.54% respectively. Feeding cows with a supplement of soya bean oil/tuna oil (70:30, w/w) increased the proportions of eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) from 0% to 0.86% and from 0% to 1.41% respectively. These changes were accompanied with a reduction of the saturated fatty acids myristic acid (tetradecanoic aicd, C14:0) and palmitic acid (hexadecanoic acid, C16:0) but no marked reduction in the proportion of stearic acid (octadecanoic acid, C18:0).

A study by Collomb showed changes in the composition of milk fat with dietary supplements of ground rapeseed, sunflower seed and linseed (Collomb *et al* 2004). The findings of the study are presented in Table 2.

They show that supplementing a cow's diet with high volumes of fat (greater than 1kg per day) leads to a reduction in milk production, and a reduction in the milk fat percentage. All the supplements in the study, rapeseed, sunflower seed and linseed increased the proportion of

oleic acid (C18:1 c9) and stearic acid and reduced the proportions of myristic and palmitic acid.

Fatty acids (g $100g^{-1}$)	Diet						
		Control	RAP1	SUN1	SUN1.4	LIN1	LIN1.4
	Milk yield (kg day^{-1})	30.26	30.95	29.59	27.66	29.88	28.45
	Fat (g $100g^{-1}$)	4.02	4.05	4.13	3.69	4.19	4.18
C4:0		3.1	3.1	3.0	3.3	3.1	3.3
C6:0		2.2	2.3	2.2	2.1	2.3	2.2
C8:0		1.4	1.4	1.4	1.2	1.5	1.3
C10:0		3.6	3.3	3.1	2.5	3.5	2.8
C12:0		4.5	3.8	3.6	2.8	4.0	3.0
C14:0		12.0	11.3	10.9	9.6	11.5	9.8
C15:0		1.4	1.2	1.2	0.9	1.2	0.9
C16:0		31.1	24.5	25.1	20.7	26.2	22.1
C18:0		5.4	9.1	8.8	9.8	8.7	10.7
C18:1 c9		11.3	16.6	15.9	17.9	14.0	17.1
C18:2 c9c12		1.8	1.6	2.2	2.6	1.5	1.6
C18:3 c9c12c15		0.7	0.7	0.7	0.7	1.2	1.6

Table 2: Yield and fatty acid composition of milk from cows fed on different diets. Control diet consits of hay ad libitum and 15 kg beet, the study groups were fed the control diet but were also fed supplements: RAP1 - ground rapeseed, 1kg; SUN1 – ground sunflower seed, 1kg; SUN1.4 – ground sunflower seed, 1.4kg; LIN1 – ground linseed, 1kg; and LIN1.4 – gound linseed, 1.4kg.

A study investigating the changes in milk fat composition with the altitude at which the animal grazes has been carried out by Collomb. As the altitude at which cows graze changes, the botanical composition and diversity on the slopes also changes. Lowland pastures consist mainly of grasses and legumes and highland pastures have a higher botanical diversity with more dicotyledonous plants and fewer grasses (Collomb *et al* 2002). Lowlands are defined as having an altitude of 600 – 650m, and highlands are 1275 – 2120m. Milk from cows grazing in highlands contains 1.49 g $100g^{-1}$ more CLA than milk from lowland grazing cows, 1.3 g $100g^{-1}$ of the total CLA is of the C18:2 *c9t*11 isomer. The total percentage of *trans* fatty acids from highland milk is higher than that of lowland milk by 3.98g $100g^{-1}$.

The diets of dairy cows have also been manipulated specifically to alter the amount of CLA present in the milk. This has been done by supplementing a rich pasture diet with crushed rapeseeds and soyabeans. The CLA content of milk also changes seasonally. In areas where cows are put to pasture in the summer, the CLA content of the milk increases from 0.24 to 2.81 % (Martin, 1998). A similar increase is noticed when cows are put out to pasture in more arid regions in the winter. Variation in CLA content is shown to be highest when cows are fed on dry food concentrate, then fed at pasture in areas where there is lush vegetation rich in polyunsaturated fatty acids (PUFAs).

1.6 Olive and Vegetable Oils

1.6.1 Olive Oils – Regulation and Adulteration

The edible oil industry is highly regulated – especially for high value products such as olive oil. The market for olive oil is increasing – especially in countries where the standard of living is increasing. Olive oil is popular due to its favourable organoleptic properties (taste and smell) and its proven health benefits (Jee, 2002). Olive oil is unique in vegetable oils as it has a low percentage of saturated fatty acids (~ 15 %), a high percentage of mono-unsaturated fats, mainly oleic acid (~ 70 %) and around 15 % poly-unsaturated fat, mainly linoleic acid (Shaw *et al*, 1997). Different classes of olive oils exist, the labelling of oils is of great importance and the legislation behind the production and labelling is restrictive. According to the European Community, virgin olive oil is defined by EC regulation EC 136/66 and is amended by EC 1638/98 and EC 1513/01.

"Virgin olive oils are obtained from the fruit of the oil tree by solely mechanical, or other physical means under conditions, particularly thermal, that do not lead to alterations in the oil, such oil not having undergone any treatment other than washing, decantation, centrifugation and filtration. Thus there is the total exclusion of oils obtained using solvents or re-esterification processes, and any mixture with oils of other kinds." (EC directives EC136/66; EC directive EC1513/01; EC directive EC1638/98)

The class olive oil falls into is determined by the concentrations and relative percentages of the minor components of the oil. Olive oil is 97 – 98 % triglyceride, the remaining proportion being made up of alkanes, squalene, wax esters, aliphatic alcohols and aldehydes, sterols, triterpenes, free fatty acids, vitamins, phospholipids, polyphenols and glycosides. It is these compounds which give the oils their organoleptic properties as the triglycerides themselves are tasteless.

Olive oils are divided up into nine classifications by European Community regulations – these categories are: Extra virgin olive oil, virgin olive oil, ordinary virgin olive oil, virgin lampante olive oil, refined olive oil, olive oil, crude olive-residue oil, refined olive-reside oil and olive reside oil. These categories of olive oil are defined by their extraction process and their acidity levels (expressed as grams of free oleic acid per 100 g of oil).

As a result of the market for olive oils being so lucrative, there is a need for regulation of the industry to prevent unscrupulous individuals from fraudulently marketing oil that is not categorised correctly or has been adulterated in some way. Olive oil is subject to a set of chemical and physical tests to determine its authenticity and class, and to detect any adulteration. There are 18 tests carried out under EC regulation EC 2568/91. These tests cover all aspects of the oil from chemical tests, chromatographic separation and identification of components, spectroscopic analysis of the oils to determine trace components,

organoleptic assessments and written proof of testing and refining (EC directive 2568/91). Research from other groups have shown that ^{13}C NMR of olive oils can distinguish between different cultivars and between olive oils from different geographical locations (Zamora *et al*, 2002, p216; Brescia *et al*, 2003).

1.7 Thermal degradation

As fats are heated, they react with the oxygen and water in the atmosphere and undergo chemical changes. During the heating of edible oils, aldehydes, ketones, alcohols, dienes and acids can be formed; these will change both the flavour and appearance of the oil, as well as causing possible health problems (Yen, 2003). Investigation into exactly which chemicals are formed during the cooking process is therefore important. Detecting and analysing these chemicals can be done in various ways.

Moreno *et al* have studied the formation of carbonyl compounds in edible oils by analysis using ^{1}H NMR and quantitative FTIR (Moya Morena *et al*. 1999). They detected an increase in products such as aldehydes, ketones, FFAs and hydroxyl compounds to levels which could be harmful to human health. This study was carried out on a selection of fats and oils, including olive oil, sunflower oil, corn oil, various seed oils and lard. It was found that carbonyl compounds began to form at above 80°C.

A study by Takeoka showed that when heating cooking oil in processes such as deep fat frying, not only do carbonyl compounds form, but the triglycerides can polymerise too (Takeoka *et al*, 1997). An increase in the total polar material in the thermally treated cooking

oils was detected by column chromatography, and a decrease in the iodine value was also reported; this is indicative of a decrease in the number of double bonds in the oil and is likely to result from oxidation and polymerisation. The rate of production of dimeric and polymeric triglycerides determined by size exclusion chromatography was discovered to be similar to the rate of production of polar compounds. Takeoka's study also stated that oils were not acceptable for frying once the high molecular weight fraction reached 20%.

1.8 Oil Analysis Methods

Analysis of lipids is a vast subject, and many techniques are used, including high performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography with mass spectrometry (GC-MS), ultra-violet spectroscopy, silver ion chromatography, thin layer chromatography (TLC), solid phase extraction (SPE) and gel permeation chromatography (GPE). A number of chemical tests are also routinely used in industry, such as calculation of the iodine value (a measure of the level of unsaturation of the sample) and calculation of the total polar material (by column chromatography). A comprehensive review of the analysis of triglycerides is presented by Ruiz-Gutiérrez *et al* (1995).

1.8.1 Gas Chromatography with Mass Spectrometry

Gas chromatography is probably the most widely used technique for lipid analysis; it can be especially useful in tandem with Mass Spectrometry (MS). GC is a highly versatile technique that separates compounds based on their volatility and their interactions with the stationary phase. The columns for GC are typically about 30m in length, but can be up to 100m long, and they have an internal diameter of approximately 0.2mm (Breitmaier, 2002).

There are two main controlling factors for the separation of components of mixtures; the stationary phase and the temperature program. There are two types of temperature program commonly used; isothermal and temperature gradient. Using the isothermal method, the dominant factor determining the elution order is the interactions of the chemicals with the stationary phase, but using a temperature gradient, the volatility of the compounds also affects the elution order.

Not only are there a wide variety of columns and temperature programs that may be used with GC, but there are also a large number of detectors. The most commonly used detector is a flame ionisation detector (FID), which is highly sensitive and can be used to detect almost all chemicals containing carbon atoms. Component identification with an FID is through comparisons of retention times with known standards. The detector used throughout this work was an MS detector, which fragments the eluants from the GC and reports a mass to charge ratio (*m/z*) for each peak; this can be used to determine the structure of the compounds from the mixture.

1.8.2 High Performance Liquid Chromatography

Although GC is the technique most frequently used in fatty acid analysis, HPLC has its place when handling unusual, heat sensitive or non-volatile compounds. HPLC can also be used as a micro-preparative technique (Christie, 1997). It is a highly versatile technique; different columns (stationary phases) and different solvents (mobile phase) can be used to separate fatty acids and triglycerides by polarity, length of hydrocarbon chain, equivalent carbon number (takes into account the length of the fatty acid chains and the number of unsaturated

centres in the fatty acid or triglyceride), degree of unsaturation or to separate *cis* and *trans* isomers.

Silica columns can be used to separate polar fatty acids, such as hydroperoxides and hydroxy fatty acids, and can also be used to separate positional isomers. Fatty acids with enantiomeric centres can be separated using chiral columns. Silver ion columns can be used to simplify complex mixtures, separate *cis* and *trans* isomers of fatty acids, and can also be used to separate some positional isomers of fatty acids. Reversed-phase columns are used most frequently in HPLC analysis of fatty acids, the stationary phase is made of a material called octadecylsilyl (ODS) and is commonly used with solvents such as acetonitrile or methanol.

1.8.3 Derivatisation and limitations

There are several techniques used for the analysis of lipids; most require a derivative, rather than the triglyceride itself. Although HPLC can be used to separate triglycerides, the method is complex and time consuming and the resolution and separation can be poor, especially with complex analytes such as milk fat where the number of components is vast. High temperature GC can also be used to separate triglycerides, but complications occur with baseline bleed at such high temperatures. Analytical techniques such as HPLC and GC are much more effective when used with derivatives of triglycerides.

As triglycerides are not volatile enough to be analysed by conventional GC (temperatures up to 210°C are usually employed), derivatives need to be made. A comprehensive review of derivatives and their preparation has been carried out by Christie (Christie, 1993).

The most common type of derivative used for GC analysis of lipids is fatty acid methyl esters (FAME) as they are easy to prepare by a variety of methods. The structure of a FAME is shown in Figure 10.

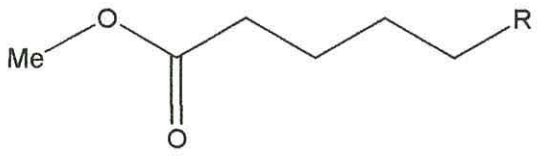


Figure 10: A methyl ester of a generic fatty acid.

Acid catalysed methods require a large excess of anhydrous methanol and an acidic catalyst (such as anhydrous hydrogen chloride) (Christie, 1990, p48). If water is present, it acts as an inhibitor, and prevents the reaction from going to completion. An alternative reagent is acetyl chloride dissolved in cooled dry methanol. For either of these reagents, the reaction mixture should either be heated under reflux for two hours, or heated to 50°C overnight in a stoppered tube.

Esterification can also be carried out using BF_3-MeOH (BTF) (Christie, 1994). This reagent has a limited shelf life and can cause the presence of 'artefacts' in the solution. BF_3-MeOH has been known to cleave cyclopropane rings in fatty acids and react with BHT (butylated hydroxy toluene, an antioixidant commonly added to lipid samples up to concentrations of 100ppm) in natural samples to produce spurious peaks in chromatograms (Christie, 1994). At high concentrations of BTF, approximately 50%, it has been known for methanol to add across the double bonds of unsaturated fatty acids.

Triglycerides are transesterified rapidly in anhydrous methanol with a basic catalyst such as sodium methoxide (Christie, 1990, p 79). If water is present in the reaction mixture, the lipids undergo hydrolysis, and are converted to FFA, which are not esterified under these conditions. This reaction is much more rapid than the acid catalysed method and can be performed at room temperature in minutes if toluene, or another non-polar solvent such as hexane is added to solubilise the triglycerides in the methanolic sodium methoxide solution.

The derivatisation of triglycerides has two drawbacks: the first is that during the process, trace components can be lost or a sample can be contaminated; the second is that when the residues are cleaved from the glycerol backbone, there is no way to know which position they occupied on the glycerol, so no information about the regio-chemistry can be determined. There are two ways this second problem can be overcome, the whole triglyceride could be analysed or the fatty acid residues can be cleaved depending on their position on the glycerol backbone; this can be done using pancreatic lipase.

1.8.4 Pancreatic lipase methods

Pancreatic lipase is an enzyme which only removes fatty acids from the *sn*-1 and *sn*-3 positions on a triglyceride. These free fatty acids can then be removed by column chromatography and esterified to produce fatty acid methyl esters for analysis by GC or GCMS. The remaining 2-monoglycerides can be transesterfied to form fatty acid methyl esters for analysis.

This pancreatic lipase method is a standard method as stated in European Community directives for assessing the distribution of fatty acid chains on a triglyceride (Official Journal of The European Community, L248, 1991). Olive oils that have had re-esterified fatty acids added to them or are made entirely of re-esterified triglycerides will have saturated fats in the *sn*-2 position because the re-esterification process is not regiospecific, unlike biosynthesis which will only place unsaturated fatty acids on the *sn*-2 position. Regio-specific analysis of triglycerides can detect this type of adulteration.

1.8.5 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy (NMR) is a technique widely used in organic chemistry for the structural identification of compounds. It is not *commonly* used to analyse complex mixtures such as natural lipid samples. NMR is not widely used in industry to analyse lipid samples because it is a relatively specialised technique with very high initial outlays to buy the equipment. Despite these disadvantages, NMR is a versatile technique, which has the benefits of being non-destructive, structure sensitive and flexible with respect to the nuclei being analysed. NMR does not suffer from common chromatographic problems such as retention time drift or blocked columns.

There are three nuclei that are of particular interest to scientists studying lipids, ^{1}H, ^{13}C and ^{31}P, and as NMR is sensitive to any spin active nucleus, all three of these nuclei can be studied using this technique.

^{1}H NMR is not regularly used to analyse complex mixtures such as those found in dairy products and other natural lipid samples as every single ^{1}H atom in the sample gives rise to a

resonance. A large proportion of these resonances overlap making spectra difficult to interpret to the same level as a single compound (Belloque and Ramos, 1990). The hydrogen atoms on the acyl chain have slightly different, but overlapping chemical shifts, giving rise to broad signals with large integrals. However, these one-dimensional spectra can present quantitative data on a sample as the area under each signal can be integrated and calibrated to provide information on the ratios of components within the mixture. ^{1}H NMR can give information on the types of fatty acids present e.g. saturated, mono-unsaturated etc, but cannot give specific details of which fatty acid is present e.g. oleic acid, stearic acid etc.

Analysis of ^{13}C nuclei gives different data to that of ^{1}H NMR. Each resonance in a ^{13}C NMR spectrum represents a different carbon environment. Data from ^{13}C spectra can give details on the specific fatty acids within the triglyceride (or other lipid sample) as each fatty acid has characteristic signals.

NMR can also be carried out to determine the presence of phospholipids within a sample. ^{31}P NMR will not identify the fatty acids present within a sample, but will show a single peak for each different phosphorus environment, meaning that the number of different phosphorus containing groups can be identified. Comparison of the chemical shifts of these signals can identify which types of phospholipids are present in a sample.

The naming of fatty acids

Fatty acids and their related compounds can be described in three ways, their systematic name, their generic name or by a short-hand label. All three of these naming systems will be used throughout this report. For ease of reference, the systematic and generic names of fatty

acids are presented below, along with their short-hand name, in order of the number of carbons and double bonds present.

Short hand	Generic Name	Systematic Name
C4:0	Butyric acid	Butanoic acid
C5:0		Pentanoic acid
C6:0	Caproic acid	Hexanoic acid
C7:0		Heptanoic acid
C8:0	Caprylic acid	Octanoic acid
C9:0		Nonanoic acid
C10:0	Capric acid	Decanoic acid
C10:1		Decenoic acid
C11:0		Undecanoic acid
C12:0	Lauric acid	Dodecanoic acid
C13:0		Tridecanoic acid
C14:0	Myristic acid	Tetradecanoic acid
C14:1	Myristoleic acid	Tetradecenoic acid
C15:0		Pentadecanoic acid
C16:0	Palmitic acid	Hexadecanoic acid
C16:1	Palmitoleic acid	Hexadecenoic acid
C17:0	Margaric acid	Heptadecanoic acid
C17:1		Heptadecenoic acid
C18:0	Stearic acid	Octadecanoic acid
C18:1 (9c)	Oleic acid	*Cis*-9, Octadecenoic acid
C18:1 (6c)	Petroselenic acid	*Cis*-6-Octadecenoic acid
C18:2 (9c, 12c)	Linoleic acid	*Cis*-9, *cis*-6, octadecadienoic acid
C18:2 (c9,11t)	Conjugated linoleic acid	*Cis*-9, *trans*-11, octadecadienoic acid
C18:2 (t10,c12)	Conjugated linoleic acid	*Trans*-10, *cis*-12, octadecadienoic acid
C18:3	α-Linolenic acid	9,12,15-octadecatrienoic acid
C18:3	γ-Linolenic acid	6,9,12-octadecatrienoic acid
C20:5 (c5,c8,c11,c14,c17)	EPA	*Cis*-5,8,11,14,17- eicosapentaenoic acid
C22:6 (c4,c,7,c10,c13,c16,c19)	DHA	*Cis*-4,7,10,13,16,19 - Docosahexaenoic acid

2 NMR Method Development

2.1 Introduction

NMR is a versatile technique, which also has the benefits of being non-destructive, structure sensitive and flexible with respect to the nuclei being analysed (Belloque, 1999). It is a technique widely used in organic chemistry for the structural identification of compounds, but not commonly used to analyse multi-component samples, such as natural lipids, due to their complex nature.

The analysis of triglycerides is usually carried out by HPLC involving long run times, broad peak shapes and poor separation. Quantitative identification of components is also limited (Gonzalez, 2001; Ruiz-Giitierrez, 1995; Stefanoudaki *et al*, 1997). Alternative analytical methods involve derivatisation of the triglycerides; either free fatty acids for analysis by HPLC, or fatty acid methyl esters for analysis by GCMS (Chistie, 1995). However, these widely used techniques are not regio-selective and information about the distribution of fatty acids on the triglyceride cannot be acquired from analysis of the derivatives. An HPLC chromatogram of extra virgin olive oil triglycerides is shown in Figure 11. It can be seen that several peaks represent more than one triglyceride, making this technique less than ideal for analysis of complex mixtures.

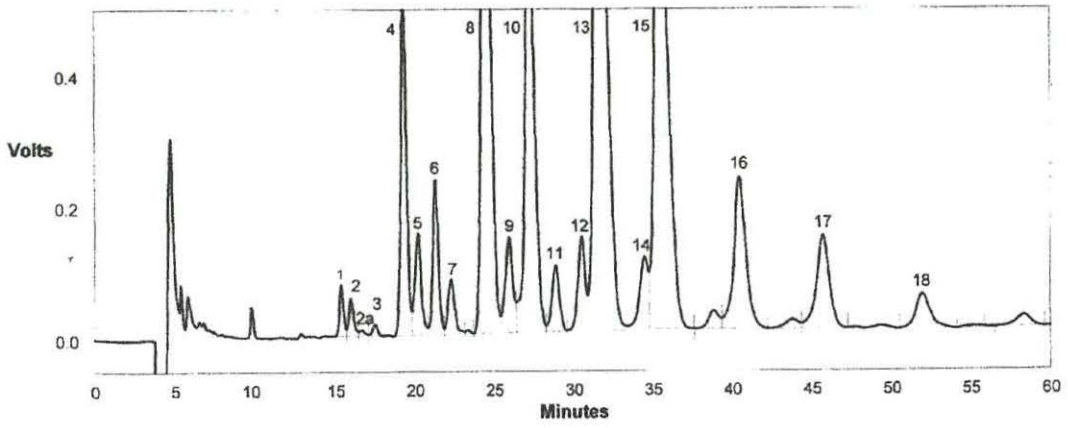


Figure 11: HPLC chromatogram of triglycerides from virgin olive oil.

Peaks represent : 1) LLL, 2) OLLn+PoLL, 3) PLLn, 4) OLL, 5) OOLn+PoOL, 6) PLL+PoPoO, 7) POLn+PPoPo+PPoL, 8) OOL+LnPP, 9) PoOO, 10) SLL+PLO, 11) PoOP+SPoL+ SOLn+SPoPo, 12) PLP, 13) OOO+PoPP, 14) SOL, 15) POO, 16) POP, 17) SOO, 18) POS+SLS. Where P = Palmitate, Po = Palmitoleate, L = linoleate, Ln = Linolenate, O = Oleate and S = Stearate. (www.cyberlipid.org)

Treatment of the triglyceride with pancreatic lipase enzymes cleaves the fatty acids from the *sn*-1 and *sn*-3 positions (α-position) on the glycerol, and leaves the residue on the *sn*-2 position (β-position) in place. Information can then be attained regarding the structures of the residues on the *sn*-1 and *sn*-3 positions and the 2-monoacylglycerol can be derivatised further to analyse the fatty acids on the *sn*-2 position. This method is used to authenticate olive oils and to detect adulteration in other high value natural oils (Official Journal of The European Community, L248, 1991).

Natural lipid samples contain a large number of fatty acids esterified to glycerol backbones. These samples give rise to complex ^{1}H NMR spectra due to the large number of overlapping resonances along the acyl chains. These broad, overlapping signals contain information on the sample an integration of these signals provides information on the ratios of components species within the mixture.

NMR analysis of ^{13}C nuclei gives different, complementary, data to that of ^{1}H NMR. Each resonance in a ^{13}C NMR spectrum represents a different carbon environment. Data from ^{13}C spectra can give details on the specific fatty acids within the triglyceride (or other lipid sample), as each fatty acid has characteristic signals. Detailed data on the chemical shifts of individual carbons within triglycerides has previously been published by Lie Ken Jie (Lie Ken Jie *et al*, 1995, p187; Lie Ken Jie *et al*, 1995, p 15; Lie Ken Jie, 1995, p1).

A standard, (power gated) ^{13}C spectrum can give detailed qualitative data, but the resonances cannot be integrated to attain quantitative data as they can in ^{1}H NMR due to long relaxation times and the Nuclear Overhauser Effect (NOE). To remove the complication of carbon resonances being split by adjacent hydrogen nuclei, ^{13}C NMR spectra are acquired using a technique known as broad-band decoupling. But broad-band decoupling also has the effect of increasing the NOE. NOE has the effect of increasing the height of the carbon resonances by different amounts depending on their environment. The number of protons coupled to a carbon atom directly alters the gyromagnetic ratio, which, in turn, changes the intensity of the carbon signal. This effect can cause signal intensities to be increased up to threefold (Breitmaier, 2002).

To reduce the NOE, the broad-band decoupling can be switched off while data is not being acquired (i.e. during the delay before the pulse). This is referred to as inverse gated ^{13}C NMR acquisition. The differences between inverse gated and power gated pulse sequences can be seen in Figure 12.

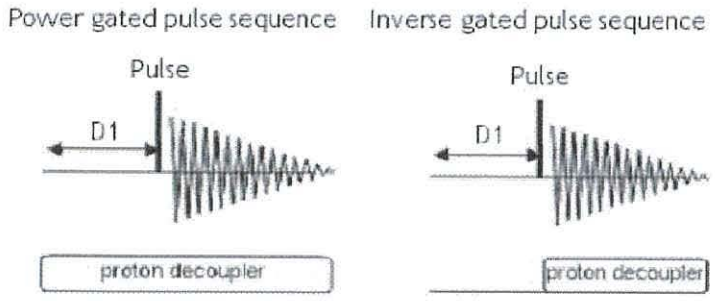


Figure 12: Pulse sequences for power gated and inverse gated ^{13}C NMR acquisition. D_1 is the delay between pulses. For power gated NMR, D_1 = 2 seconds.

2.2 Development of a quantitative method

Analysis of a sample using ^{13}C NMR gives useful information about the number of different carbon environments within that sample. This data is qualitative, unlike ^{1}H NMR, which can be integrated to give quantitative data. Quantitative ^{13}C NMR is possible if the acquisition parameters of the NMR are altered to allow for parameters such as spin lattice relaxation time (T_1) and NOE.

It is important to know the spin lattice relaxation time of the nuclei within a molecule if quantitative ^{13}C NMR is to be carried out, as the delay between pulses has to be long enough to allow all nuclei to relax between pulses. The delay in standard, power gated NMR is 2 seconds. The relaxation time can be measured by carrying out an inversion recovery experiment. This measures the spin lattice relaxation time of all the carbon nuclei in the molecule, and this data can then be used to calculate an appropriate delay between pulses. It is generally accepted that for quantitative ^{13}C NMR, the delay between pulses (D_1) must be

at least three times that of the T_1 of the slowest relaxing carbon nucleus. The formula relating these terms is shown in Equation 1 (Clarridge, 1999).

$$D_1 \geq 3 \times T_{1max}$$

Equation 1: Equation governing the length of delay (D_1) between pulses for quantitative NMR (T_{1max} - spin lattice relaxation time of the slowest relaxing carbon nucleus).

The spin lattice relaxation times of some nuclei in triglycerides and other lipid molecules are much longer than the D_1 used in 'normal' carbon NMR (2 seconds). It is because of this, that some signals, particularly the carbonyl signals, appear much lower in intensity than those of other carbons with the same abundance in the sample.

The spin lattice relaxation times of two triglycerides, tristearin and triolein were measured by inversion recovery; these results are presented in Tables 3 and 4.

Carbon Environment	Chemical Shift (ppm)	T_1 (seconds)
Carbonyl	174	3.956
Glycerol	62	0.570
Alpha to Glycerol	31	3.195
Methylene	29	4.061
Terminal Methyl	14	4.568

Table 3: Inversion recovery data for Tristearin

Carbon Environment	Chemical Shift (ppm)	T_1 (seconds)
Carbonyl	172	8.514
Olefinic	130	1.622
Glycerol	62	0.345
Alpha to Glycerol	34	0.672
Methylene	29	1.458
Alpha to methyl	22	4.689
Terminal methyl	14	4.313

Table 4: Inversion recovery data for Triolein

It can be seen that T_{1max} for triglycerides is significantly longer than the D_1 in standard carbon NMR. A large proportion of the carbon nuclei in the triglycerides do not have time to fully relax between pulses meaning that any results are not quantitative and cannot be reliably integrated.

To decrease the relaxation times of nuclei in organic molecules, a relaxation agent, such as $Cr(acac)_3$ can be added. According to literature, the addition of relaxation agents such as $Cr(acac)_3$ at concentrations of up to 0.1M does not alter the chemical shifts of the analyte, but the literature does not state the limits of 'acceptable' chemical shift change (Berger, 1998).

As part of this research, a study was carried out to investigate how the concentration of $Cr(acac)_3$ alters the ^{13}C NMR spectra. ^{13}C NMR spectra were taken of a sample of tristearin (0.011 mmol in 1.000 ml $CDCl_3$) with varying amounts of $Cr(acac)_3$ from 5 to 400 mmol. Results show that high concentrations of relaxation agent (greater than 200 mmol) alter the way the nuclei relax to such an extent that the spectra acquired become unrecognisable. The addition of $Cr(acac)_3$ at amounts of 5 – 200 mmol changes the chemical shifts from those acquired with no relaxation agent. The less relaxation agent added, the smaller the chemical

shift difference. This relationship between change in chemical shift and concentration of $Cr(acac)_3$ is shown in Figure 13. Tristearin was used for this study, as it is the triglyceride with the highest molecular weight likely to be encountered in the analysis of naturally occurring lipids.

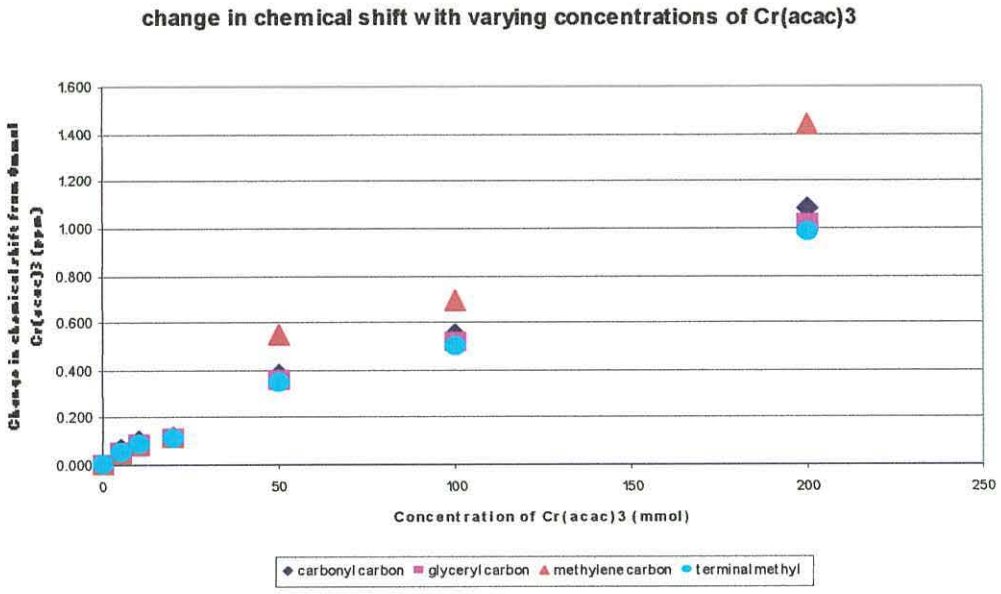


Figure 13: Change in chemical shift difference of a variety of carbon nuclei in tristearin with varying amounts of $Cr(acac)_3$.

As the change in chemical shift is an undesirable side-effect of the relaxation agent, an optimum concentration to gain fully quantitative data has been found to be 0.02 M (20 mmol) a ratio of 2000:1 $Cr(acac)_3$:triglyceride. This was determined by repeating the inversion recovery experiments with $Cr(acac)_3$ added. At concentrations less than 20 mmol/ml, the data acquired was not quantitative – the ratio of relaxation agent to analyte was thought to be insufficient to induce the increased relaxation of all nuclei.

To confirm that the amount of relaxation agent was sufficient to gain fully quantitative spectra, inversion recovery experiments were carried out on tristearin and triolein, with $Cr(acac)_3$ (20 mmol) to calculate the enhanced relaxation times of each type of carbon within the lipid sample. The results of these experiments are shown in Tables 5 and 6.

Carbon Environment	Chemical Shift (ppm)	T_1 (seconds)	
		No $Cr(acac)_3$	20 mmol $Cr(acac)_3$
Carbonyl	174	3.956	1.721
Glycerol	62	0.570	0.307
Alpha to glycerol	31	3.195	1.871
Methylene	29	4.061	0.820
Terminal methyl	14	4.568	1.786

Table 5: Spin lattice relaxation times for tristearin with and without $Cr(acac)_3$.

Carbon Environment	Chemical Shift (ppm)	T_1 (seconds)	
		No $Cr(acac)_3$	20 mmol $Cr(acac)_3$
Carbonyl	172	8.514	0.808
Olefinic	130	1.622	0.723
Glycerol	62	0.345	0.248
Alpha to glycerol	34	0.672	0.454
Methylene	29	1.458	0.875
Alpha to methyl	22	4.689	1.010
Terminal methyl	14	4.313	1.201

Table 6: Spin lattice relaxation times for triolein with and without $Cr(acac)_3$.

In an inversion recovery experiment, nuclei are exposed to a 180° pulse, followed by a 90° pulse after a delay (D_1). Nuclei that have a spin-lattice relaxation rate (T_1) faster than the delay, will be detected after the 90° pulse, and will show a positive signal on the spectrum. Nuclei with a T_1 less than D_1 will not be detected after the 90° pulse, so will show a negative peak. Nuclei with a T_1 equal to D_1 will not show a peak at all. During an inversion recovery experiment, the delay between the 180° pulse and the 90° pulse is changed to gain a full

spectrum of data points for each carbon environment. The inversion recovery plot of triolein is shown in Figure 14.

The data in Table 5 and Table 6 show that with an inverse gated ^{13}C pulse sequence with a D_1 of five seconds, the acquisition would be quantitative as T_{1max} is less than or equal to three times that of D_1 when $Cr(acac)_3$ is present at 0.02 M. The largest differences in relaxation time are for the carbonyl signals, especially for triolein. This is to be expected as quaternary carbons have the slowest spin-lattice relaxation time.

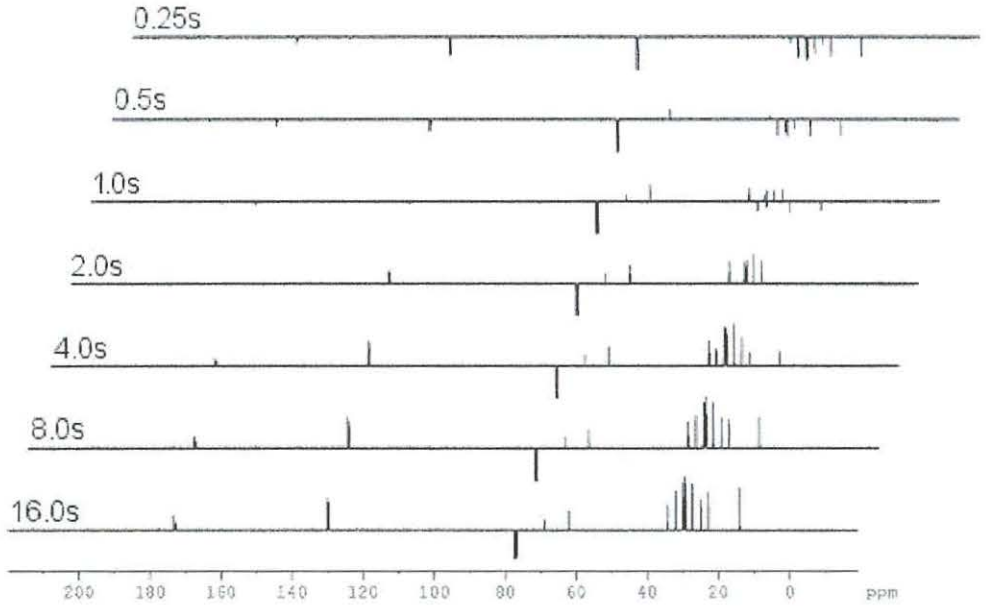


Figure 14: Inversion recovery plot of Triolein. D_1 in seconds ranges from 16 to 0.25 seconds. Positive peaks represent nuclei with a T_1 less than D_1, negative peaks represent a nuclei with a T_1 greater than D_1, missing peaks show $T_1 = D_1$.

2.3 Limit of Quantitation of the ^{13}C NMR Method

Samples of different ratios of tristearin and tributanoin were analysed to establish the lowest level at which a component in a mixture could be quantified. Samples of tributanoin were prepared with 10%, 2%, 1.4% and 1% (molar percentage) tristearin included. These sample were analysed using quantitative ^{13}C NMR and were integrated to compare characteristic peaks. It was shown that components could be detected at 1%, but not quantified below 1.4%.

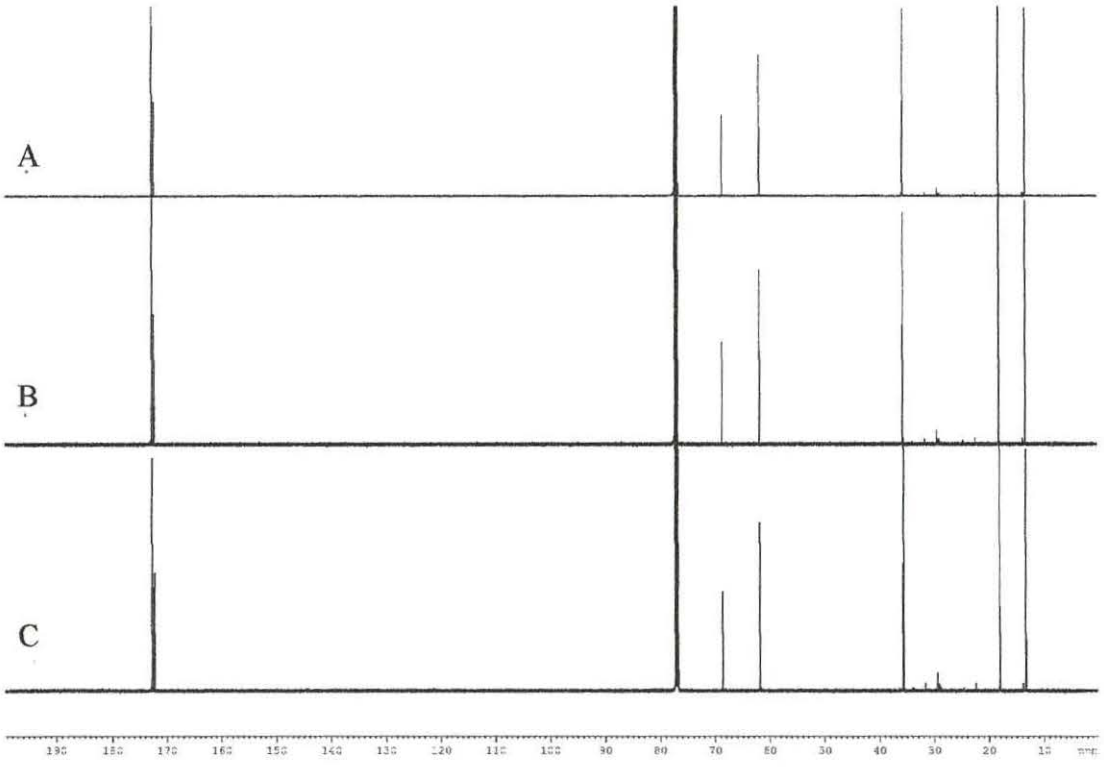


Figure 15: Quantitative ^{13}C NMR of tributanoin with 1.0% (A), 1.4% (B) and 2.0% (C) tristearin added.

The quantitation limit could be lowered by several methods. If a sample with an intermediate concentration in the mixture was added, this could be used as the calibration peak so the integration of the lowest concentration peak would be more accurate. Standard addition could also be used to quantify components present at low concentrations; a quantitative spectrum would be acquired and the integrals measured. A known amount of the smallest component could then be added and the spectrum recorded again and the difference in the integrals measured. This would give a more accurate measure of the amount of the lowest concentration component. This method is not practical however for identifying the concentration of a large number of components present in a mixture at low concentrations as the amount of instrument time would be large – a new spectrum would need to be acquired for each component, and as the over-all concentration of the sample increases, the ratio of $Cr(acac)_3$ to analyte would change and could effect the complete relaxation of all nuclei.

2.4 ^{13}C NMR chemical shift and integral data for triglycerides

A ^{13}C NMR spectrum of a triglyceride appears highly complex due to the large number of carbon signals; however, a great deal of information can be extracted. The quantitative ^{13}C NMR spectrum for trilinolenin is shown in Figure 16; expansions of each region of interest in this spectrum will be presented in this chapter. The chemical shift of each signal along with its integral is presented in Table 7. The structure of trilinolenin is shown in Figure 17.

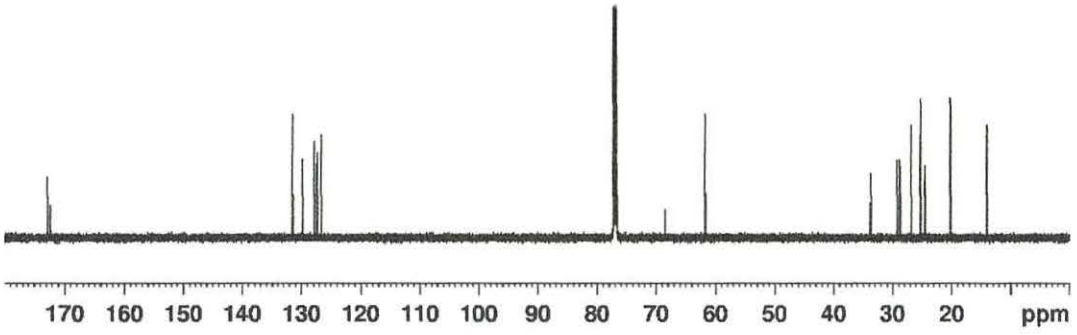


Figure 16: ^{13}C NMR spectrum of trilinolenin acquired using a quantitative pulse program.

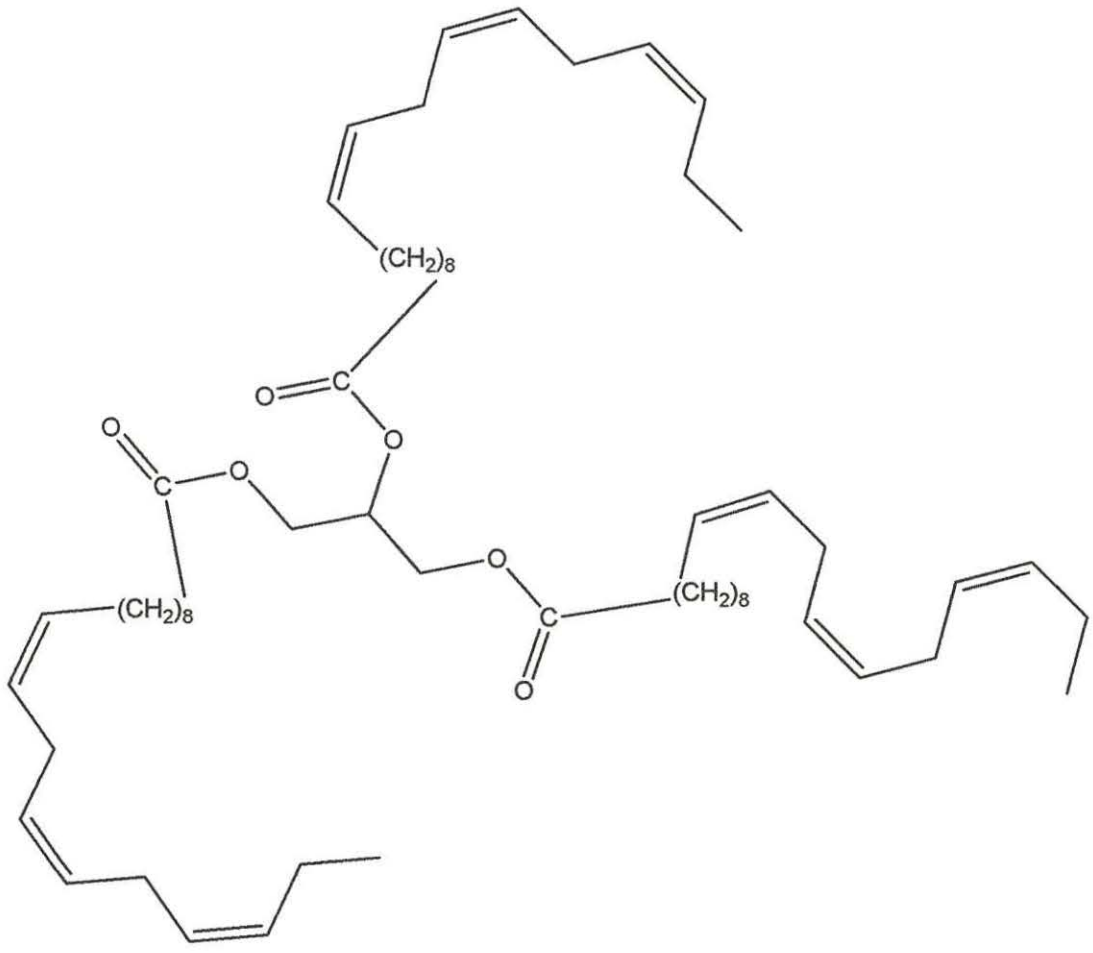


Figure 17: The structure of trilinolenin.

Carbon Number	Chemical Shift (ppm)	Integral	Carbon Number	Chemical Shift (ppm)	Integral
Carbonyl Region			Fingerprint Region		
C-1 α	172.830	2.17	C-7 β	29.208	0.97
C-1 β	172.418	1.06	C-7 α	29.195	2.09
Olefinic Region			C-6 β	29.799	1.23
C-9 α + β	131.555	2.97	C-6 α	28.799	2.08
C-10 α	129.826	2.05	C-5 β	28.729	1.16
C-10 β	129.799	1.15	C-5 α	28.716	2.03
C-12 β	127.906	1.10	C-4 α	28.684	2.36
C-12 α	127.897	2.12	C-4 β	28.645	1.14
C-13 α	127.844	2.10	Acyl Chain Region		
C-13 β	127.831	1.05	C-8 α + β	26.811	2.89
C-15 β	127.379	1.04	C-11 α + β	25.227	2.76
C-15 α	127.361	1.99	C-14 α + β	25.140	2.95
C-16 α + β	126.724	3.14	C-3 β	24.477	1.05
Glycerol Region			C-3 α	24.438	1.98
β	68.490	0.91	C-17 α + β	20.170	2.86
α	61.712	2.00	Terminal Methyl Group		
Acyl Chain Region			C-18 α + β	13.911	2.95
C-2 β	33.790	0.98			
C-2 α	33.627	2.05			

Table 7a: Chemical shifts and integrals of all ^{13}C resonances for trilinolenin normalised to the carbon signal for the beta-carbons in the glycerol backbone.

Carbon Number	Chemical Shift (ppm)	Integral	Carbon Number	Chemical Shift (ppm)	Integral
Carbonyl Region			Fingerprint Region		
C-1 α	172.830	2.39	C-7 β	29.208	1.07
C-1 β	172.418	1.17	C-7 α	29.195	2.30
Olefinic Region			C-6 β	29.799	1.35
C-9 α + β	131.555	3.27	C-6 α	28.799	2.29
C-10 α	129.826	2.26	C-5 β	28.729	1.28
C-10 β	129.799	1.26	C-5 α	28.716	2.23
C-12 β	127.906	1.21	C-4 α	28.684	2.60
C-12 α	127.897	2.33	C-4 β	28.645	1.25
C-13 α	127.844	2.21	Acyl Chain Region		
C-13 β	127.831	1.15	C-8 α + β	26.811	3.18
C-15 β	127.379	1.14	C-11 α + β	25.227	3.04
C-15 α	127.361	2.19	C-14 α + β	25.140	3.25
C-16 α + β	126.724	3.45	C-3 β	24.477	1.15
Glycerol Region			C-3 α	24.438	2.18
β	68.490	1.00	C-17 α + β	20.170	3.15
α	61.712	2.20	Terminal Methyl Group		
Acyl Chain Region			C-18 α + β	13.911	3.24
C-2 β	33.790	1.08			
C-2 α	33.627	2.25			

Table 7b: Chemical shifts and integrals of all ^{13}C resonance for trilinolenin normalised to the carbon signal for the alpha-carbon in the glycerol backbone.

The chemical shifts of all carbon resonances detected in a sample of trilinolenin are presented in Tables 7a and 7b. It can be seen that in table 7a, the integrals have been calibrated to the alpha-glycerol carbon signal, which has been set to represent 2.00 carbon nuclei. In Table 7b, the integrals have been calibrated again the beta-glycerol carbon signal, which has been set to represent 1.00 carbon nuclei. The values for these integrals are almost whole integers – being representative of either one, two or three carbons. Where the integral represents three carbons, the signal is from carbon nuclei on both the alpha- and beta-positions. If a signal represents two carbons, these are from carbon nuclei on the alpha-positions, and a signal representing one carbon nucleus is from the beta-position.

The values from each method of integration show a similar level of error. The error in these values can be attributed to three factors, one being the accuracy of the integration software, the second is change in temperature in the magnet during the acquisition period and the third is the drift in the magnetic field, which changes depending on the age of the magnet and can be as much as a few Hertz per day. As the software used for interpretation of NMR spectra is not designed specifically for integration of carbon spectra, automatic integration was not used, as this did not allow for integration of close or overlapping signals. Manual integration was used as the accuracy and precision was greater. **Throughout this work, ^{13}C NMR spectra were calibrated against the beta-carbon in the glycerol backbone.** All results from ^{13}C NMR spectra and all integrals shown in figures and tables are from spectra where the glycerol carbon in the beta-position has been set to 1.00.

The carbonyl region (δ = 174 – 170 ppm) gives information about the regiochemistry of the triglycerides – saturated fatty acids will show at different chemical shifts to mono-unsaturated

and poly-unsaturated fatty acids, and the alpha- and beta-positions are grouped together separately. The ^{13}C NMR of the carbonyl region of trilinolenin is shown in Figure 18. The signal at δ = 172.830 ppm represents the carbonyl carbons on the *sn*-1 and *sn*-3 positions, and the signal at 172.418 ppm represents the carbonyl carbon on the *sn*-2 position. The integrals of the signals are representative of two (2.30) and one (1.13) carbons respectively.

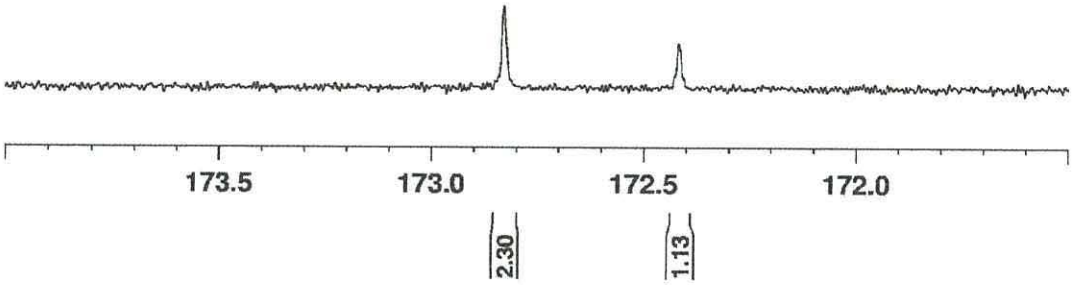


Figure 18: ^{13}C NMR Spectrum of the carbonyl region (174 - 170 ppm) of trilinolenin. α carbonyl chemical shift: δ=172.85, β carbonyl chemical shift: δ= 172.40.

The alkene region (δ = 132 – 126 ppm) can be used to identify which olefinic fatty acids are present in a triglyceride sample. The ^{13}C NMR spectrum of trilinolenin is shown in Figure 19. Analysis of this olefinic region not only allows identification of which unsaturated fatty acids are present in a triglyceride, but it is also possible to identify which position on the triglyceride the fatty acid is on, as the fatty acids on each position have distinct and characteristic chemical shifts.

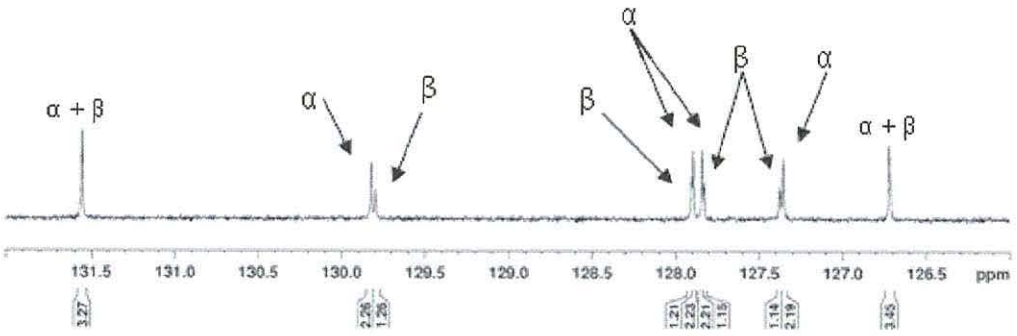


Figure 19: ^{13}C NMR Spectrum of the olefinic region (132 - 126 ppm) of trilinolenin. Signals labelled to show which position on the glycerol backbone they represent.

The carbons on the glycerol backbone have chemical shifts of approximately 68 ppm (β position) and 62 ppm (α position). These signals have integrals corresponding to one and two carbons respectively. The ^{13}C NMR spectrum of the glycerol region of trilinolenin is shown in Figure 20.

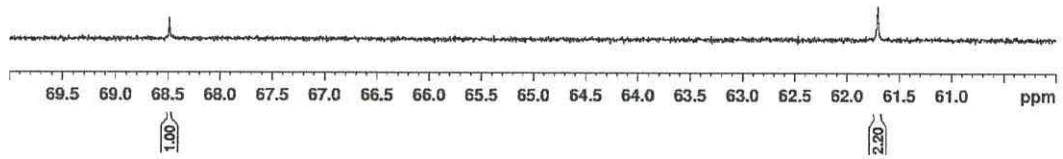


Figure 20: ^{13}C NMR spectrum of the glycerol region (70 - 60 ppm) of trilinolenin.

The methylene region (δ = 40 – 20 ppm) can be used as a fingerprint to identify the individual fatty acids in a mixture, as clusters of signals and integrals are diagnostic of each fatty acid reside. The ^{13}C NMR of this region is shown in Figure 21. Soon Ng and Ping Tou Gee have used the area under the peaks in this region to calculate the average molecular weight of the mixture of triglycerides, and from this, calculate the iodine value. These calculated iodine values were compared to official recognised standard iodine values and have been shown to be in good agreement (Ng and Gee, 2001).

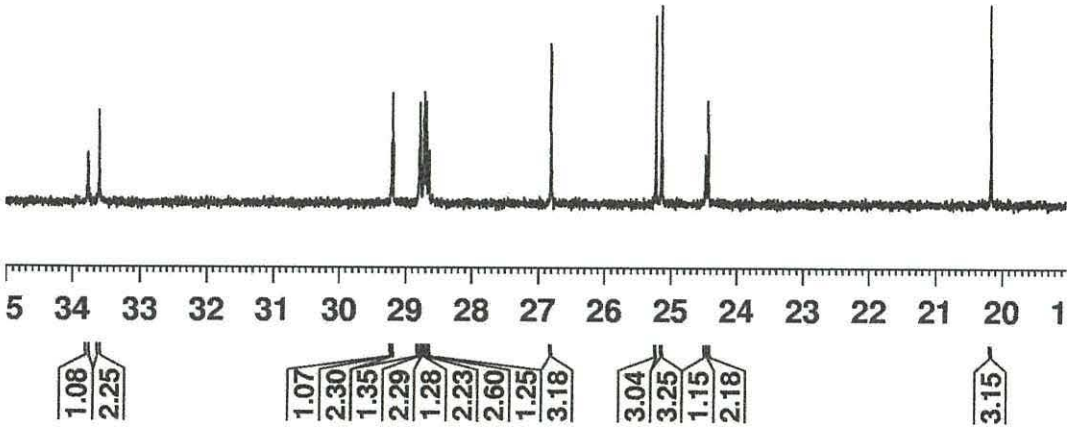


Figure 21: ^{13}C NMR spectrum of trilinolenin showing the region 35 - 19 ppm (the methylene region).

The region between δ = 30 – 28 ppm contains the largest number of overlapping signals, this region represents the middle of an acyl chain and signals from both the α- and β-chains are found in this region. Analysis of this region can give information on the number of carbons in the chain meaning the identity of individual saturated fats can be ascertained, as well as an indication of which position the fatty acid occupies on the glycerol backbone. The quantitative ^{13}C NMR spectra of this 'fingerprint' region and can be seen in Figure 22.

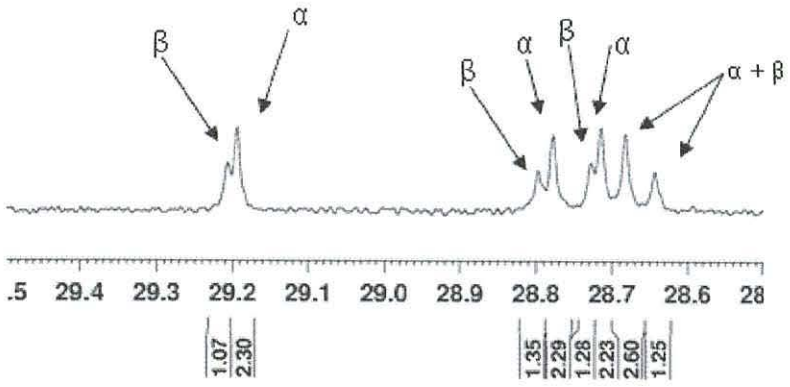


Figure 22: ^{13}C NMR Spectrum on the methylene region (30 - 28 ppm) of trilinolenin.

The terminal methyl carbons have similar chemical shifts in the region δ = 13 – 15 ppm, and for triglycerides containing fatty acids with 10 or more carbons in the chain, the signals for the terminal methyl carbons on the α- and β-chains have the same chemical shifts. The ^{13}C NMR spectrum of the terminal methyl group of trilinolenin is shown in Figure 23.

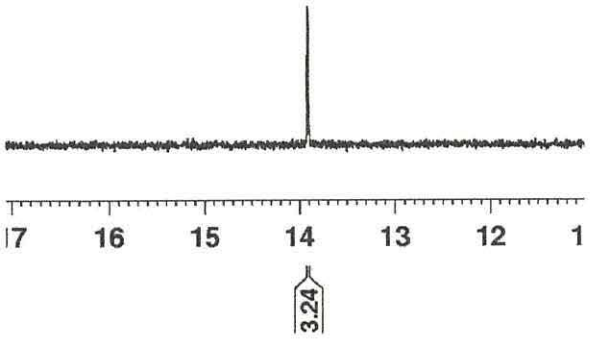


Figure 23: Terminal methyl region (20 -10 ppm) of trilinolenin.

2.5 Chemical shifts of triglyceride standards

Quantitative and qualitative ^{13}C NMR spectra were recorded for several pure and mixed triglyceride standards. The chemical shifts have been published by previous authors, Lie Ken Jie *et al* (1995, p1, p15, p187) but not in such detail – resolution of some signals has been shown here which has not previously been reported. These chemical shifts were used for comparison purposes throughout this research. ^{13}C NMR spectra of triglyceride standards are shown in detail in Chapter 10.

The chemical shifts of saturated triglycerides of the type AAA are shown in Table 8. Triglycerides analysed are tributyrin, trihexanoin, trioctanoin, tridecanoin, trilaurin, trimyristin, tripalmitin and tristearin. The chemical shifts of unsaturated triglycerides of the form AAA are shown in Table 9.

			Glycerol	C-1	C-2	C-3	C-4	C-5	C-6
C4:0	tributyrin	α	61.596	172.161	35.567				
		β	68.392	172.558	35.418				
C6:0	trihexanoin	α	61.660	172.384	33.714	24.101			
		β	68.441	172.795	33.555	24.077			
C8:0	trioctanoin	α	61.668	172.391	33.773	24.454	28.570	28.472	
		β	68.445	172.802	33.609	24.418	28.612	28.463	
C10:0	tridecanoin	α	61.673	172.391	33.785	24.469	26.635	28.842	29.000
		β	68.448	172.802	33.619	24.428	28.672	28.825	28.982
C12:0	tridodecanoin	α	61.648	172.788	33.598	24.407	28.655	28.808	29.011
		β	68.416	172.377	33.764	24.448	28.619	28.829	29.019
C14:0	tritetradecanoin	α	61.679	172.820	33.630	24.439	28.691	28.845	29.070
		β	68.444	172.408	33.795	24.480	28.654	28.866	29.050
C16:0	trihexadecanoin	α	61.674	172.809	33.625	24.436	28.687	28.842	29.049
		β	68.439	172.399	33.789	24.477	28.650	28.849	29.053
C18:0	trioctadecanoin	α	61.716	172.872	33.836	24.526	28.920	29.128	29.261
		β	68.476	172.461	33.671	24.484	28.897	29.105	29.250

			C-7	$(CH_2)_m$	ω-5	Ω-4	ω-3	ω-2	ω-1
C4:0	tributyrin	α						17.850	13.053
		β						17.878	13.116
C6:0	trihexanoin	α					30.735	21.831	13.427
		β					30.782		
C8:0	triotcanoin	α					31.210	22.157	13.613
		β							
C10:0	tridecanoin	α				28.825	31.428	22.231	13.664
		β							
C12:0	tridodecanoin	α	28.881		28.881	29.155	31.458	22.232	13.663
		β							
C14:0	tritetradecanoin	α	29.196	29.260	29.231	28.935	31.504	22.272	13.700
		β		m=2					
C16:0	trihexadecanoin	α	29.234	29.271	29.196	28.937	31.503	22.270	13.696
		β		m=4					
C18:0	trioctadecanoin	α	29.289	29.331	28.702	28.992	31.556	22.323	13.753
		β		m=6	28.739				

Table 8: Chemical shifts of saturated triglycerides of the form AAA, where A is a saturated fatty acid with an even number of carbons. Chemical shifts recorded with 100 mg triglyceride, 7.5 mg $Cr(acac)_3$, 0.625 ml $CDCl_3$.

		Glycerol	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C8	C9
triolein	α	61.657	172.364	33.747	24.438	Miss	Miss	29.264	29.328	28.884	129.556
	β	68.43	172.773	33.584	24.4						129.569
tripetroselinin	α	61.666	172.639	33.476	24.022	28.71	29.214	128.48	130.088	29.214	29.298
	β	68.453	172.239	33.635	24.06	28.66		128.465			
trilinolein	α	61.624	172.731	33.542	24.355	28.601	28.634	28.696	28.873	26.723	129.721
	β	68.402	172.321	33.705	24.394	28.562	28.648	28.717			
trilinolenin	α	61.712	172.83	33.627	24.438	28.684	28.716	28.779	29.195	26.811	131.555
	β	68.49	172.418	33.79	24.477	28.645	28.729	29.799	29.208		

		C10	C-11	C-12	C-13	ω-5	ω-4	ω-3	ω-2	ω-1	
triolein	α	129.256	28.884	29.328	29.277	26.779	26.727	31.472	22.253	13.693	
	β	129.229			29.264						
tripetroselinin	α	29.25	29.14	29.25	29.298	26.812	26.353	31.489	22.261	13.693	
	β										
trilinolein	α	129.511	25.152	127.605	127.422	26.712	29.131	31.051	22.109	13.618	
	β	129.484		127.588	127.41		29.14				
trilinolenin	α	129.826	25.227	127.897	127.844	25.14	127.361	126.724	20.17	13.911	
	β	129.799		127.906	127.831		127.379				

Table 9: Chemical shifts of triglycerides of the form AAA where A is an unsaturated fatty acid with 18 carbons. Spectra recorded with 100 mg triglyceride, 7.5 mg $Cr(acac)_3$, 0.625 ml $CDCl_3$.

		Glycerol	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
1,3-distearoyl-2-oloeyl-glycerol	α	62.078	173.237	34.04	24.856	29.647				29.692	29.692
	β	68.867	172.796	34.187	24.876	29.647			29.757	29.112	130.003
1,2-distearoyl-3-oloeyl-rac-glycerol	1	62.193	173.197	34.026	24.848	29.651				29.69	29.69
	2	68.886	173.167	34.005	24.826	29.651				29.69	29.69
	3	62.193	172.79	34.191	24.888	29.651			29.374	29.301	129.968
		C-10	C-11	C-12	C1-13	ω-5	ω -4	ω -3	ω -2	ω -1	
1,3-distearoyl-2-oloeyl-glycerol	α	29.692	29..692	29.692	29.692			31.916	22.674	14.084	
	β	129.663	29.112	29.757		27.215	27.163	31.895	22.674	14.084	
1,2-distearoyl-3-oloeyl-rac-glycerol	1	29.69	29.69	29.69	29.69			31.912	22.667	14.072	
	2	29.69	29.69	29.69	29.69			31.912	22.667	14.072	
	3	129.67	29.301	29.374		27.201	27.15	31.889	22.667	14.072	

Table 10: ^{13}C NMR chemical shifts of mixed triglycerides. Spectra recorded with 10 mg triglyceride, 0.75 mg $Cr(acac)_3$, 0.250 ml $CDCl_3$.

Vlahov states that the NMR of triglycerides can be broken down into four regions – the carbonyl region (172 – 174 ppm), the olefinic region (124 – 134ppm), the glycerol backbone region (70 – 60 ppm) and the aliphatic region (10 – 35 ppm) which fits well for the more common, long to medium chain fatty acids (Vlahov, 1999). It does not however, show the whole picture for polyunsaturated fatty acids such as eicosapentanoic acid and docosahexaenoic acid which contain five and six double bonds respectively and due to the large number of olefinic carbons, each with different chemical shifts, they do not fit within the region outlined by Vlahov for olefinic signals. Similarly, with short chain fatty acids such as butyric acid, the aliphatic carbons may appear outside of the range defined by Vlahov, and have chemical shifts above 35 ppm.

2.6 The use of NMR for regio-specific analysis of triglycerides

NMR can be used to investigate the positions of fatty acid residues on a triglyceride. This technique is particularly useful for authentication of high value products such as olive oils, as natural products will only have certain fatty acid residues in certain positions. For example, vegetable oils will have saturated fatty acids almost exclusively on the *sn*-1 and *sn*-3 positions, with saturated fatty acids on the *sn*-2 positions being present at less than 0.5% making them too small to detect (Mannina *et al*, 1999). Oils that have been re-esterified from free fatty acids and glycerol will have random positioning of the fatty acids on the glycerol, and this can be detected by NMR. The importance of the positioning of the fatty acid residues can be seen by comparing cocoa butter and lard, which have similar fatty acid compositions (mainly palmitic acid (P), stearic acid (S) and oleic acid (O)) (Creamer and MacGibbon, 1996). The physical properties of the fats are quite different however, mainly

due to the positioning of the fatty acids on the glycerol; lard having mainly OPS (oleic, palmitic and stearic acid on the *sn*-1, 2 and 3 position respectively) and cocoa butter being mainly POS (palmitic, oleic and stearic acid on the *sn*-1, 2 and 3 positions respectively).

Carbonyl signals (chemical shift 172 – 174 ppm) are used to gain information on which species of fatty acid is present on which position, but analysis of the olefinic signals (124 – 134 ppm) can also be used. A study by S. Simova *et al* (2003) showed the differing chemical shifts of the carbonyl signals of triglycerides with a variety of arrangements of oleic and palmitic acid as the sole fatty acid components. Their findings are presented in Table 11.

	Triglyceride composition					
Position	OOO	PPP	POP	OPO	OOP	PPO
1	173.229	173.270	173.285	173.254	173.250	173.281
2	172.815	172.858	172.838	172.868	172.836	172.865
3	173.229	173.270	173.285	173.254	173.282	173.250

Table 11: ^{13}C chemical shifts in ppm of carbonyl carbons in triglycerides comprised of oleic and palmitic acid, where O corresponds to oleic acid and P to palmitic acid. E.g. POP is 1,3-palmitoly-2-oleoyl glycerol.

It can be seen that oleate on the sn-1 and sn-3 position has a chemical shift of ~173. 25 ppm and 172.82 ppm on the sn-2 position. Regardless of what the rest of the triglyceride is made up of, α-oleate will always have a chemical shift of ~ 173.25 ppm. The same applies to all fatty acids esterified to a glycerol backbone, for example, this would apply for mixtures of linoleate and palmitate, or any other combination of acyl chains.

Although only the carbonyl signals are studied in Table 11, the olefinic and fingerprint regions of ^{13}C NMR can be used to identify the position of a fatty acid on the glycerol backbone. This

is because each fatty acid has characteristic peaks on both the alpha- and beta-positions that can be used for identification purposes. Taking triolein as an example, in the olefinic region, there are four signals detected. These are representative of carbons 9 and 10 in the acyl chain. The four signals are grouped into two pairs, each pair having one signal with an integral of 1.0 and the other an integral of 2.0 representing the carbons on the beta- and alpha-positions respectively.

As individual saturated fats cannot be identified by examination of the carbonyl region, and they have no signals in the olefinic region, the fingerprint region (30 – 28 ppm) can be used to identify the fatty acid and its position on the glycerol backbone. Taking tristearin and an example, a signal at 28.702 ppm is representative of a carbon nucleus on the alpha position and its corresponding partner on the beta-position has a chemical shift of 28.739 ppm. These signals have integrals of 1.0 and 2.0 respectively.

Short chain fatty acids – those with four carbons or less, can be identified quickly and easily. Also, their position on the glycerol backbone can be identified by examination of the terminal methyl carbon nuclei. As the terminal methyl carbon is so close to the glycerol backbone, the chemical shifts for the alpha- and beta-positions differ quite significantly. The alpha-position can be identified as the integral of the signal equals 2.0 and the beta-position carbon nuclei integral equals 1.0. Triglycerides with 6 and 8 carbon fatty acids present in them also show different signals for the terminal methyl carbons. Although there is no separation of the alpha- and beta-positions, the chemical shifts are lower than those with ≥ 10 carbons. As these short chain fatty acids have characteristic terminal methyl signals, they can be used in

comparison with the terminal methyl signal for the longer chain fatty acids to calculate a ratio of long and medium chain: short chain fatty acids present in a sample.

2.7 ^{1}H NMR of Lipids

2.7.1 Interpretation of ^{1}H NMR spectra

^{1}H NMR shows a signal for every proton environment present in a sample. ^{1}H NMR of a triglyceride shows around 13 signals. The terminal methyl groups are represented by one or two signals; one for medium and long chain saturated fatty acids and unsaturated fatty acids with the first double bond more than three carbons away from the end of the chain. Short chain fatty acids and ω-3 unsaturated fatty acids exhibit terminal methyl signals at a different chemical shift to other fatty acids (Knothe and Kenar, 2004). There is no distinction between terminal methyl groups on the alpha- and beta-positions.

A broad signal for the protons on the acyl chain is the largest signal in a ^{1}H NMR spectrum of a triglyceride. This signal is made up of many overlapping resonances, and not much information can be acquired from this signal alone.

2.7.2 Mathematical analysis of integrals of ^{1}H NMR

A standard solution of saturated, mono-unsaturated, bis-unsaturated and poly-unsaturated fatty acids was analysed by ^{1}H NMR, this spectrum is shown in Figure 24. Signals are marked v, w, x, y and z. These signals are representative of; v = protons on a carbon adjacent to a double bond, w = protons on a carbon adjacent to a carbonyl group, x = protons on a carbon between two double bonds in a bis-unsaturated system, y = protons on a carbon

between two double bonds in a tris-unsaturated system and z = olefinic protons. It was hypothesised that mathematical manipulation of the integrals of these five signals would enable the ratios of fatty acid species to be calculated.

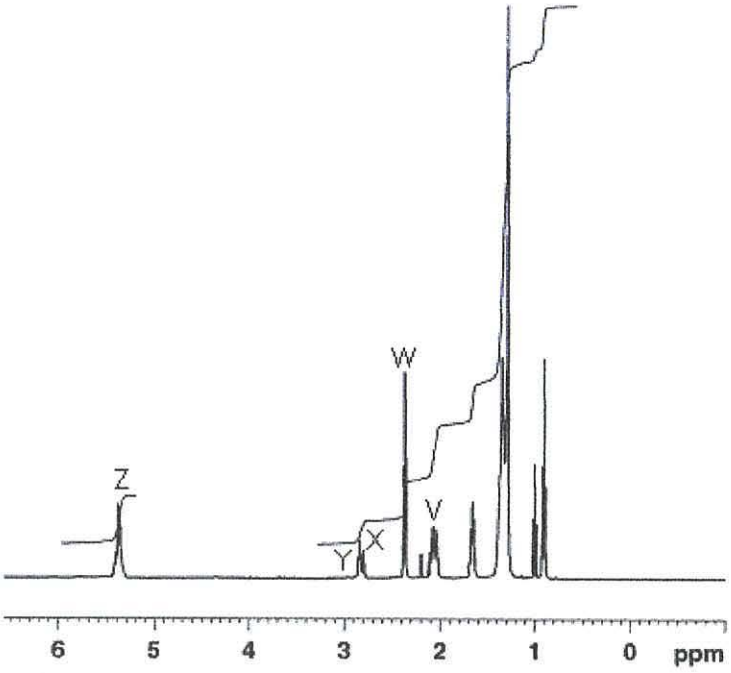


Figure 24: ^{1}H NMR of a mixture of fatty acid standards showing each signal used in the calculations.

From careful analysis and integration of a ^{1}H NMR spectrum that contains saturated and skipped conjugated, cis-unsaturated fatty acids, it is possible to calculate the percentage of saturated, mono-unsaturated, bis-unsaturated and tris-unsaturated fatty acids present in the sample. This method applies to samples of triglycerides, free fatty acids and fatty acid methyl esters as long as no fatty acids have trans or conjugated fatty acids are present. ^{1}H NMR can determine not only he degree of unsaturation (iodine value), but can distinguish between the different acyl groups present in the triglyceride (Guillen and Ruiz, 2003).

Table 12 shows the number of each type of proton present in each type of fatty acid. For example, all fatty acids contain two protons that are adjacent to a carbonyl group, and all unsaturated fatty acids contain four protons on carbons adjacent to unsaturated centres.

These values were put into a set of equations which were re-arranged to form equations 2 – 5 which can be used to calculate the percentages of saturated, mono-, bis-, and poly-unsaturated fats in a lipid sample by inserting the numerical values of each integral into these equations. For triglycerides, the integral values must be divided by three before inserting them into the equations.

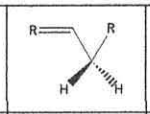
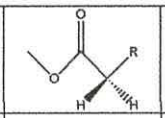
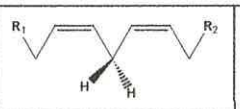
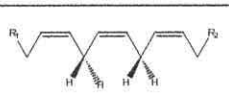
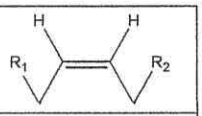
Type of proton					
Fatty acid	v	w	x	y	z
Sat	0	2	0	0	0
Mono	4	2	0	0	2
Bis	4	2	2	0	4
Tris	4	2	0	4	6

Table 12: Table showing the number protons in each environment in saturated, mono-unsaturated, bis-unsaturated and tris-unsaturated fatty acids.

$$Saturated\ fat = (w/2) - (v/4)$$

Equation 2: Simultaneous equation to calculate the amount of saturated fat present in an oil sample where v = ∫ ~ 2.0 ppm and w = ∫ ~ 2.3 ppm

$$Mono\text{-}unsaturated\ fat = (v - 2x - y)/4$$

Equation 3: Simultaneous equation to calculate the amount of mono-unsaturated fat present in an oil sample v = ∫ ~ 2.0 ppm, , x = ∫ ~ 2.7 ppm, and y = ∫ ~ 2.8 ppm.

$$Bis\text{-}unsaturated\ fat = x/2$$

Equation 4: Simultaneous equation to calculate the amount of bis-unsaturated fat present in an oil sample where x = ∫ ~ 2.7 ppm.

$$Tris\text{-}unsaturated\ fat = y/4$$

Equation 5: Simultaneous equation to calculate the amount of tris-unsaturated present in an oil sample where y = ∫ ~ 2.8 ppm.

Throughout this work, this method has been applied to many samples, from fatty acid methyl esters to triglycerides. It has been of great use in the study of extra virgin olive oils.

As stated previously, there is an inherent error present when integrating NMR signals. It is generally understood that these errors add up to a variance in data of ± 5 %. Even when this error is taken into account, the quick and easy method of assessing the fatty acid species present in a triglyceride sample makes it a useful technique. Work has been published showing reliable quantitation of linolenic acyl groups at concentrations of 0.4% and higher (Guillen and Ruiz, 2003, p502), and reliable quantitation of ω-3 fatty acids have been quantified using 1H NMR by Igarashi *et al* (2006).

The main advantages are the lack of derivatisation required – analysis can be carried out on the crude triglyceride, and the speed in which the data can be acquired is great – a ^{1}H NMR spectrum takes only a few minutes to record, integration can be carried out automatically by software, and interpretation of these integrals can be facilitated by the use of spreadsheets,

enabling easy data handling and comparison of data from different samples. This method offers a quick overview of the sample as a whole, so not only will fatty acid components be identified, but contaminants could be easily identified which may not be found if chromatographic analysis was carried out. For example, if a contaminant is not volatile, or if it binds irreversibly with the stationary phase, it may not be detected by GCMS. NMR would 'see' these contaminants and maybe even allow for identification and quantification of the impurity.

There are of course, disadvantages to this method. Because the error is comparatively large (± 5 %) when contrasted to other analytical techniques such as GC (where figures are often quoted to two decimal places), trace components may not be detected, or may be incorrectly quantified. In addition, the individual fatty acids in the triglyceride are not identified, and their positions on the glycerol backbone are not assigned by ^{1}H NMR.

The results attained by GCMS analysis of methyl esters of triglycerides will be compared with that from the ^{1}H NMR of triglycerides in Chapter 3, and the systematic errors in the method will be discussed further with respect to the analysis of olive oil.

So far, this method has been used for the analysis of lipids containing saturated and *cis*-unsaturated fatty acids only. Natural lipid samples can contain fatty acid species other than saturated and *cis*-unsaturated. For example, some food products such as dairy products contain conjugated systems where both *cis* and *trans* double bonds are present, and other food such as meat from ruminant animals contain some *trans* fatty acids. It is here that the

complication lies. The signals present in ^{1}HNMR for *trans* and *cis-trans* conjugated systems overlap.

As it stands, the integral of the olefinic signal relating to the *trans* fat can be compared to the integral of the olefinic signal for *cis*-unsaturated fats and a ratio of *cis* to *trans* could be calculated, but this does not give information on whether the *trans*-unsaturated system is a trans fatty acid or a *cis-trans* conjugated fatty acid.

In practice, knowing that only one type of *trans* system is present in a sample is not achievable without using some other analytical technique such as chromatography of triglyceride derivatives. As this ^{1}H NMR technique was designed to prevent the need for derivatisation and long chromatographic acquisition times, this is not practical. As it stands, this method of interpreting ^{1}H NMR spectra to determine the ratios of saturated and *cis* mono-, bis- and poly-unsaturated fat present in a sample is easy to apply to samples such as olive and vegetable oils as their triglycerides are composed entirely of saturated and *cis*-unsaturated fat.

3.0 Analysis of extra virgin olive oils

3.1 Introduction

The analysis of vegetable oils to determine the acyl chain composition is of paramount importance due to the importance of oils and fats in many food products.

Oils and fats play a huge part in the characteristics of many foodstuffs, ranging from organoleptic properties to the resistance of rancidity or oxidation. Understanding the composition of vegetable oils allows a greater knowledge of how particular oils can be used in the food industry to enhance the flavour and mouth-feel of products, their shelf life and stability and the health benefits of foodstuffs.

It is for these reasons that the study of vegetable oils is important, and the development of quick, easy, cheap quantitative methods of analysis is necessary. In this chapter, the acyl chain composition of olive oils was studied using conventional chromatographic techniques and quantitative NMR methods.

In the past, many papers have been published focusing on the analysis of fatty acids in olive oils, using chromatography as the main analytical technique. The reason for extending the research previously carried out was to apply the techniques of ^{1}H NMR and qualitative and quantitative ^{13}C NMR to the field of fatty acid analysis with respect to olive oil. It was hypothesised that NMR, particularly ^{13}C NMR would add an extra dimension to the data acquired, by identifying the positions of the fatty acids on the glycerol backbone as well as quantifying the individual fatty acids present.

The aim of this research, therefore, was to compare the already well-established technique of gas chromatography to that of NMR analysis of lipids. A selection of extra virgin olive oils were analysed by ^{1}H NMR and ^{13}C NMR. These results were then compared with the data from GCMS of methyl esters prepared from the triglycerides for verification purposes.

The use of ^{1}H NMR was expected to provide a quick measure of the ratios of species (saturated, mono-unsaturated, bis-unsaturated and poly-unsaturated) of fatty acids within the whole triglyceride. A ^{1}H NMR spectrum can be acquired in minutes, and the integration of key, characteristic peaks and manipulation of their integrals gives a quick overview of the fatty acid species present.

Analysis to show the degree of unsaturation in olive oil using ^{1}H NMR was carried out in 1999 by Vlahov (1999). This work compared the iodine values of the olive oils with the ^{1}H NMR and noted a linear relationship between the two. However, this work just presented an overall value for unsaturation and did not state separate values for mono-, bis-, and poly-unsaturated fats present.

The chromatographic analysis of free fatty acids and methyl esters from olive oil triglycerides is accurate, precise and quantitative, but it gives information only on the composition of the oil sample as a whole, and does not convey any positional information with respect to the fatty acids on the glycerol backbone of the triglyceride. Using pancreatic lipase to selectively cleave the fatty acids from the alpha-position can give this information, but it is anticipated that ^{13}C NMR can show this positional information from the whole triglyceride, without the use

of enzymes and chemicals for derivatisation. Using both quantitative and qualitative ^{13}C NMR, the individual fatty acids present in the olive oil sample can be identified AND quantified by comparing the chemical shifts with those of known standards and by integrating the signals to calculate the amounts of each fatty acid present. As fatty acids show different chemical shifts on the alpha- and beta-positions of the triglyceride, it is possible to assign the position of a fatty acid on the triglyceride as well as its proportion on each position. This is important for the detection of adulteration in high value products and has health implications as the fatty acids on the alpha- and beta-positions are metabolised differently.

It has been reported in several sources that it is possible to identify the position of the different species of fatty acid on the glycerol backbone of a triglyceride by careful analysis of the carbonyl region of a ^{13}C NMR spectrum (Mannina *et al*, 1999; Mannina *et al* 2003). This however, only indicates the ratios of saturated, mono- and poly-unsaturated fatty acids on each position, and does not identify which individual fatty acids are present. This is nevertheless, a useful analytical tool for the analysis of olive oils, as the beta-position in unadulterated olive oil is almost exclusively occupied by unsaturated fatty acids, so high levels of saturated fatty acids detected on the *sn*-2 position would be an indication that the oil is either spiked with another vegetable oil (typically hazelnut oil), or has been formed from a re-esterified mixture of the correct ratio of free fatty acids (Li-Chan, 1994). Incorrect identification of unsaturated fatty acids with the double bond a long way away from the carbonyl group (i.e. on the 11 position) can be erroneously identified as saturated fatty acids using this method.

3.2 Comparison of ^{1}H NMR of triglycerides with GCMS

Investigation of olive oil composition using ^{1}H NMR and GCMS has shown that the data acquired from ^{1}H NMR analysis is comparable with that from GCMS analysis. The data from these techniques is outlined in this chapter. Special attention was paid to comparing olive oils of different geographical origin, and from different cultivars.

A selection of extra virgin olive oils was bought from local supermarkets. The crude triglycerides were analysed by ^{1}H and ^{13}C NMR. The triglycerides were then transesterified using sodium methoxide and the resultant methyl esters were analysed by GCMS.

Analysis of the methyl esters by GCMS showed a spectrum of 11 fatty acids to be present, of which only 4 were present in all samples. The methyl esters of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1) were detected in all extra virgin olive oil samples. Methyl esters of heptadecenoic acid (C17:1)[1], linoleic acid (C18:2) conjugated linoleic acid (C18:2), linolenic acid (C18:3), and eicosanoic acid (C20:0) were also found in some extra virgin olive oil samples. The average fatty acid composition of extra virgin olive oils from Italy, Spain, Australia and Greece as determined in this research is outlined in Table 13.

[1] The presence of odd-chain fatty acids in olive oil is not unprecedented (Codex Standard for olive oils and olive pomace oils Codex stan 33-1981 (Rev. 2-2003)).

	Italian	Spanish	Australian	Greek
C16:0	17.0	13.5	13.1	12.2
C16:1	1.1	1.1	0.8	0.7
C17:0	0.0	0.0	0.0	0.0
C17:1	0.9	0.3	0.0	0.0
C18:0	3.4	3.0	2.1	2.7
C18:1 cis	75.0	77.6	74.0	78.0
C18:2	1.9	4.1	9.2	78.0
C20:0	0.2	0.2	0.2	0.2
C18:3	0.1	0.3	0.4	0.4
C18:2 CLA	0.1	0.0	0.0	0.0
Sat	20.5	16.6	15.4	15.0
Mono	76.9	78.9	74.9	78.6
Bis	2.0	4.1	9.2	5.8
Tris	0.6	0.4	0.6	0.6

Table 13: Composition of extra virgin olive oils from Italy, Spain, Australia and Greece as determined by GCMS (samples analysed twice and an average taken). Where sat is saturated fat, mono is mono-unsaturated, bis is bis-unsaturated and tris is tri-unsaturated.

Codex Alimentarius quotes the composition of virgin olive oils as determined by gas chromatography shown in Table 14.

Fatty Acid	Composition (% total fatty acids)
C14:0	0.0-0.05
C16:0	7.5-20.0
C16:1	0.3-3.5
C17:0	0.0-0.3
C17:1	0.0-0.3
C18:0	0.5-5.0
C18:1	55.0-83.0
C18:2	3.5-21.0
C18:3	0.0-1.5
C20:0	0.0-0.6
C20:1	0.0-0.4
C22:0	0.0-0.2
C24:0	0.0-0.2
C18:1t	0.0-0.05
C18:2t + C18:3t	0.0-0.05

Table 14: Standard composition limits for virgin olive oil. (Codex Alimenarius 33-1981 Rev 2-2003)

Oleic acyl chains made up the bulk of olive oil samples, varying in concentration from 72.5% to 82.7%, with the average being 77.1%. The second most abundant acyl chain is palmitic

acid, present at an average concentration of 14.1%, but ranging from 8.8% to 18.5%. Of the 4 fatty acids present in all samples, stearic acid is the next most abundant at 2.9% followed by palmitoleic acid at an average of 1.0%.

The following fatty acids, although not present in all the extra virgin olive oil samples analysed were found in the following proportions: Linoleic acid is present at 3.6% on average, heptadecenoic acid is present at 0.4%, eicosanoic acid and linolenic acid are present at approximately 0.2%.

Some olive oils contained notably high levels of linoleic acid, especially the olive oil from Cobram Estate. This is of significance as it is the only olive oil in the study, which is of non-European origin. This Australian oil contained below average amounts of palmitic, palmitoleic, stearic, oleic, and eicosanoic acid, with an above average concentration of linolenic, and levels of linoleic acid three times that of the average.

Extra virgin olive oils Arbequina, Hojiblanca, Picual, Rustica and San Leandro were all of Spanish origin. On average, these Spanish olive oils had less saturated and tris-unsaturated fatty acids and slightly more mono-unsaturated and bis-unsaturated fatty acids than the average of the whole selection of olive oils. Notably, conjugated linoleic acid was not found in any of these Spanish extra virgin olive oils.

Arbequina, Hojiblanca and Picual olive oils were all made by the same producer, but from different single cultivars of olives. The palmitic acid content in these oils varied quite considerably – from 10.9 % to 18.5 %, and the quantity of linoleic acid also varied

considerably, with Arbequina containing only 1.0 %, but Hojiblanca and Picual containing 4.3 and 6.3 % respectively. Antonio Sacco *et al* have previously shown that analysis of the fatty acid composition of olive oils can discriminate between different cultivars of olives (Sacco *et al*, 2002).

Extra virgin olive oils Duano Basso, Colline Etrusche, Montolivo, Filippo Berio, Olio del Castello, Safeways, Carapelli Firenze and Toscano were all of Italian origin. Italian olive oils, on average, had more saturated and tris-unsaturated fatty acids and less mono-unsaturated and bis-unsaturated fatty acids than the average of the whole selection of olive oils. The amount of bis-unsaturated fatty acids, linoleic and conjugated linoleic acid was almost half of that found in Spanish olive oil.

Greek extra virgin olive oil from Tesco's Finest range had a similar composition to the mean of Spanish extra virgin olive oil, with the exception of the tris-unsaturated fatty acids, which were present at nearly twice the level to that found in the Spanish oil.

Tesco did not state the country of origin of their extra virgin olive oil on the label, only that it was produced in the EU. This olive oil had the lowest proportion of saturated fatty acids, and the highest quantity of mono-unsaturated fatty acids seen in any of the extra virgin olive oils studied. The ratios of bis- and tris-unsaturated fatty acids matched those of Spanish extra virgin olive oil almost exactly. From this data alone, it was not possible to predict the origin of the extra virgin olive oil from Tesco.

Determination of the fatty acids present in an olive oil sample is a good indicator of whether the oil is in fact derived from olives, and can also give an indicator of the geographical origin. The triglyceride composition of olive oil can be used to identify not only the country of origin, but also the region within that country that the olive was from (Stefanoudaki *et al*, 1997). A study by Stefanoudaki *et al* on the triglyceride of Cretan olive oils from five locations and two different cultivars showed discernible differences in the make-up of the cultivars. Differences in geographic morphology (the altitude, slope and orientation) of the olive groves also affected the lipid composition of the olive oil.

Throughout this work, the ratios of fatty acid species of a selection of olive oils was determined by two methods, by interpretation of the integrals of the ^{1}H NMR spectrum of the triglycerides, and by GCMS analysis of the fatty acid methyl esters formed from the whole triglycerides by transesterification. The GC data was summed to give totals for saturated, mono-, bis- and poly-unsaturated fatty acids to allow a direct comparison with the data from ^{1}H NMR. The results from GCMS give results as a weight percentage and those from NMR give results as a molar percentage. In the case of the composition of olive oil, where the molecular weights of components do not differ by large amounts (acyl chain length varies from C16 – C18), these percentages can be considered comparable

Interpretation of the integrals from the ^{1}H NMR spectra of the triglycerides of the extra virgin olive oils showed the ratios of saturated, mono-unsaturated, bis-unsaturated and tris/poly-unsaturated fatty acids. The ratios of the different species of fatty acids as determined by ^{1}H NMR simultaneous equations are shown in Table 15.

Examination of the ^{1}H NMR spectra of extra virgin olive oil triglycerides showed no *trans* or short chain fatty acids to be present at concentrations detectable by NMR. GCMS analysis of methyl esters formed from these triglycerides shows no short chain fatty acids to be present, but does detect CLA in extra virgin olive oil from Safeways. This is, however, at a concentration too low to be detected and quantified by ^{1}H NMR.

It should be noted that the total composition of each olive oil does NOT add up to 100%; this is due to the inherent error in the integration of NMR spectra, which can be taken to be ±5%. This error is due to inaccuracies in manual integration, the signal to noise ratio, resolution, which is some cases, is not to the baseline and inconsistencies during acquisition such as change in temperature and drift of the magnetic field.

The ratios of fatty acid species as determined by GCMS are shown in Table 16.

The data from GCMS and ^{1}H NMR data can be directly compared as, in theory, they should show the same results.

Oil	sat	mono	bis	poly	total
Arbequina	13.0	82.5	6.0	0.5	102.0
Carapelli Firenze	14.5	81.0	9.0	0.5	105.0
Cobram Estate	14.0	73.0	11.0	0.5	98.5
Colline Etrusche	15.0	80.0	7.5	0.5	103.0
Duano Basso	12.0	85.0	7.0	0.5	104.5
Fillipo Berio	16.0	76.0	11.0	0.5	103.5
Hojiblanca	18.0	74.5	10.5	0.5	103.5
Montolivo	15.0	78.0	9.0	0.5	102.5
Picual	15.5	77.0	8.0	0.5	101.0
Rustica	14.0	84.0	5.0	0.5	103.5
Safeways	14.0	80.5	7.5	0.5	102.5
San Leandro	15.0	83.0	6.5	0.5	105.0
Tesco's	13.0	83.5	5.5	0.5	102.5
Tesco's Finest Greek	14.5	80.0	7.0	0.5	102.0
Toscano	12.0	83.5	7.0	0.5	103.0

Table 15: Molar percentages of fatty acid species as determined by ^{1}H NMR simultaneous equations. Note that values are rounded to the nearest 0.5 %.

Oil	sat	mono	bis	poly	total
Arbequina	14.5	78.0	6.5	0.0	100.0
Carapelli Firenze	15.5	75.5	8.0	1.0	100.0
Cobram Estate	15.5	75.0	9.0	0.5	100.0
Colline Etrusche	16.0	77.5	6.0	0.5	100.0
Duano Basso	16.0	76.5	7.0	1.0	100.5
Fillipo Berio	14	74.0	11.5	0.0	100.0
Hojiblanca	14.0	70	11.0	0.5	100.0
Picual	16.5	77.0	6.5	0.5	100.5
Rustica	15.0	79.5	4.5	1.0	100.0
Safeways	15.0	79.0	6.0	0.0	100.0
San Leandro	16.0	79.0	4.5	0.0	99.5
Tesco's	12.5	83.0	4.0	0.5	100.0
Tesco's Finest Greek	15.0	78.5	6.0	0.5	100.0

Table 16: Ratios of fatty acid species as determined by GCMS analysis of detected FAMEs quoted as weight percentages.

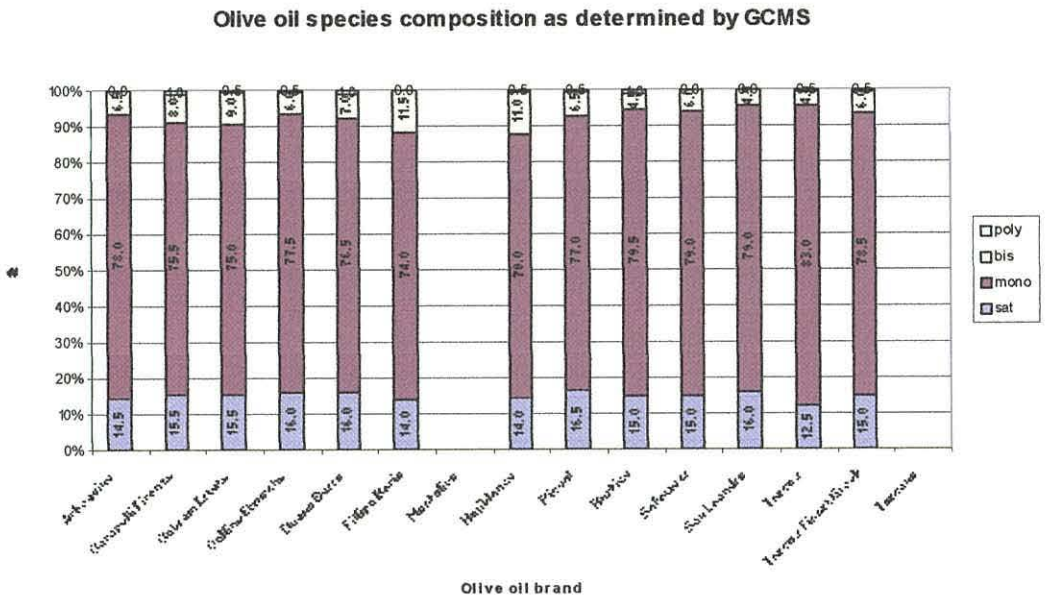


Figure 25: Olive oil species composition as determined by GCMS quoted as weight %.

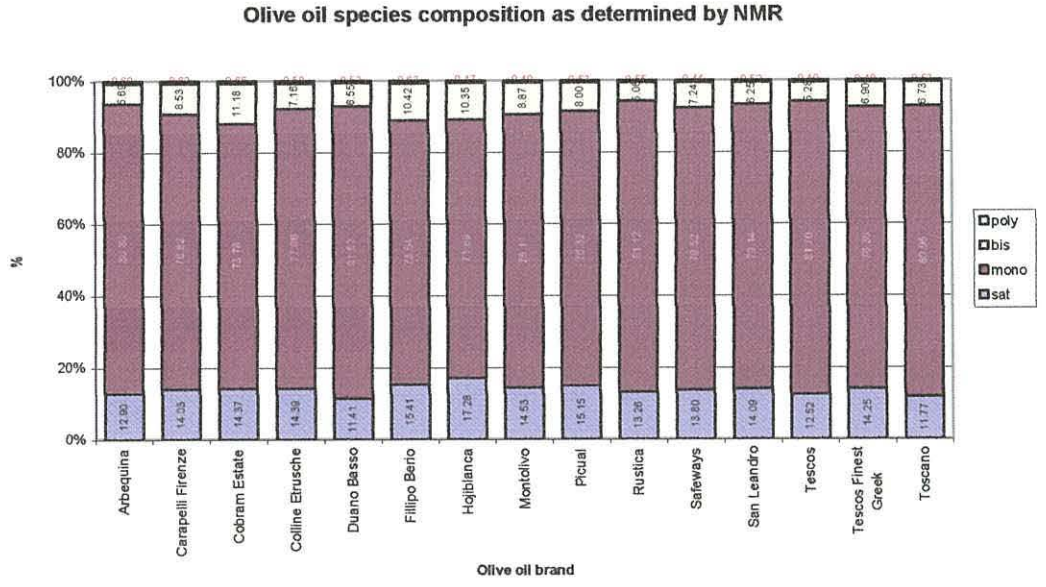


Figure 26: Olive oil species composition as determined by NMR quoted as molar %.

GCMS analysis of the fatty acid methyl esters formed from olive oil triglycerides gives an overall profile of the individual fatty acids present. This information is not available from the ^{1}H NMR as the chemical shifts are so similar for the protons on the acyl chains, that signals become broad and cannot be separated or identified for each individual component. The individual fatty acid breakdown of each extra virgin olive oil sample as determined by GCMS is shown in Table 17.

Charts showing the fatty acid species present in extra virgin olive oils as determined by GCMS and ^{1}H NMR are shown in Figures 25 and 26.

Graphs showing how the concentration of each individual fatty acid changes from brand to brand of olive oil are shown in Figure 27 to Figure 30.

Figure 27 to Figure 30 show the percentage of palmitoleic acid, stearic acid, oleic acid and eicosanoic acid present in each extra virgin olive oil sample. The variation in the concentration of these fatty acids is very small, and each fatty acid is present in all olive oils analysed except eicosanoic acid, which was not detected in two olive oils (Safeways and San Leandro).

Figure 31 shows the concentration of palmitic acid. This varies in concentration from 8.8 to 20.23 %. Montolivo extra virgin olive oil, which contained the highest concentration of palmitic acid, also contained the highest concentration of stearic acid.

Figure 32 shows how the concentration of linolenic acid varied within different olive oils. Linoleic acid was not detected in Monolivo olive oil. FIlippo Berio extra virgin olive oil contained the highest amount of linoleic acid at 11.6 %.

Figure 33 shows the linolenic acid content of extra virgin olive oils. Linolenic acid was only detected in half of the olive oils studied. The concentration of linolenic acid detected in half of the oils varied very little ranging from 0.33 to 0.5 %.

The amount of conjugated linoleic acid detected in olive oils is shown in Figure 34, it can be seen that CLA was only detected in one sample, that from Safeways.

FA	Arbequina	Carapelli Firenze	Cobram Estate	Colline Etrusche	Duano Basso	Fillipo Berio	Hojiblanca	Montolivo	Picual	Rustica	Safeways	San Leandro	Tescos	Tescos Finest Greek
C16:0	12.0	11.7	13.1	13.7	11.6	10.8	10.2	20.2	13.7	11.0	12.7	13.1	8.9	12.2
C16:1	1.4	1.3	0.8	0.9	0.9	1.3	0.7	1.4	1.2	0.8	0.9	1.2	0.5	0.7
C17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C17:1	0.8	1.8	0.0	0.0	0.0	1.2	0.2	2.2	0.3	0.0	0.0	0.0	0.0	0.0
C18:0	2.3	3.6	2.1	2.0	3.9	3.2	3.5	4.9	2.7	3.8	2.6	2.6	3.3	2.6
C18:1 cis	75.8	72.5	74.0	76.6	75.4	72.0	69.2	70.3	75.3	78.9	77.9	78.6	82.7	78.0
C18:2	6.5	8.0	9.2	5.8	7.0	11.5	11.0	0.0	6.3	4.4	5.6	4.4	4.1	5.8
C20:0	0.2	0.3	0.2	0.3	0.6	0.0	0.4	0.2	0.1	0.3	0.0	0.0	0.2	0.2
C18:3	0.0	0.0	0.4	0.5	0.0	0.0	0.4	0.0	0.3	0.5	0.0	0.0	0.3	0.4
C18:2 CLA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0
sat	14.5	15.5	15.4	15.9	16.0	14.0	14.0	25.4	16.5	15.0	15.2	15.8	12.4	15.0
mono	78.0	75.6	74.9	77.5	76.3	74.5	70.0	73.9	76.9	79.7	78.8	79.8	83.2	78.6
bis	6.5	8.0	9.2	5.8	7.0	11.5	11.0	0.0	6.3	4.4	5.9	4.4	4.1	5.8
tris	0.0	0.0	0.4	0.5	0.0	0.0	0.4	0.0	0.3	0.5	0.0	0.0	0.3	0.4

Table 17: Individual fatty acid composition of extra virgin olive oil s as determined by GCMS.

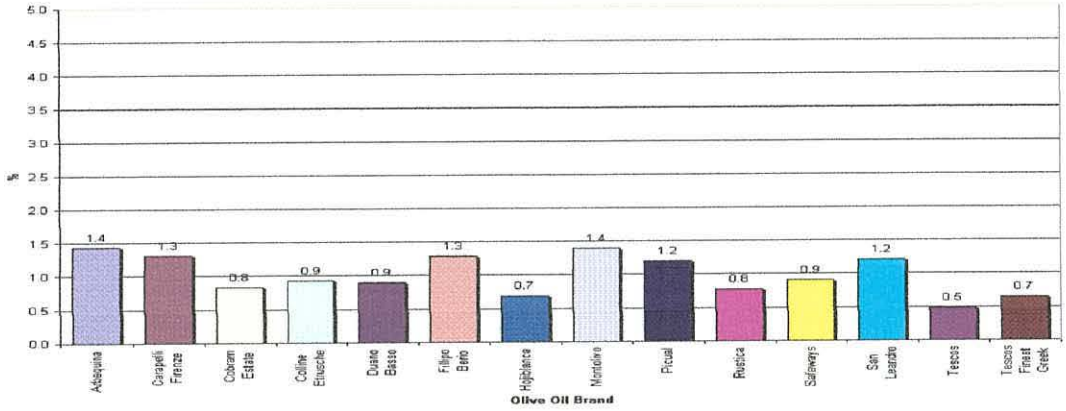


Figure 27: Palmitoleic acid content of extra virgin olive oils as detected by GCMS.

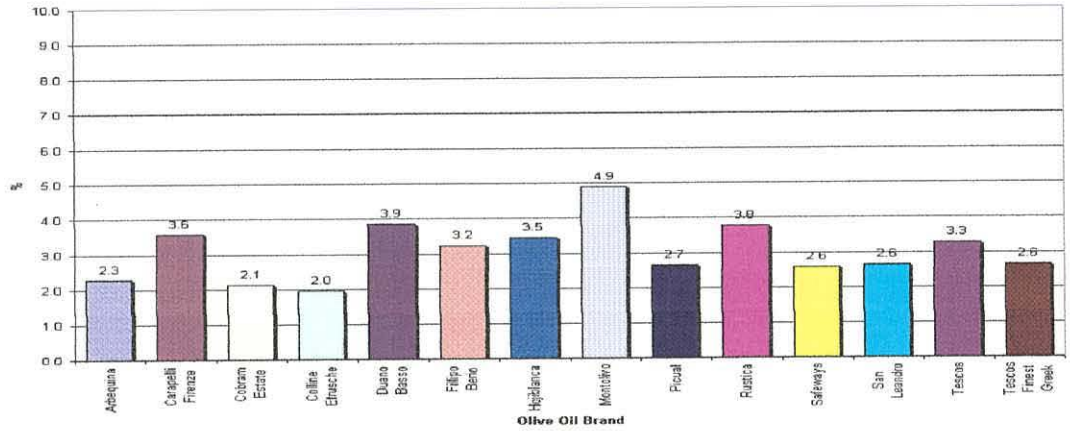


Figure 28: Stearic acid content of extra virgin olive oils as detected by GCMS.

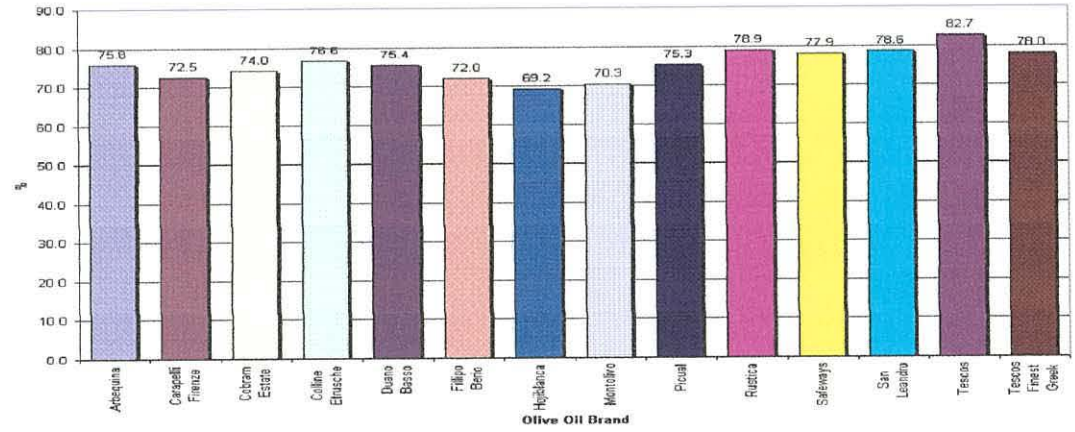


Figure 29: Oleic acid content of extra virgin olive oils as detected by GCMS.

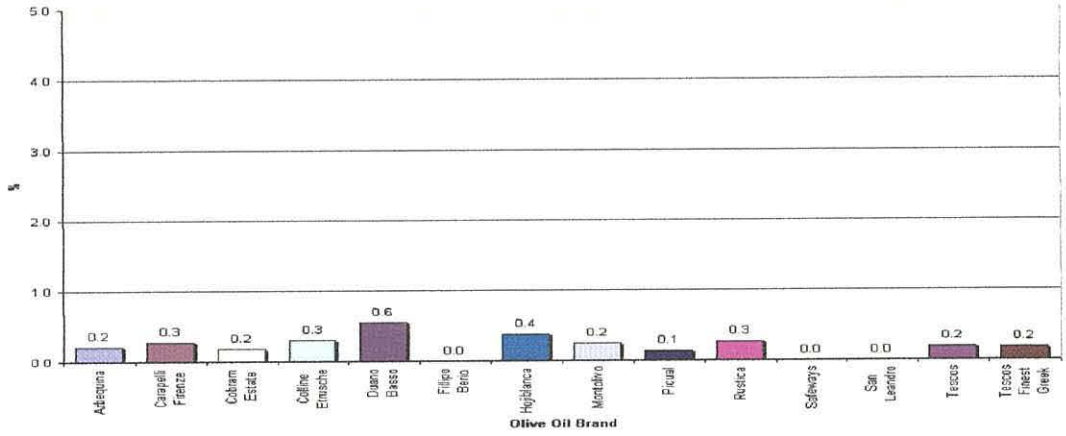


Figure 30: Eicosanoic acid content of extra virgin olive oils as detected by GCMS.

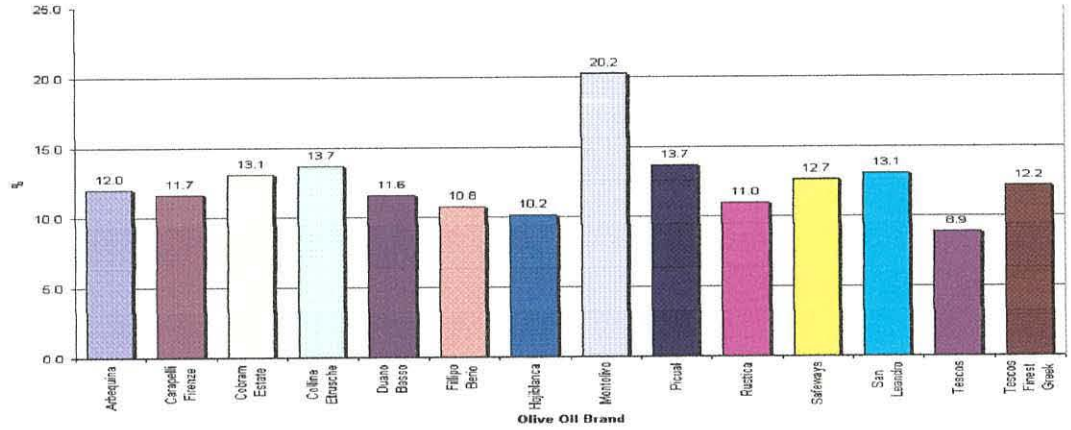


Figure 31: Palmitic acid content of extra virgin olive oils as detected by GCMS.

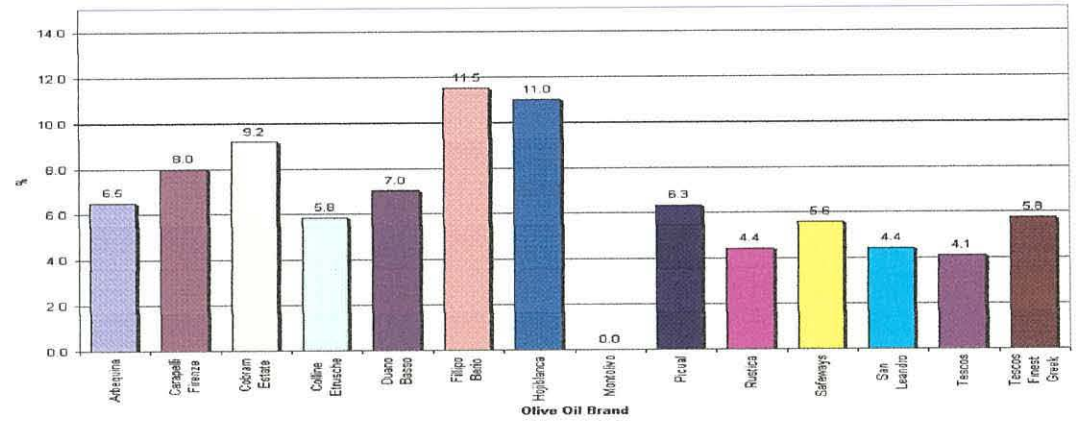


Figure 32: Linoleic acid content of extra virgin olive oils as detected by GCMS.

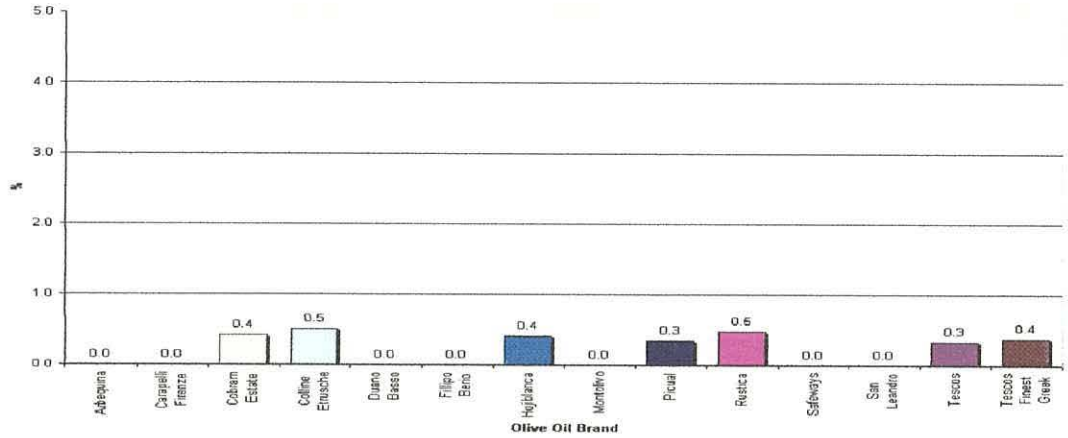


Figure 33: Linolenic acid content of extra virgin olive oils as detected by GCMS.

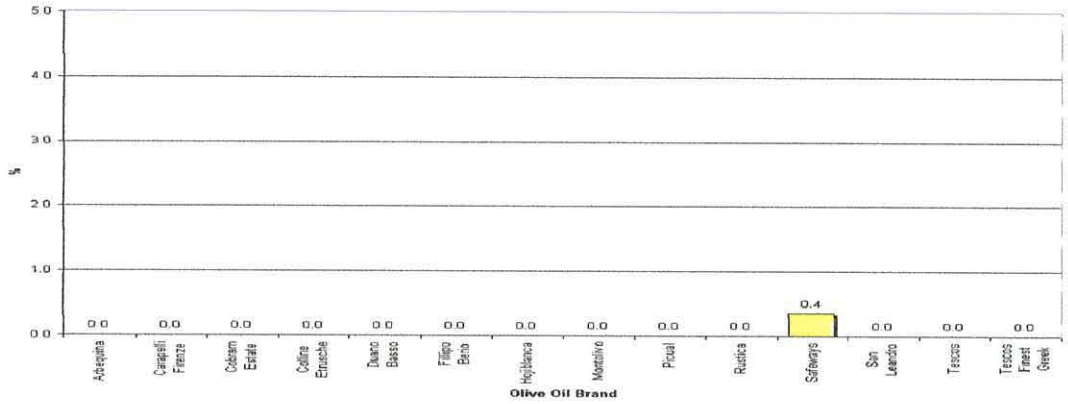


Figure 34: Conjugated linoleic acid content of extra virgin olive oils as detected by GCMS.

3.3 ^{13}C NMR of Olive Oils

Quantitative and qualitative ^{13}C NMR spectra were acquired for each olive oil. Quantitative spectra were integrated using X-Win NMR and the integral at ~68 ppm (the beta-carbon in the glycerol) calibrated to represent one carbon. These integral values were used for calculating the molar percentage of each fatty acid present. Qualitative ^{13}C NMR was used to assign the chemical shifts of each component as the addition of $Cr(acac)_3$ alters the chemical shift as explained in Chapter 2.

Each region of the ^{13}C NMR spectrum will be discussed individually below and the results combined, discussed and compared with those from the literature at the end of the chapter.

3.3.1 Interpretation of carbonyl region of ^{13}C NMR

The data that can be acquired from analysis of the carbonyl region is limited to looking at the positional distribution of the species of acyl chains on the glycerol backbone. No information on individual fatty acids can be obtained from this region. Analysis of the carbonyl region (174 – 170 ppm) showed two groups of signals in all olive oil samples, representing the carbonyl carbons on the alpha-positions (173.5 – 172.8 ppm) and beta-positions (172.7 – 172.0 ppm) on the glycerol backbone. The beta-position (*sn*-2) showed only unsaturated fatty acids to be present. The alpha-position showed saturated, mono- and poly-unsaturated fatty acids. An example of the carbonyl region of an olive oil is shown in Figure 35.

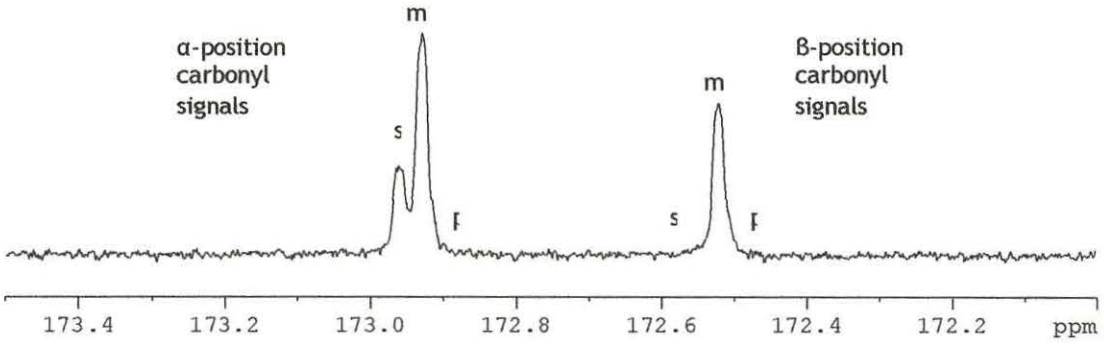


Figure 35: Carbonyl region of a ^{13}C NMR of Cobram Estate extra virgin olive oil showing the regions for carbonyl signals on the alpha- and beta-positions. s = saturated, m = mono-unsaturated and p = polyunsaturated. Where no signal is detected for saturated or poly-unsaturated fat in this sample, the letters indicate where a signal is expected to be.

As discussed in Chapter 2, the fatty acids on each position fall into two groups of peaks, one group for those on the alpha-position and one group for the beta-position. Saturated fatty acids have a distinct chemical shift on each position, which is well resolved from the unsaturated fatty acids. Only one carbonyl signal for saturated fatty acids was detected in each oil, and this was located on the alpha-position. Poly-unsaturated fatty acids were more difficult to detect due to poor resolution of the mono- and poly-unsaturated fat signals. The carbonyl signals for mono- and poly-unsaturated fatty acids, particularly on the beta position are not well resolved; they are represented by a broad peak for the mono-unsaturates with a small shoulder for the poly-unsaturates. Resolution enhancement (zero-filling and line-broadening) separates the two peaks better, allowing them to be integrated separately. This will be discussed in more detail in Section 3.3.2.

The molar percentages of each species of fat on the alpha- and beta- positions are presented in Table 18.

Although analysis of the carbonyl region of ^{13}C NMR gives no information on the individual fatty acids present in the triglyceride, information on the positioning of the fatty acid species on the glycerol backbone can be acquired. To attain this information using chromatographic techniques (the recognised method for this type of analysis) a lengthy process of enzymatically directed cleavage of fatty acids on the alpha-position would be required followed by steps of separation and derivatisation. Using NMR to acquire this data removes the need for chemical and enzymatic reactions as the triglyceride is directly analysed.

	Sat	Mono	Poly	*Total*	Sat	Mono	Poly	*Total*
Arbequina	32.5	59.5	7.5	*99.5*	0.0	74.5	22.5	*97.0*
Carapelli Firenze	28.0	70.5	2.5	*101.0*	0.0	84.0	17.0	*101.0*
Cobram Estate	28.5	71.0	0.0	*99.5*	0.0	98.5	10.5	*109.0*
Colline Etrusche	28.5	71.5	0.0	*100.0*	0.0	100.0	0.0	*100.0*
Duano Basso	21.5	78.5	0.0	*100.0*	0.0	97.0	4.0	*101.0*
Hojiblanca	22.0	76.0	4.0	*102.0*	0.0	84.0	20.0	*104.0*
Olio Del Castello	21.0	79.5	4.0	*104.5*	0.0	95.0	14.0	*109.0*
Picual	28.0	67.5	8.5	*104.0*	0.0	84.0	21.0	*105.0*
Rustica	23.0	76.0	5.0	*104.0*	0.0	90.0	13.0	*103.0*
Safeways	26.5	73.5	3.0	*103.0*	0.0	88.0	11.0	*99.0*
San Leandro	27.0	73.5	0.0	*100.5*	0.0	93.0	7.0	*100.0*
Tescos	24.5	72.5	3.0	*100.0*	0.0	92.0	11.0	*103.0*
Tescos Finest Greek	31.5	78.5	0.0	*110.0*	0.0	100.0	5.0	*105.0*
Toscano	27.0	73.0	0.0	*100.0*	0.0	94.5	5.5	*100.0*

Table 18: Molar percentage of saturated (sat), mono-unsaturated (mono) and poly-unsaturated fat (poly) found on each position on the triglyceride backbone as determined by analysis of the carbonyl region of quantitative ^{13}C NMR of extra virgin olive oil. Values rounded to the nearest 0.5 %.

According to GCMS results, poly-unsaturated fatty acids should be present at between 0.8 and 9.8%. The carbonyl signal for poly-unsaturated fatty acids on the beta-position should be quantifiable at 2 %. Cobram Estate extra virgin olive oil contains the highest amount of poly-unsaturated fatty acids (9.8%w/w); this data is corroborated by the ^{13}C NMR which shows poly-unsaturated fatty acids to be present at 10.5 % (molar). When the differences in analytical methodologies are taken into consideration, the values from ^{13}C NMR and GCMS are to be considered to be with acceptable limits.

^{13}C NMR not only shows the same results as GCMS analysis of methyl esters prepared from the triglycerides but it allows identification of the fatty acids present on the glycerol backbone which is not possible by GCMS alone. T Marvomoustakos *et al* have shown that quantitative

analysis of the olefinic region of olive oils can detect the adulteration of olive oils with other oils in a semi-quantitative manner (Marvomoustakos *et al*, 2000).

3.3.2 Interpretation of olefinic region of ^{13}C NMR

Two fatty acids (oleate and linoleate) were detected in the ^{13}C NMR of each olive oil – both fatty acids were detected on the alpha- and beta-positions in the olefinic region. Both oleate and linoleate show distinct signals for each position on the triglyceride. Linoleate shows two signals for each position which do not overlap with any other signals. These signals can be integrated separately and an average value can be determined for each position allowing the determination of the distribution of linoleate. Oleate only has one pair of signals (one from the alpha-position and one from the beta-position), which do not overlap with others, but as this is well resolved, the signals can be integrated to calculate the distribution of oleate on the backbone.

All quantitative ^{13}C NMR spectra were processed using resolution enhancement techniques with line broadening set at 0.1 Hz and zero-filling at 256k. The increase in resolution by decreasing line broadening and increasing zero filling can be shown in Figure 36 and Figure 37. It can be seen that these resolution enhancement techniques increase the signal to noise ratio, but also allow more accurate integration of signals as the peak separation is closer to base-line resolution.

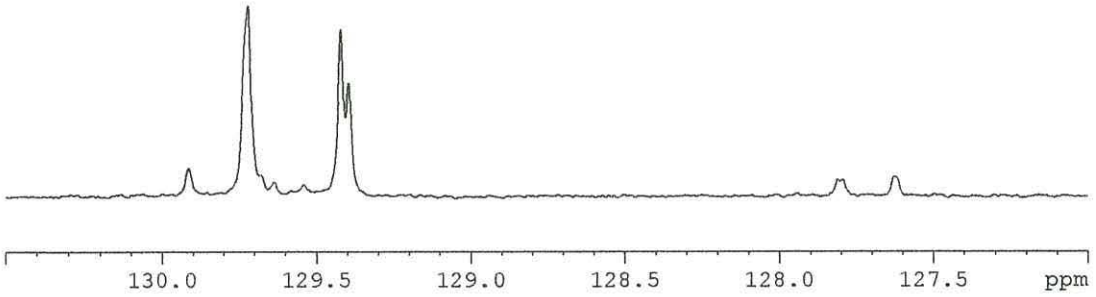


Figure 36: Olefinic region of an extra virgin olive oil. Line broadening 1.0Hz, zero filling 128k.

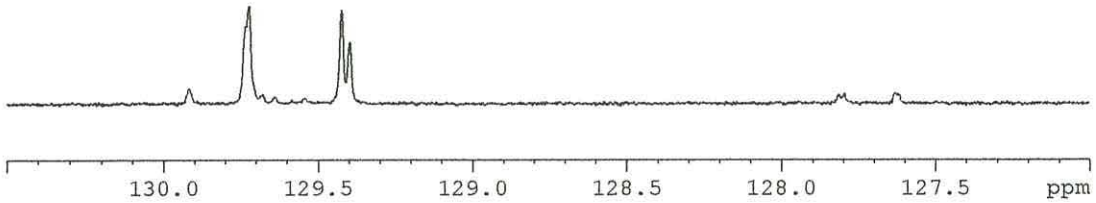


Figure 37: Olefinic region of an extra virgin olive oil. Line broadening 0.1Hz, zero filling 256k.

Investigating each region of the ^{13}C NMR spectrum separately (i.e. separate spectra for the carbonyl, olefinic and fingerprint regions) would reduce the need for these resolution enhancement techniques, as a smaller spectral width and a higher number of time domain points would increase the resolution. This would, however, increase the amount of time taken to acquire a full spectrum.

The signals for oleate and linoleate on the alpha- and beta-positions are shown in Figure 38.

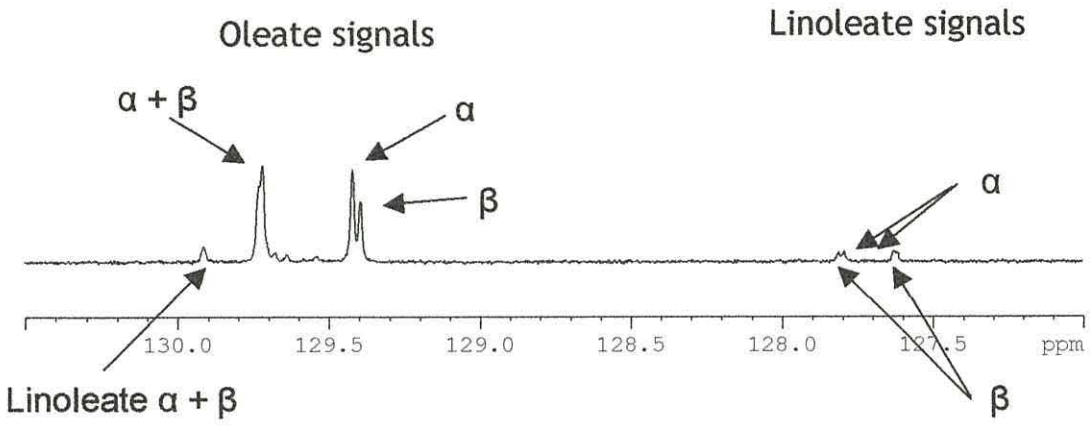


Figure 38: Olefinic region of ^{13}C NMR of an extra virgin olive oil showing the signals for the oleate and linoleate on the alpha- and beta-positions. The signal at 129.7 ppm is representative of oleate and linoleate on the alpha- and beta-positions, for this reason it cannot be integrated to calculate the ratios of either fatty acid present.

The distribution of oleate and linoleate on the triglyceride backbone of each olive oil is shown in Table 19. The values shown represent the percentage of each position occupied by each fatty acid.

	Oleate		Linoleate		Total	Total
	α	β	α	β	α-unsat	β-unsat
Arbequina	66.0	87.5	7.5	16.5	73.5	104.0
Carapelli Firenze	76.5	92.0	8.0	12.0	84.5	104.0
Cobram Estate	57.0	83.0	7.5	14.5	64.5	97.5
Colline Etrusche	69.0	86.5	5.5	11.0	74.5	97.5
Duano Basso	72.5	92.0	8.0	11.5	80.5	103.5
Hojiblanca	76.0	95.0	2.5	9.5	78.5	104.5
Picual	72.5	90.0	6.0	13.0	78.5	103.0
Rustica	75.5	99.0	4.0	9.5	79.5	108.5
Safeways	72.5	94.0	5.5	11.5	78.0	105.5
San Leandro	72.5	87.0	5.0	9.0	77.5	96.0
Tesco's	74.0	97.0	3.5	6.5	77.5	103.5
Tesco's Finest Greek	75.0	98.0	5.5	11.0	80.5	109.0
Toscano	79.5	98.0	5.5	11.0	85.0	109.0

Table 19: Unsaturated fat composition as determined by interpretation of the olefinic region of ^{13}C NMR spectra of extra virgin olive oils. Values represent the percentage of each position occupied by each fatty acid. Values are rounded to the nearest 0.5 %.

Taking Colline Etrusche extra virgin olive as an example, 69 % of all the alpha-positions in the triglyceride mixture are occupied by oleic acid and 5.5 % are esterified to linoleic acid. This means that 74.5 % of all fatty acids on the *sn*-1 and *sn*-3 positions are unsaturated. The *sn*-2 position is occupied almost exclusively by unsaturated fats, and 97.5 % of the beta-positions have been accounted for; 86.5 % are esterified to oleate and 11 % to linoleate. Bearing in mind that integration normally has an error of approximately 5 %, and the fact that no saturated fat signal was detected on the beta-position in the carbonyl region, it can be presumed that either the remainder of the *sn*-2 positions are occupied by other unsaturated fats or only oleate and linoleate are present. Comparison with the results from the GCMS of the methyl esters of Colline Etrusche extra virgin olive oil shows that another unsaturated fat (linolenate) was present at a total of 0.7 % which is too small to detect using ^{13}C NMR with these acquisition parameters. These smaller components would be detectable if longer acquisition times were used. If these two poly-unsaturated fats were only present on the *sn*-2 position, then that would account for 98.2 % of all the fatty acids on the beta-position.

In the ^{13}C NMR spectra of some olive oils, a signal in the olefinic region is detected that corresponds with that of palmitolein. Unfortunately, the resolution of this peak is poor, so there is no separation between the alpha- and beta- position signals, and quantitation of palmitoleate and identification of its distribution on the triglyceride backbone was not possible at such low concentrations.

Table 19 shows the percentages of the alpha- and beta-positions occupied by unsaturated fatty acids (both mono- and poly-unsaturated). The total figures for the beta-position do no add up to 100 % as would be expected. The range of values for the occupation of the beta-

position ranges from 96 – 109 %, which equates to a maximum error of 9 %. This error comes from several sources. The integration of the signals was done manually, which can induce error, the drift in the magnet, the signal to noise ratio, the change in temperature of the sample during acquisition all combine to increase the error in the readings taken.

These errors could be minimised by standardising the integration against a different signal from the beta-glycerol carbon, and by further increasing the number of scans and time domain points carried out during the analysis. This does however, increase the acquisition time, and this method was designed to be a quick look at the composition of the triglyceride.

3.3.3 Analysis of the 'fingerprint' region of ^{13}C NMR

The large number of signals present in the region of 40 – 20 ppm makes this region more challenging to interpret, but by careful comparison with the spectra of known standards, all the individual saturated fatty acids present at quantifiable concentrations (≥ 2 %) can be identified and their position on the glycerol backbone can be assigned.

Identification of fatty acids and their positions in this region has been carried out by Mannina *et al* (1999) but was performed by measuring the spectrum of a vegetable or olive oil, then adding a known standard to the sample and measuring the spectrum again. Identification of components was carried out by observing which peaks increased in intensity. This data was not quantitative however.

Throughout the analysis of the carbonyl and olefinic region of the extra virgin olive oil ^{13}C NMR spectra, the *sn*-2 position on the triglyceride had been fully characterised, i.e. the

percentage occupancy of the *sn*-2 position was determined to be ~ 100 %. All the fatty acids on the *sn*-2 position were determined to be unsaturated meaning that saturated fats are only present on the *sn*-1 and *sn*-3 positions. This makes the identification of fatty acids present in the fingerprint region much easier as it is possible to exclude all chemical shifts corresponding to saturated fats on the beta-position.

The main saturated fats found in olive oil are palmitate and stearate, found at 11.9 % and 2.9 % respectively as determined by GCMS of the fatty acid methyl esters prepared from the triglycerides. These are consistently found on the alpha-position, so signals for these two saturated fats were found which did not overlap with any other fatty acid found in the olive oils. A signal at 29.016 ppm was selected for palmitate, and one at 29.174 ppm for stearate. The data was handled and processed in the same way as that from the carbonyl and olefinic regions to give values which represent the percentage of the alpha position occupied by each fatty acid. Calculations showing how the integrals were manipulated are shown in Chapter 9. The distribution of saturated fats on the triglyceride is shown in Table 20. Only palmitate and stearate were present at concentration high enough to be detectable and quantifiable using this method. According to GCMS of the methyl esters, eicosanoate is present at a maximum concentration of 0.55 %, which is too low to detect using these acquisition parameters.

	Palmitate		Stearate		Total	Total
	α	β	α	β	α -sat	β -sat
Arbequina	21.0	0.0	4.5	0.0	25.5	0.0
Carapelli Firenze	19.5	0.0	7.8	0.0	27.3	0.0
Cobram Estate	21.5	0.0	4.5	0.0	26.0	0.0
Colline Etrusche	24.0	0.0	8.0	0.0	32.0	0.0
Duano Basso	14.5	0.0	3.5	0.0	18.0	0.0
Hojiblanca	15.0	0.0		0.0	15.0	0.0
Picual	20.0	0.0	3.5	0.0	23.5	0.0
Rustica	17.0	0.0	3.5	0.0	20.5	0.0
Safeways	19.5	0.0	3.5	0.0	23.0	0.0
San Leandro	20.5	0.0	5.5	0.0	26.0	0.0
Tesco's	15.5	0.0	4.0	0.0	19.5	0.0
Tesco's Finest Greek	19.5	0.0	3.0	0.0	22.5	0.0
Toscano	20.0	0.0	4.5	0.0	24.5	0.0

Table 20: Saturated fat composition as determined by interpretation of the 'fingerprint' region of ^{13}C NMR spectra of extra virgin olive oils. Values represent the percentage of each position occupied by each fatty acid. Values are quoted to the nearest 0.5 %.

So far in this work, four fatty acids; palmitate, stearate, oleate and linoleate have been characterised in all extra virgin olive oil samples. The distribution of these fatty acids on the triglyceride backbone is shown in Table 21.

	Palmitate		Stearate		Oleate		Linoleate		Total	
	a	ß	a	ß	a	ß	a	ß	a	ß
Arbequina	21.0	0.0	4.5	0.0	63.5	86.0	7.0	17.5	96.0	103.5
CarapelliFirenze	19.5	0.0	7.8	0.0	76.5	92.0	8.0	12.0	111.8	104.0
CobramEstate	21.5	0.0	4.5	0.0	57.0	83.0	7.5	14.5	90.5	97.5
CollineEtrusche	24.0	0.0	8.0	0.0	69.0	86.5	5.5	11.0	106.5	97.5
DuanoBasso	14.5	0.0	3.5	0.0	72.5	92.0	8.0	11.5	98.5	103.5
Hojiblanca	15.0	0.0	3.0	0.0	76.0	95.0	2.5	9.5	96.5	104.5
Olio DelCastello	16.0	0.0	3.0	0.0	78.5	101.0	6.0	8.0	103.5	109.0
Picual	20.0	0.0	3.5	0.0	72.5	90.0	6.0	13.0	102.0	103.0
Rustica	17.0	0.0	3.5	0.0	75.5	99.0	4.0	9.5	100.0	108.5
Safeways	19.5	0.0	3.5	0.0	72.5	94.0	5.5	11.5	101.0	105.5
SanLeandro	20.5	0.0	5.5	0.0	72.5	87.0	5.0	9.0	103.5	96.0
Tesco's	15.5	0.0	4.0	0.0	74.0	97.0	3.5	6.5	97.0	103.5
Tesco'sFinestGreek	19.5	0.0	3.0	0.0	75.0	98.0	5.5	11.0	103.0	109.0
Toscano	20.0	0.0	4.5	0.0	79.5	98.0	5.5	11.0	109.5	109.0

Table 21: Positional analysis of the most abundant fatty acids found in samples of extra virgin olive oil as determined by quantitative ^{13}C NMR.

Other fatty acids, namely palmitoleate are present at levels in some olive oils that should be detectable by ^{13}C NMR.

Four fatty acids are found to be present in all samples. Other fatty acids such as palmitoleate and eicosanoate were detected by GCMS analysis of the fatty acid methyl esters prepared from the triglycerides, but they were present at levels too low to be detected using the acquisition parameters used throughout this research.

Again, it is important to notice the error in the total values quoted for the alpha- and beta-positions. This error, although significant, does not detract from the fact that the data that can be acquired in two hours gives an over-all view of the major fatty acids present in olive oil

triglycerides, and their distribution on the glycerol backbone, a process which previously could only be done by multi-stage enzymatic reactions, derivatisation and chromatography.

3.4 Comparison of analytical methods

The data produced throughout this research has been acquired in several different ways, but they can all be compared as the molar percentage data is comparable with the weight percentage data. The data from the carbonyl region of the ^{13}C NMR can be compared with that from the ^{1}H NMR as they both show the percentage of the fatty acid species within the triglyceride. This can also be compared with the GCMS data, and data from the olefinic and fingerprint regions of the ^{13}C NMR. Although the data from ^{13}C NMR is specific to the positions on the glycerol backbone, the information from each position can be combined to give an overall molar percentage of each fatty acid or species present*.

The saturated fatty acids in a triglyceride can be quantified using GCMS, ^{1}H NMR and by investigating the carbonyl and fingerprint regions of ^{13}C NMR. The data obtained by each of these methods is shown in Table 22.

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* Values from carbonyl, fingerprint and olefinic regions of ^{13}C NMR were calculated as follows: Total = ((2 x alpha value) + beta value)/3

	^{1}H NMR	GCMS	^{13}C NMR CARBONYL	^{13}C NMR FINGERPRINT
Arbequina	12.7	14.5	21.7	17.0
Carapelli Firenze	13.8	15.5	18.6	18.2
Cobram Estate	14.2	15.5	19.0	17.3
Colline Etrusche	14.6	16.0	19.0	21.3
Duano Basso	11.5	16.0	14.3	12.0
Hojiblanca	17.4	14.0	14.4	15.7
Picual	15.3	16.5	17.9	12.0
Rustica	13.5	15.0	14.7	13.7
Safeway	13.7	15.0	17.1	15.3
San Leandro	14.3	16.0	17.9	17.3
Tesco	12.7	12.5	16.3	13.0
Tesco Finest Greek	14.2	15.0	19.0	15.0

Table 22: Comparison of the data for saturated fat content of extra virgin olive oils ^{13}C NMR (carbonyl and fingerprint regions), ^{1}H NMR and GCMS.

The data from the carbonyl region generally shows a higher percentage of saturated fat than that from the fingerprint region, the ^{1}H NMR and GCMS. It is expected to be higher than the results from the fingerprint region as the signal in the carbonyl region represents all the saturated fatty acids present, and the data from the fingerprint region only represents a combined value from palmitate and stearate. As other saturated fatty acids are present at levels too low to quantify, they are not included in the value for the fingerprint region. The only olive with a measurement of saturated fat higher in the fingerprint region than the carbonyl region is Colline Etrusche.

The mono-unsaturated fatty acids present in olive oils were quantified using ^{1}H NMR, GCMS and ^{13}C NMR concentrating on the carbonyl and olefinic regions. The data produced from each of these analytical methods is presented in Table 23. The difference between the values acquired by each method is less than that for the saturated fat; the standard deviation

is much lower on average. It is important to remember that the values quoted for ^{13}C NMR of the olefinic region are combined to give a total value for all mono-unsaturated fat present in the triglycerides. The data acquired from this region also conveys information on the positional distribution of the mono-unsaturated fats on the glycerol backbone.

	^{1}HNMR	GCMS	^{13}C NMR CARBONYL	^{13}C NMR OLEFINIC
Arbequina	80.9	78.0	65.5	72.0
Carapelli Firenze	77.1	75.5	74.1	80.5
Cobram Estate	74.1	75.0	77.4	66.4
Colline Etrusche	77.7	77.5	81.0	75.6
Duano Basso	81.3	76.5	84.3	78.0
Hojiblanca	72.0	70.0	76.6	81.0
Picual	76.2	77.0	79.2	77.5
Rustica	81.2	79.5	69.9	80.7
Safeway	78.5	79.0	77.9	78.0
San Leandro	79.0	79.0	77.2	78.5
Tesco	81.5	83.0	79.7	80.6
Tesco Finest Greek	78.4	78.5	78.1	80.0

Table 23: Comparison of the data for mono-unsaturated fat content of extra virgin olive oils ^{13}C NMR (carbonyl and olefinic regions), ^{1}H NMR and GCMS.

The percentages of poly-unsaturated fatty acids were calculated in the same way as those for mono-unsaturated fatty acids. The data acquired for the percentage of poly-unsaturated fatty acids by each analytical method is presented in Table 24. Again, the standard deviation of these values appeared to be less than for the saturated fatty acids. Once more, its is vital to remember that the data from ^{13}C NMR analysis does not just convey the total amount of each fatty acid and species present, but identifies its position on the glycerol backbone.

	^{1}HNMR	GCMS	^{13}C NMR CARBONYL	^{13}C NMR OLEFINIC
Arbequina	6.4	6.5	12.8	10.3
Carapelli Firenze	9.1	9.0	7.1	9.2
Cobram Estate *	1.7	9.5	3.5	10.0
Colline Etrusche *	7.8	6.5	0.0	7.4
Duano Basso *	7.2	8.0	1.3	9.0
Hojiblanca	10.6	11.5	9.0	3.0
Picual	8.4	7.0	12.1	7.9
Rustica	5.3	5.5	7.4	5.6
Safeway	7.8	6.0	5.6	7.3
San Leandro *	6.7	4.5	2.3	6.5
Tesco *	5.9	4.5	5.6	4.4
Tesco Finest Greek *	7.4	6.5	1.6	7.0

Table 24: Comparison of the data for poly-unsaturated fat content of extra virgin olive oils ^{13}C NMR (carbonyl and olefinic regions), ^{1}H NMR and GCMS.

There appear to be large inconsistencies in the data acquired for determining the amount of poly-unsaturated fatty acids present in olive oil. In the highlighted brands of olive oil, no poly-unsaturated fat was detected on the alpha-position in the carbonyl region. This in turn, leads to a lower value than expected for poly-unsaturated fatty acids being detected in the carbonyl region.

3.5 Comparison with data from the literature

Many papers have been published investigating the composition of olive oils. Comparing the data from these papers can be difficult as they all use slightly different methods for the analysis so direct comparisons cannot be easily made.

Marvomoustakos *et al.* (1999) studied the composition of oils from the main olive oil producing regions of Greece. They used ^{1}H NMR and GCMS to analyse these oils; ^{1}H NMR to analyse the triglycerides and GCMS for analysis of methyl esters produced by

transesterification of the triglyceride. Results were comparable to a satisfactory degree taking into account the different errors in the analytical and preparatory methods. Their results are shown in Table 25.

Fatty Acid	NMR Molar %	GCMS Weight %
Saturated	16.7	15.03
Oleic	74.7	73.38
Linoleic	7.1	8.17

Table 25: GCMS and NMR results for the average composition of Greek olive oils, Marvomoustakos *et al* (1999) .

A review article published by Li-Chan (1994), quotes ranges for the percentages of each fatty acid (along with other non-lipid components) found in a variety of olive oil samples. Oleic acid was most abundant at 63 - 83 %, linoleic acid 3.5 - 20.0 %, palmitoleic acid 0.5 - 3.0 %, palmitic acid 7.5 - 18.0 %, stearic acid 0.5 - 3.0 %, myristic acid was not detected, linolenic acid 0.1 - 0.6 %, eicosanoic acid 0.1 - 0.8 % and docosanoic acid trace – 0.8 % (Li-Chan, 1994).

Shaw *et al* (1997), published general figures for olive oil, not stating how they were established. They found that olive oil generally contained 15 % saturated fat, 70 % mono-unsaturated fat (mainly oleate) and 15 % poly-unsaturated fat (mainly linoleate) (Shaw *et al*, 1997).

Guillén *et al* (2003), published general results for olive oil and a more specific breakdown of the comparison between different brands of extra virgin olive oils and 'normal' olive oil. They found saturated fat to be present at 14.5 % with oleate, linoleate and linolenate present at

75.5 %, 7.5 % and 1.0 % respectively (Guillen and Ruiz, 2003). A more comprehensive study of olive oil and extra virgin olive oil is summarised in Table 26.

Olive oil	Saturated	Oleate	Linoleate	Linolenate
EV 1	10.9	84.5	4.1	0.5
EV 2	12.2	82.9	4.2	0.7
EV 3	12.5	81.7	5.3	0.5
EV 4	11.8	82.6	5.1	0.6
EV 5	10.9	84.5	4.3	0.4
EV 6	13.7	80.3	5.1	0.9
EV 7	17.0	71.4	10.7	1.0
EV 8	12.1	78.5	9.1	0.3
N1	14.0	77.8	7.6	0.6
N2	11.7	80.1	7.5	0.7
N3	12.1	79.6	7.6	0.8
N4	12.3	80.0	7.3	0.5
EV mean	12.6	80.8	6.0	0.6
'normal' mean	12.5	79.4	7.5	0.7

Table 26: Composition of olive oil and extra virgin olive oil as published by Guillén *et al*. EV denotes extra olive oil and N denotes 'normal' olive oil.

Comparing the published data outlined above shows a general recognition that saturated fat is present at 10 – 15 %, mono-unsaturated fat is present at 70 – 80 % and around 10 % is poly-unsaturated fat. This is in general agreement with the results acquired throughout these studies and the results from GCMS are in harmony with those from ^{1}H and ^{13}C NMR.

Of the studies outlined above, few of them discuss the positional distribution of fatty acids on the glycerol backbone, and those that do look at distribution, do not do so quantitatively.

3.6 Conclusion

This research shows that a new method has been developed for the analysis of olive oils.

- ^{1}H NMR can be used as a quick measure of the species of fatty acids present in olive oil

- qualitative ^{13}C NMR can be used to determine the individual fatty acids present in the olive oil
- qualitative ^{13}C NMR can establish the positional distribution of fatty acids on the glycerol backbone
- quantitative ^{13}C NMR can give information on the molar percentage of individual fatty acids present on each position. With extra scans, all fatty acids (i.e. those present at less than 2 %) would be detectable, and standard addition techniques could be used to quantify these components at low concentrations.

The information collated in this chapter has given an overview of the positional distribution of fatty acids found in olive oil. It has compared the data acquired by NMR techniques with that from widely accepted chromatographic methods. This data is backed up with that from the literature and agrees with the general composition of olive oil as published over the past 10 years.

The quantitative ^{13}C NMR method adds a new dimension to the data already available as it gives information not only on the fatty acids present and their positions on the glycerol backbone, but also quantifies the molar percentage of each fatty acid present on each position.

The method used throughout this research to analyse the fatty acids present in olive oil has many applications – not only to the field of olive oil, but to the analysis of triglycerides from any source.

There are already many established methods for analysing olive oils and fatty acid methyl esters and other derivatives from olive oils. The triglycerides can be analysed as a whole by HPLC or high temperature GC, or derivatives such as methyl esters can be analysed by GC. HPLC of triglycerides separates out each individual triglyceride present, so a large number of signals are detected. Interpretation of these chromatograms can be complicated as comparison of retention times with known standards is required for the identification of components. LCMS can be used to assist this identification, but differentiation between positional isomers such as POP and PPO would be difficult using this method. If a triglyceride was detected which was not identifiable by comparison with known standards and couldn't be identified by mass spectrometry, then the use of ^{13}C NMR could identify which fatty acids were present and their distribution on the glycerol backbone.

Hyphenation of analytical techniques is becoming increasingly popular, and using a flow system NMR with HPLC, identification of each component of a triglyceride would be possible. This, coupled to a mass spectrometry system would hopefully solve all possible structural identification problems that could be encountered for the analysis of triglycerides. If flow NMR was not available, preparative HPLC could be used to isolate the unknown fraction which could then be analysed by ^{13}C NMR to identify the structure of the triglyceride.

The quantitative positional analysis of olive oils as presented in this chapter investigates only a small amount of the data available from such spectra. If more instrument time was available for acquisition of spectra, then finer detail would be available for interpretation. With

spectra acquired over a longer time, fatty acids present in trace amounts (at levels of 1 % or less) would be detectable, and possibly even quantifiable.

Because ^{13}C NMR is specific to an isotope that is present only at 1 % abundance, being able to detect and quantify fatty acids at levels of approximately 2 % of a crude triglyceride mixture is quite an achievement, and as technology advances and magnets become larger and stronger, it is my hope that one day ^{13}C NMR will be a widely used technique for the quantitative analysis of complex mixtures such as natural lipid samples.

4 Analysis of Milk from Aber Ogwen Farm

4.1 Introduction

The fatty acid composition of milk has been studied for several years by various reseach groups. The purpose of continuing the investigation into the lipid composition of milk was to apply ^{1}H NMR and quantitative and qualitiative ^{13}C NMR to the field, to allow the identification and quantification of the positional distribution of fatty acids on the glycerol backbone. The results from this study were compared with those from the more standard method of analysis, namely chromatography.

The purpose of the study outlined in this chapter was to establish a method to investigate any seasonal variations in the fatty acid composition of the milk.

It is known that milk fat is complicated in its fatty acid and triglyceride composition (Gunstone, 2000). Not only does it contain the usual C16 and C18 fatty acids commonly found in most food products, it contains a number of shorter chain fatty acid (C4 to C14), as well as *trans*-fatty acids (mainly mono-unsaturated), and small quantities of branched and oxygenated fatty acids.

The average composition of whole cows milk as defined by www.nutrition.org is shown in Table 27.

Sat %	64.8	Mono %	28.2	Poly %	6.9
C4:0	2.15	C16:1	0	C18:2	3.69
C6:0	2.15	C18:1	24.92	C18:3	2.5
C8:0	2.15	C20:1	0	C18:4	0
C10:0	2.15	C22:1	0	C20:5	0
C12:0	2.15			C22:5	0
C14:0	0.92			C22:6	0
C16:0	25.54				
C18:0	11.08				

Table 27: Average composition of whole cows milk. (nutrition.org)

As discussed previously, results aquired by NMR are quoted as molar percentages and those from GCMS are quoted as weight percentages. In the study of olive oil outlined in Chapter 3, the acyl cahins were all between 16 and 18 carbons in length, but in milk, the length of the acyl chains vary from between 4 carbons to 22 carbons, meaning the molecuar weights vary greatly. This means the molar percenatage results acquired from ^{13}C and ^{1}H NMR cannot be directly compared with those from GCMS.

To study the seasonal variation of milk fat composition, milk samples were taken in the last week of each month for one year from Aber Ogwen Farm, (Tal-y-bont, Gwynedd). Milk samples were collected from August 2004 to July 2005. The milk was taken from the storage tank, two to three hours after milking, and so was representative of the milk from the whole herd. This milk was not homogenised or pasteurised before sampling, but was stirred well before samples were taken for analysis to ensure a homogeneous sample.

The lipids were extracted from the milk using a Folch-style extraction. The crude lipid was then analysed by ^{1}H NMR and qualitative and quantitative ^{13}C NMR.

Butyl esters were formed from the triglycerides by transesterification using sodium butoxide. These fatty acid esters were separated and identified using GC/MS carried out on an HP5890 GCMS with a CP Select-FAME column. The identificaion of components was by comparison with known standard retention times and library comparisons with a mass spectrometry database.

It was necessary to produce fatty acid butyl esters from fats from dairy triglyceride sources as they contain shorter chain fatty acids, for example butyric and hexanoic acids, which when transesterified to form methyl esters become highly volatile and can easily be lost whilst working up the reaction mixture and through general sample handling. The loss of these short chain fatty acids is avoided by preparing butyl esters, although this presents its own problem. The total removal of butanol from the butyl esters is not easy, and the residual butanol can mask the butyl butyrate in the chromatogram as they have similar retention times. The signals from these two components can be deconvoluted in a mass spectrometer using selective ion monitoring.

The herd at Aber Ogwen farm are fed on a food concentrate while they are being milked twice daily. In the spring and summer months they are allowed out to pasture and in the autumn months they spend the night indoors, and during the winter, they remain inside throught the day. During the study period, the cattle were kept in sheds from October 22nd to April due to serious flooding of the local area. This caused the cows to come in for the winter earlier than usual.

The food concentrate fed to the dairy herd was supplied by HJ Lea Oakes Ltd (Cheshire) and is described as ACTION HDF 18 DAIRY NUTS. This food concenrate is made up of:

Citrus pulp; Wheatfeed; Palm Kernel Expeller; Low Glcosinolate Rapeseed*, extracted; Soya*, ext toasted; Molasses; Confectionary by-product; Distillers Dk Grains; Breakfast cereal; Soya Hulls; Wheat; Maize; Gluten Feed; Calcium carbonate; Vegetable oil blend*; Magnesium Oxide; Sodium Chloride; Vitamin Premix.*

The nutrition information of the feed pellets is outlined in Table 28.

Component	Composition
Oil	5.5 %
Protein	18.0 %
Fibre	10.5 %
Ash	8.5 %
Moisture	14 %
Magnesium	0.75 %
Vitamin A (as retinol)	10000µg/kg
Vitamin D3 (as cholecalciferol)	2500µg/kg
Vitamin E (as alpha-tocopherol)	35µg/kg
Selenium (as sodium selinate and Sel-Plex)	0.6mg/kg
Copper (as copper sulphate, carbonate and Bioplex)	60mg/kg

Table 28: Nutrition composition of Action HDF 18 Dairy Nuts.

When the cattle were not in stalls and allowed out to pasture, they eat grass and other plants found naturally in the pasture *ad libitum*, they also had access to silage, hay and straw throughout the year, even when kept inside.

Milk fat contains a large number of fatty acids, and there are considered to be eleven that are found in appreciable amounts (over 1 %). These are butanoic acid (C4:0), hexanoic acid (C6:0), octanoic acid (C8:0), decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0),

* Components marked are sources of lipids.

pentadecanoic acid (C15:0) palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1 c9), linoleic acid (C18:2), conjugated linoleic acid (C18:2 c9,t11) and linolenic acid (C18:3) (Creamer and MacGibbon 1996).

4.2 Results of GCMS analysis of butyl esters

Butyl esters of fatty acids were produced by transesterificaition of the triglycerides of milk fat. These butyl esters were analysed by GCMS. The data collected from the butyl esters of milk from Aber Ogwen farm supports that found by Creamer *et al* (1996) and is shown in Table 29. The way the species of fatty acids varies within milk month-to-month can be seen in Figure 39.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	*Mean*
C6:0	2.64	1.24	1.64	2.95	1.23	1.48	1.59	1.23	1.17	1.12	1.05	5.43	*1.9*
C8:0	0.2	1.03	1.76	2.12	1.06	0.83	2.08	2.33	1.28	1.32	1.14	5.76	*1.7*
C10:0	3.65	2.18	0.96	0.13	2.44	1.68	1.43	0.62	0.94	1.34	2.98	5.49	*2.0*
C12:0	4.64	3.47	4.08	4.15	4.11	3.36	3.43	3.86	3.89	4.2	4.4	4.94	*4.0*
C14:0	14.74	12.59	12.36	12.42	12.13	11.76	12.29	13.84	13.91	13.45	13.36	13.24	*13.0*
C14:1	ND	1.16	1.56	1.69	0.22	0.18	ND	ND	0.16	0.42	0.56	1.23	*0.8*
C15:0	ND	ND	0.17	1.25	1.15	0.16	0.11	0.98	1.43	1.19	1.39	ND	*0.9*
C16:0	42.67	36	34.6	33.89	30.8	32.01	34	36.93	30.03	32.53	33.76	34.69	*34.3*
C16:1	1.78	1.79	1.72	1.66	1.61	2.18	2.31	3.56	3.06	2.84	2.22	1.81	*2.2*
C18:0	12.27	13.56	15.8	12.53	12.56	13.14	14.6	18.9	15.32	12.1	11.03	8.76	*13.4*
C18:1 trans	ND	2.03	1.03	1.41	3.56	3.95	3.17	2.96	2.48	2.23	2.3	ND	*2.5*
C18:1 cis	17.4	23.84	24.63	24.84	26.28	27.69	27.6	29.8	26.2	24.3	23.41	16.83	*24.4*
C18:2	ND	0.89	0.74	0.41	1.61	1.05	0.92	0.87	1.28	1.43	1.59	1.81	*1.1*
C18:2 CLA	ND	0.22	0.07	0.54	0.34	0.13	ND	ND	0.21	0.28	0.32	ND	*0.3*
C18:3	ND	ND	ND	ND	0.77	0.38	ND	ND	0.16	0.32	0.48	ND	*0.4*

Table 29: Fatty acid composition of milk from Aber Ogwen Farm as determined by GCMS analysis of butyl esters quoted as weight percentage. ND = none detected.

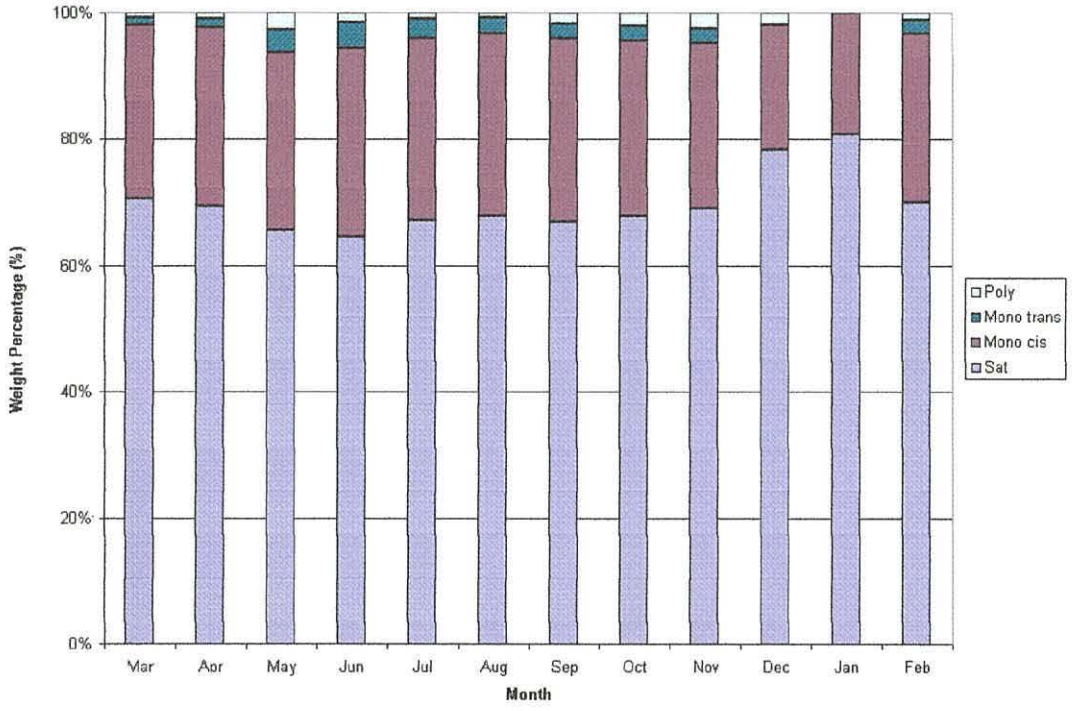

Figure 39: Species composition of milk as determined by GCMS of the butyl esters formed from milk triglycerides.

Figure 39 shows how the fatty acid species present in milk fat vary from month to month. It can be seen that saturated fatty acids consistently made up the bulk of milk fat. The next most dominant species was *cis*-monounsaturated fatty acids. *Trans*-monounsaturated fatty acids and polyunsaturated fatty acids were present in small quantities in all months except January and December. Polyunsaturated fatty acids were present in December however.

A general trend can be seen that during the summer months, when the cows were out to pasture, the percentage of saturated fatty acids found in their milk was reduced and the percentage of *cis* mono- and polyunsaturated fatty acids and *trans* monounsaturated fatty acids increased.

The composition of milk from each month is represented graphically in Figures 41 to 52 and an over all summary is presented in Figure 40.

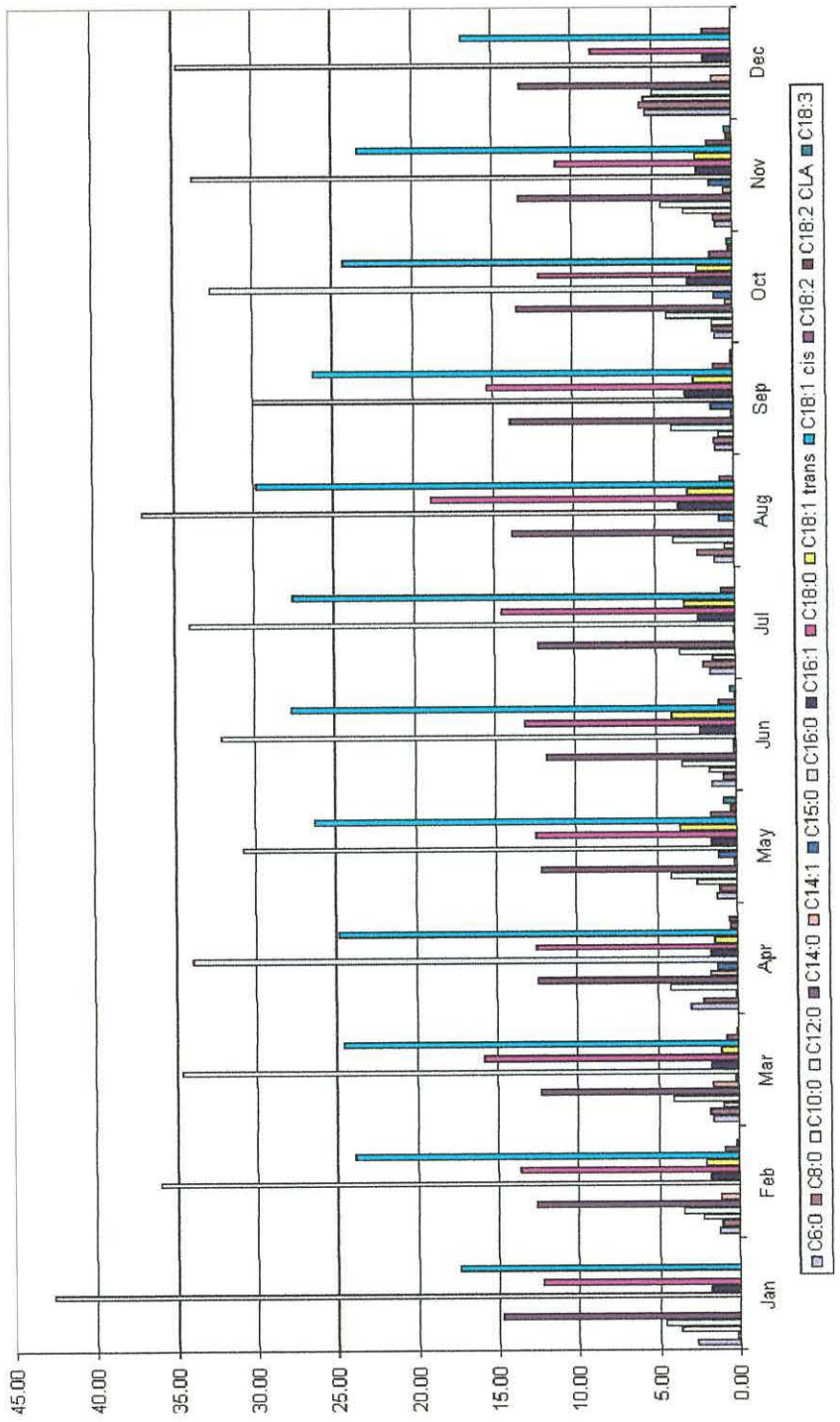

Figure 40: Monthly variation in fatty acid composition of milk from Aber Ogwen Farm as determined by GCMS.

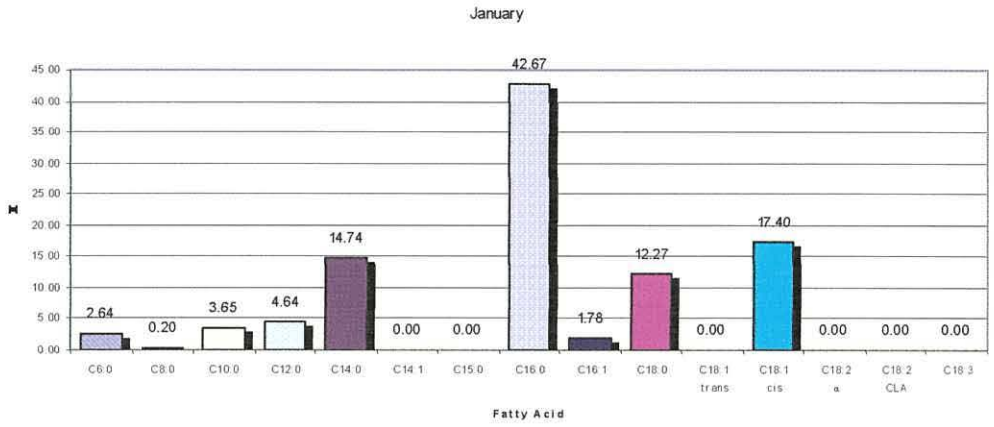

Figure 41: Composition of milk from Aber Ogwen Farm in January as determined by GCMS of butyl esters formed from milk triglycerides.

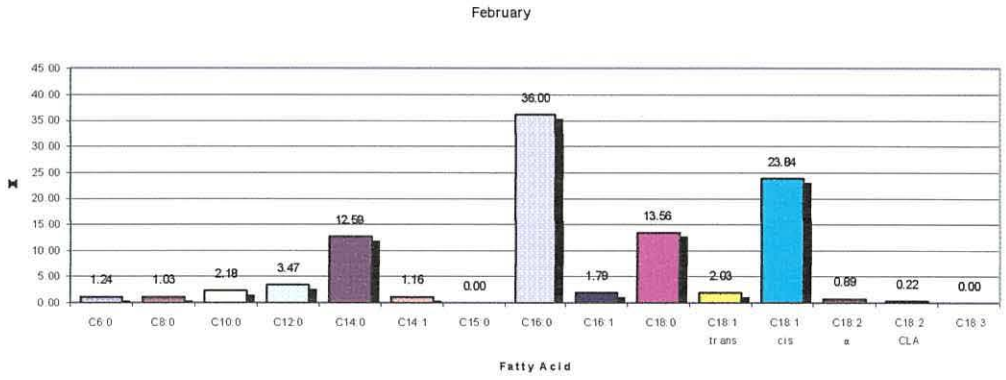

Figure 42: Composition of milk from Aber Ogwen Farm in February as determined by GCMS of butyl esters formed from milk triglycerides.

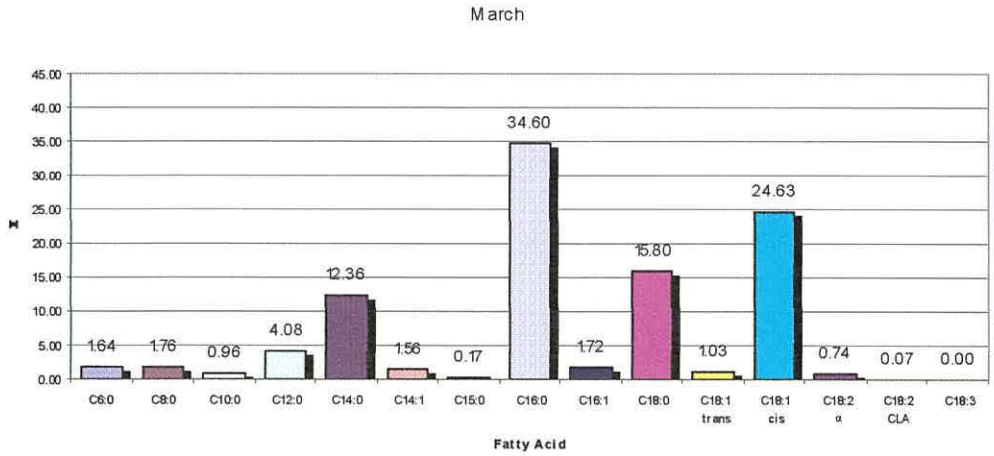

Figure 43: Composition of milk from Aber Ogwen Farm in March as determined by GCMS of butyl esters formed from milk triglycerides.

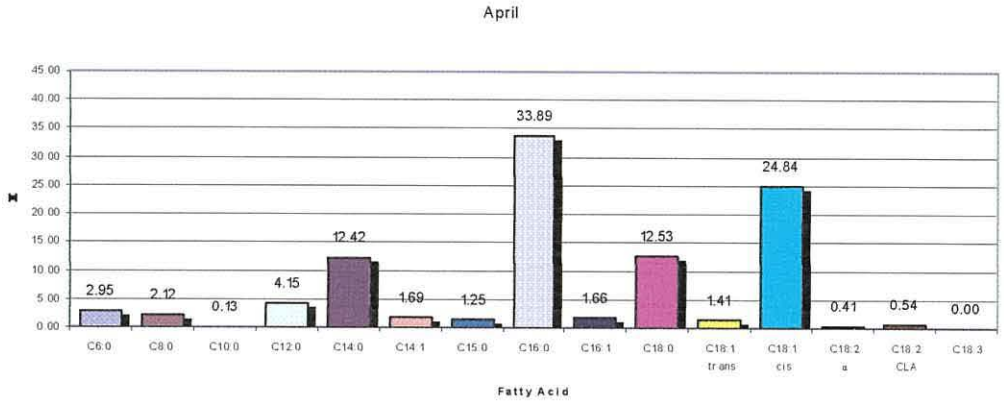

Figure 44: Composition of milk from Aber Ogwen Farm in April as determined by GCMS of butyl esters formed from milk triglycerides.

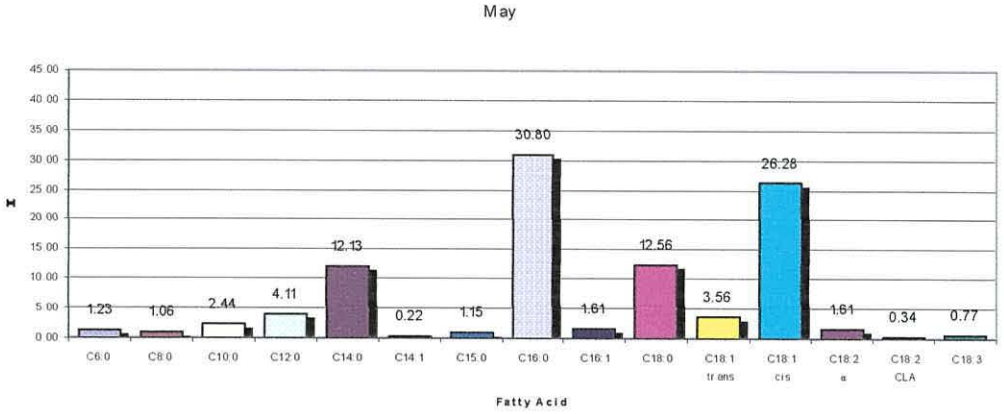

Figure 45: Composition of milk from Aber Ogwen Farm in May as determined by GCMS of butyl esters formed from milk triglycerides.

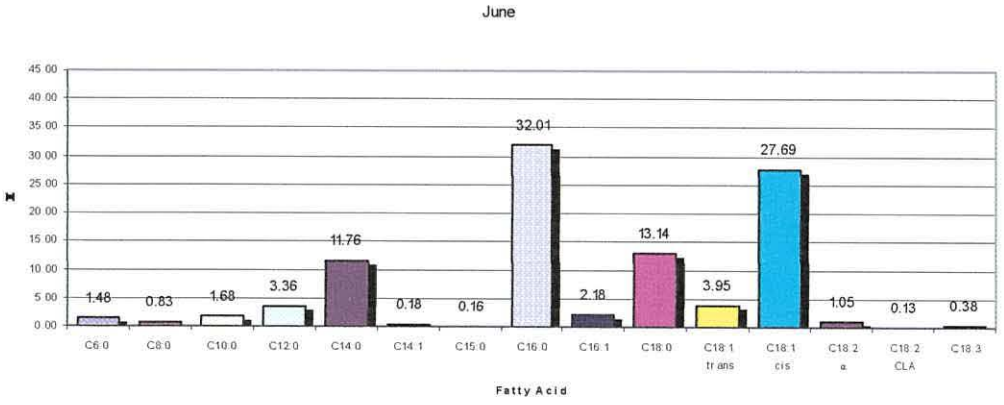

Figure 46: Composition of milk from Aber Ogwen Farm in June as determined by GCMS of butyl esters from milk triglycerides.

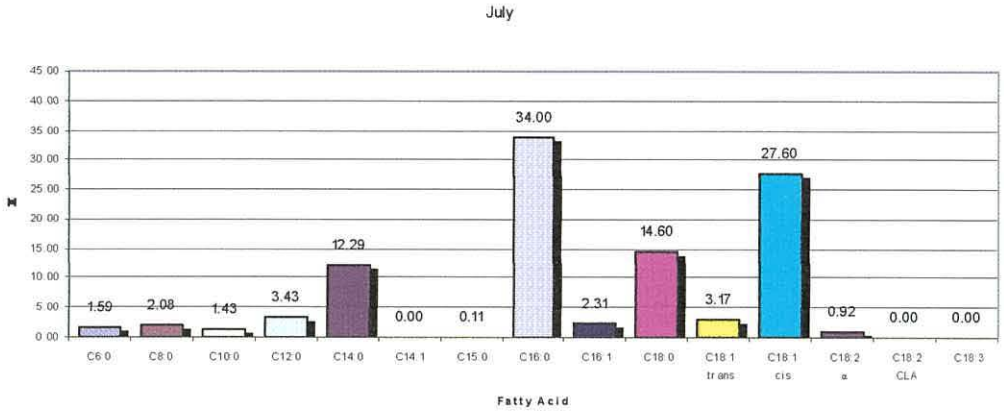

Figure 47: Composition of milk from Aber Ogwen Farm in July as determined by GCMS of butyl esters formed from milk triglycerides.

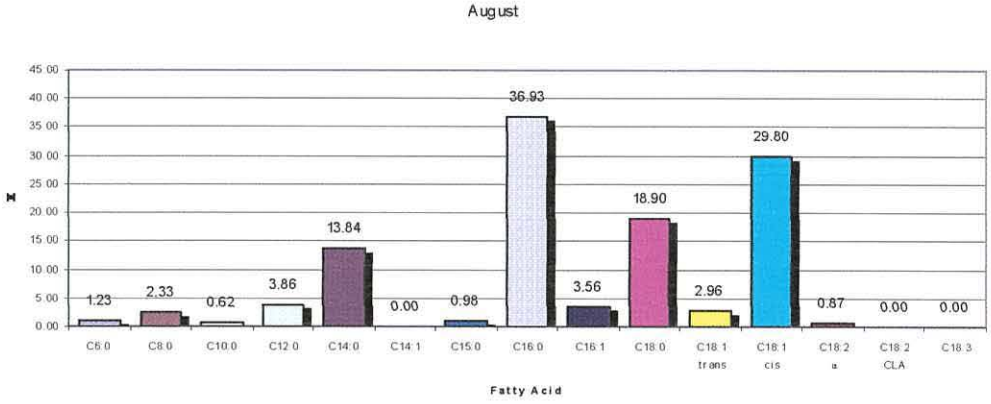

Figure 48: Composition of milk fat from Aber Ogwen Farm in August as determined by GCMS of butyl esters formed from milk triglycerides.

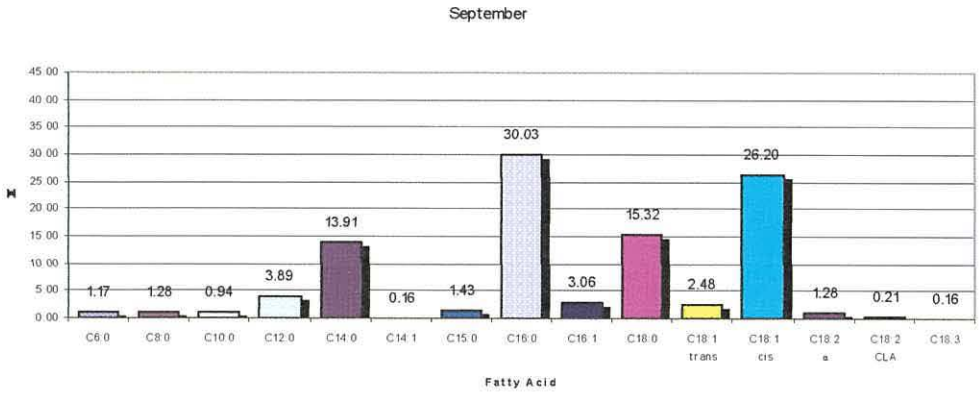

Figure 49: Composition of milk fat from Aber Ogwen Farm in September as determined by GCMS of butyl esters formed from milk triglycerides.

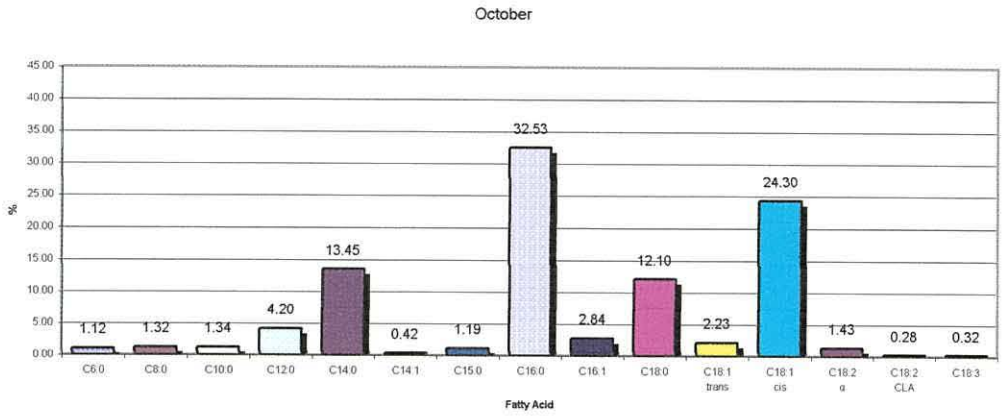

Figure 50: Composition of milk from Aber Ogwen Farm in October as determined by GCMS of butyl esters formed from milk triglycerides.

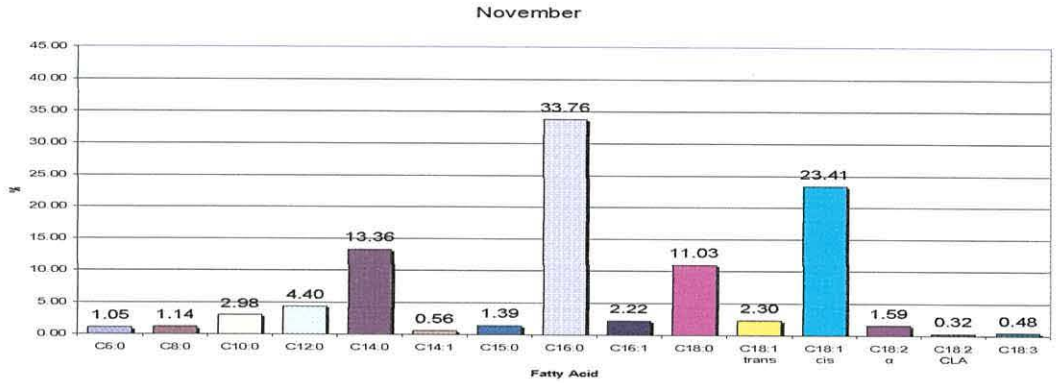

Figure 51: Composition of milk from Aber Ogwen Farm in November 2004 as determined by GCMS of butyl esters formed from milk triglycerides.

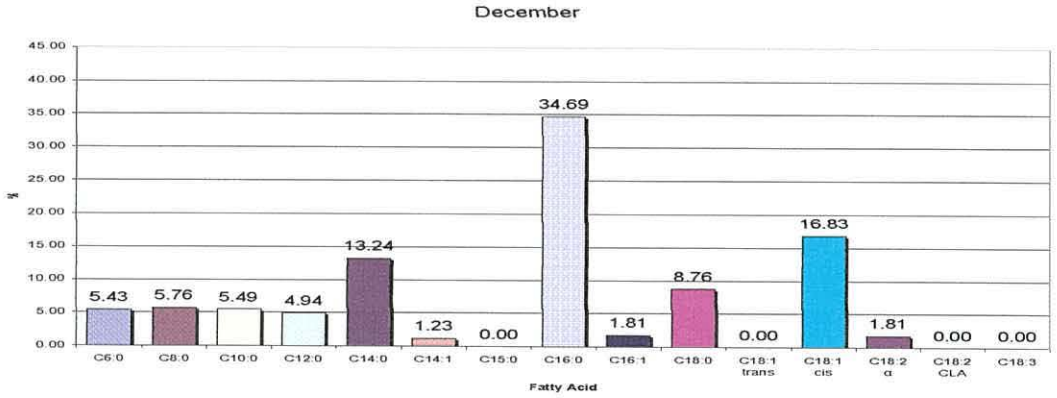

Figure 52: Composition of milk from Aber Ogwen Farm in December 2004 as determined by GCMS of butyl esters formed from milk triglycerides.

Figure 53 shows the seasonal variation in fatty acid composition of milk fat from Aber Ogwen farm as determined by GCMS analysis of butyl esters. It shows that oleate and stearate follow the same trend, as one increases, so does the other. Myristate and laurate remain roughly constant as the months go by and palmitate constantly makes up the highest proportion of milk fat.

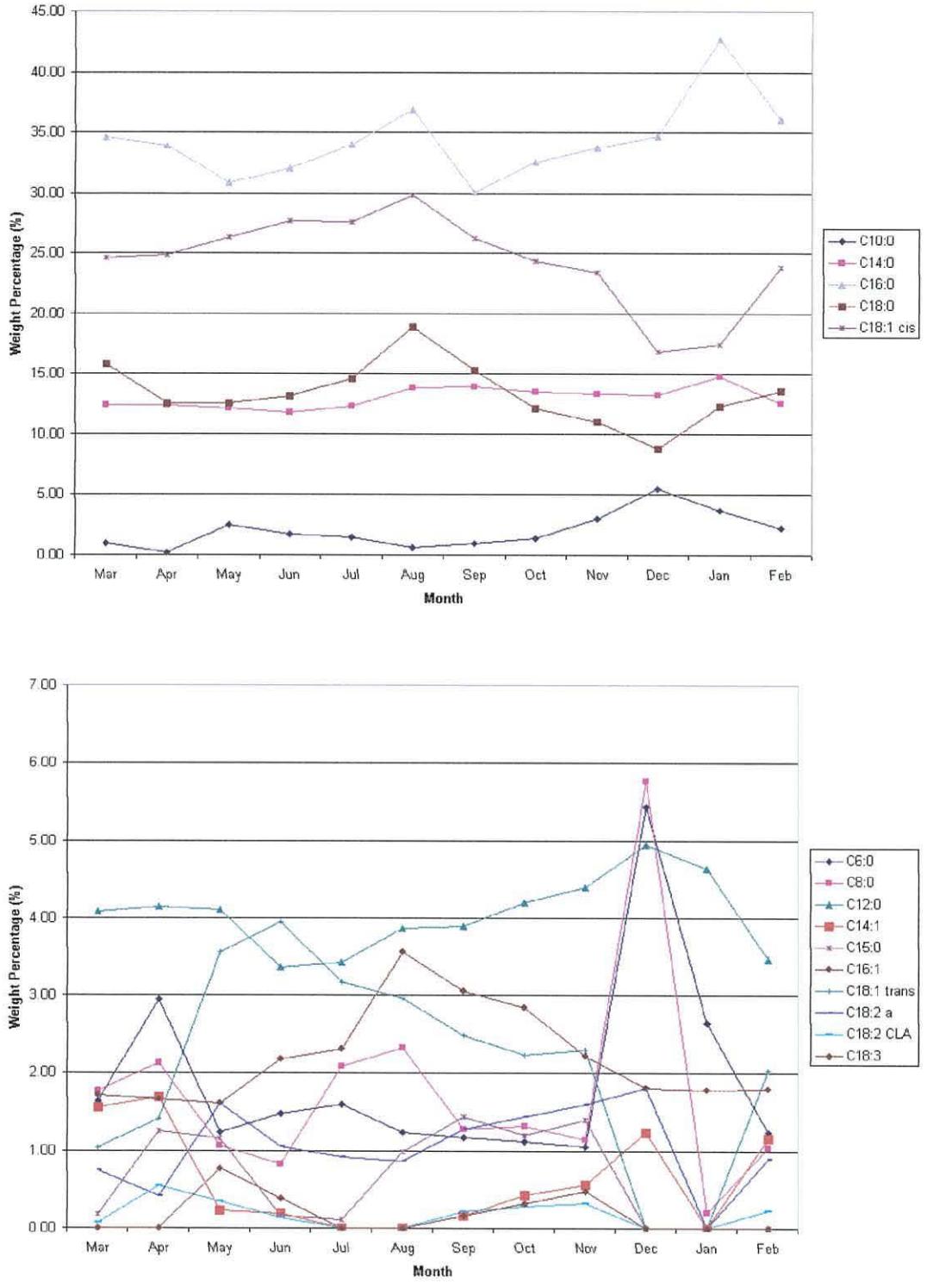

Figure 53: Seasonal variation in fatty acid composition of milk from Aber Ogwen Farm as determined by GCMS of butyl esters formed from milk triglycerides. The first shows fatty acids present in high concentrations, the second shows fatty acids present in low concentrations.

4.3 ^{1}H NMR of milk triglycerides

In the past, 2D ^{1}H NMR has been used to quantify the fatty acids present in milk samples from the cow and buffalo milk. The results allowed the milk from different species to be distinguished (Brescia *et al*, 2004). In this work, cows milk triglycerides were analysed by ^{1}H NMR and the integrals were manipulated using simultaneous equations to calculate the percentage of each species of fat present. As explained in Chapter 2, this method only accounts for saturated fat and unsaturated fat with *cis* double bonds. Milk fat, however, contains some *trans* fat and conjugated fats. The fatty acid species detected in milk fat are shown in Table 30 and Figure 54.

	sat %	mono %	bis %	poly %	Total
January	84.0	15.1	0.7	0.1	100
February	74.7	23.4	1.3	0.6	100
March	70.5	27.7	1.2	0.7	100
April	70.7	28.6	0.5	0.2	100
May	75.2	24.4	0.3	0.1	100
June	74.8	25.1	0.1	0.0	100
July	72.3	26.8	0.6	0.2	100
August	67.9	30.1	1.0	1.0	100
September	75.8	22.6	0.9	0.8	100
October	81.3	17.3	0.8	0.6	100
November	77.7	21.7	0.5	0.1	100
December	75.8	22.6	1.0	0.6	100
mean	75.1	23.8	0.8	0.4	

Table 30: ^{1}H NMR data for fatty acid species in milk triglycerides, data normalised to 100 %.

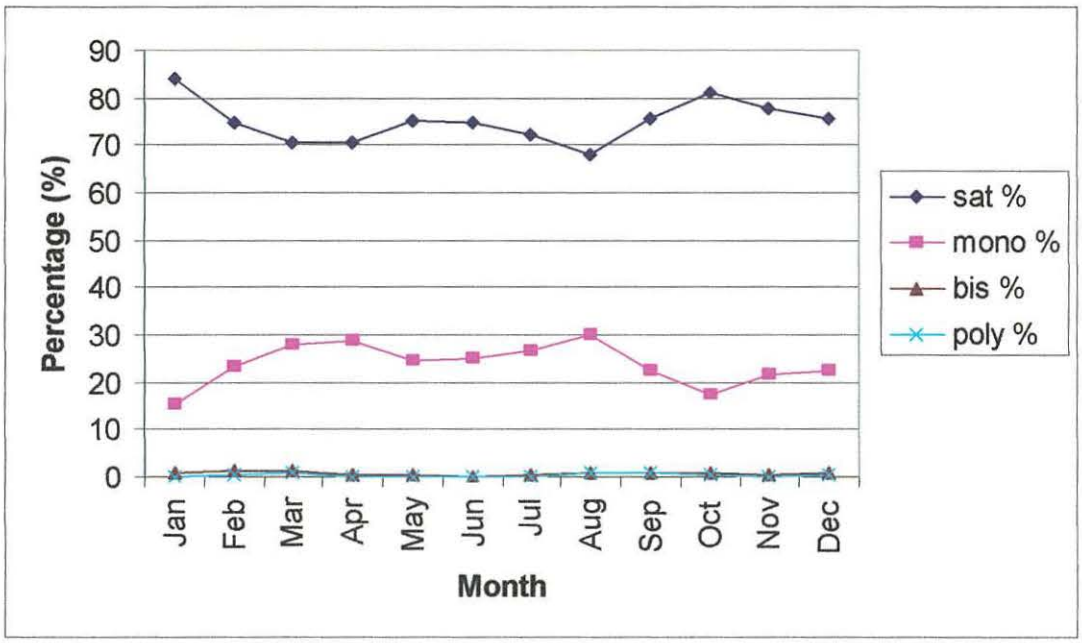

Figure 54: Graphical representation of the fatty acid species found in milk triglycerides as determines by ^{1}H NMR.

Analysis of these results show that the bis- and poly-unsaturated fat concentration stays fairly constant throughout the year with bis-unsaturated fat varying in concentration from 0.1 to 1.3 % with a mean concentration of 0.7 % and poly-unsaturated fat varying between 0.0 and 1.0 % with a mean concentration of 0.4 %.

The concentrations of saturated and mono-unsaturated fat almost exactly mirror one another; when one increases, the other decreases by the same amount. The percentage of saturated fat present in milk is between 70 and 80 % for most of the year, with the concentration dropping below 70 % only in April and August. The concentration of mono-unsaturated fat present remains in the range of 14.5 and 29.7 % for the whole year, the minimum being in the middle of winter and the maximum in the height of summer.

Comparison of the results from GCMS of butyl esters and from ^{1}H NMR shows similar results.

4.4 ^{13}C NMR of milk triglycerides

Analysis of milk triglycerides by ^{13}C NMR can provide a lot of information on which individual fatty acids are present in a milk sample, which position they occupy on the triglyceride backbone and what proportion of that position they occupy. Each region (carbonyl, olefinic, acyl chain, fingerprint and terminal methyl) can give different and complementary data. The data acquired from ^{13}C NMR has been previously been shown to distinguish between milk from different species of animals (Andreotti *et al*, 2002).

Butyric acid, as a very short chain fatty acid has chemical shifts which stand out as unique within the ^{13}C NMR of milk. As milk fat contains short chain fatty acids the ^{13}C NMR signals for butyric acid can be easily identified as they have significantly different chemical shifts from the longer chain fatty acids. From careful analysis of all the signals relating to butyric acid in the ^{13}C NMR spectra of milk triglycerides, it can be seen that butyrate is found only on the alpha-position, and it is known from the literature that this short chain fatty acid is found predominantly on the *sn*-3 position.

The ordering of fatty acids on the triglyceride backbone is not random; it is directed by enzymatic processes in the animal. Short chain fatty acids are directed to the *sn*-3 position and long chain fatty acids are directed onto the *sn*-1 position (foodsci.uogeulph.ca). Researchers at The Department of Dairy Science and Technology at the University of Guelph have shown that up to 97 % of butyric acid is found on the *sn*-3 position, and 58 % of stearic acid is to be found on the *sn*-1 position.

4.4.1 Interpretation of Carbonyl Region

The first thing to note about the carbonyl region of milk triglycerides is the carbonyl signal for butyrate, which has a chemical shift of 172.7 ppm. This is situated between the two groups of signals for the alpha- and beta-carbonyl carbons. The integral of the signals detected at 172.7 ppm in the ^{13}C NMR of milk fat from each month corresponds to the integrals of the other signals from butyric acid on the alpha-position. No other signals unique to short chain fatty acids were detected in the carbonyl region, the carbonyl signals for the other short chain fatty acids present in milk triglycerides fall in the same region as the medium and long chain fatty acids, so cannot be distinguished in this area.

No signals for poly-unsaturated fats were detected, but these are present at low quantities when the milk fat is looked at as a whole. This is to be expected as detection of poly-unsaturates in the carbonyl region at levels less than 2 % is difficult due to poor resolution of mono-unsaturated and poly-unsaturated fat carbonyl signals.

The distribution of fatty acid species as determined by analysis of the carbonyl region of ^{13}C NMR spectra of milk triglycerides is shown in Table 31 and Figure 55. It can be seen that the total values for each position do not add up to 100 %; this is due to the inherent error in the NMR method.

	a-sat	a-mono	butyrate	total	b-sat	b-mono	total
January	68.5	16.0	14.0	**98.5**	79.5	14.0	**93.5**
February	63.0	22.5	14.0	**99.5**	75.0	20.5	**95.5**
March	69.0	22.0	15.5	**106.5**	82.0	20.0	**102.0**
April	62.0	29.0	15.5	**106.5**	77.0	23.0	**100.0**
May	62.5	22.0	15.5	**100.0**	77.0	25.0	**102.0**
June	60.5	18.0	14.5	**93.0**	68.5	22.5	**91.0**
July	63.5	22.5	15.0	**101.0**	73.5	25.5	**99.0**
August	69.5	28.5	16.5	**114.5**	81.0	27.0	**108.0**
September	62.0	27.5	15.5	**105.0**	76.0	23.0	**99.0**
October	69.0	24.5	15.5	**109.0**	93.5	15.0	**108.5**
November	64.0	20.0	14.5	**98.5**	80.0	15.5	**95.5**
December	68.0	17.0	11.5	**96.5**	80.5	14.0	**94.5**

Table 31: Fatty acid species present on each position as determined by analysis of the carbonyl region of ^{13}C NMR spectra of milk triglycerides. * Figures quoted for saturated fatty acids (sat) do not include butyrate. Figures quoted are a molar percentage of each position occupied by each fatty acid species. Values are rounded to the nearest 0.5 %.

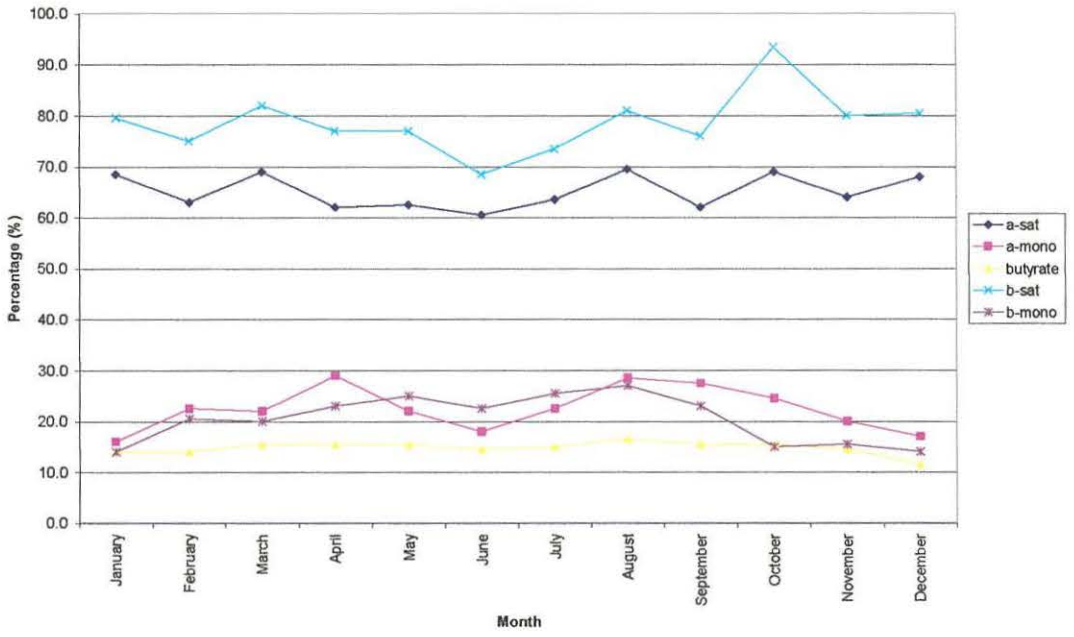

Figure 55: Seasonal variation of the fatty acid species present in milk, as determined by analysis of the carbonyl region of ^{13}C NMR. Saturated fat does not include butyrate.

From looking at the carbonyl region of the ^{13}C NMR spectra, it can be seen that saturated fat is present on the alpha-position at an average of 65.1 % and 78.6 % on the beta-position.

Mono-unsaturated fat is found on the alpha-position at an average of 22.5 % and 20.4 % on the beta-position. Butyric acid is only to be found on the alpha position at a mean concentration of 14.8 %.

4.4.2 Interpretation of Olefinic Region

The olefinic region of ^{13}C NMR spectra of milk triglycerides shows only one unsaturated fat present at levels detectable using these acquisition parameters. Oleate is detected in milk from Aber Ogwen Farm in all 12 samples analysed. Oleic acid shows different, characteristic signals when present on the alpha- and beta-positions. Because of this, the proportion of oleate present on each position can be determined. The proportions of oleate present as a percentage of the total fatty acids present on each position are shown in Table 32 and Figure 56.

	α-Oleate	β-Oleate
January	11.5	12.4
February	15.0	15.6
March	20.3	13.2
April	19.8	20.8
May	18.5	16.9
June	15.8	17.1
July	18.2	15.3
August	21.0	14.3
September	17.7	13.7
October	18.2	13.9
November	16.5	16.4
December	15.9	12.4

Table 32: Percentage of oleate present on the alpha- and beta-positions of milk triglycerides as determined by interpretation of the olefinic region of ^{13}C NMR. Each value is quoted as a percentage of the total number of fatty acids on each position occupied by oleate.

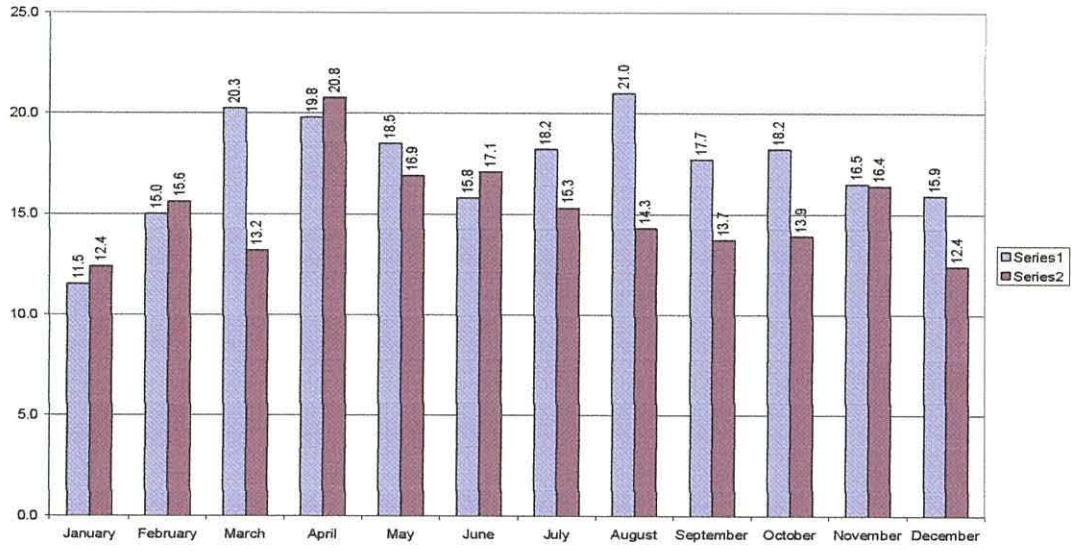

Figure 56: Monthly variation in oleate concentration on the alpha- and beta-positions in milk from Aber Ogwen farm as determined by analysis of the olefinic region of ^{13}C NMR.

^{1}H NMR of milk fat triglycerides shows bis- and poly-unsaturated fats to be present at a mean total of 1.2 % of the total fat which is too small to detect using ^{13}C NMR as this technique examines an isotope which is only present at 1 % of the total number of carbon atoms present. If it were one single poly-unsaturated fat component present at 1.2 %, it would be detectable, but probably not quantifiable with these acquisition parameters.

4.4.3 Detection of butyrate in milk triglycerides

Butyric acid was detected in all twelve milk samples from Aber Ogwen Farm. As butyric acid contains only 4 carbons, its ^{13}C NMR spectrum is much simpler than that of other fatty acids. A ^{13}C NMR spectrum of tributyrin shows one signal for every carbon environment present in the molecule (i.e. 15 carbons, 10 environments). Tributyrin is the only triglyceride studied in the course of this research that exhibits separate signals for the alpha- and beta-positions of

the terminal methyl carbon. The carbonyl signals for tributyrin also come at markedly different chemical shifts to that of triglycerides containing longer chain fatty acids.

The four signals detected for butyrate on the alpha position in milk triglycerides come at 172.7 ppm for the carbonyl carbon, 36.6 ppm and 17.9 ppm for the two carbons in the acyl chain and 13.2 ppm for the terminal methyl carbon. These signals correspond directly to those detected on the alpha position of tributyrin. The integral of each carbon can be measured and directly compared as they all come from the same fatty acid in the triglyceride. The integrals of each carbon environment for butyrate are detected in milk from each month are shown in Table 33.

Month	1	2	3	4	Mean	SD
January	15.0	14.8	14.2	14.2	14.6	0.4
February	14.2	14.4	14.1	14.0	14.2	0.2
March	15.2	14.2	15.2	15.4	15.0	0.5
April	15.8	14.6	14.2	17.1	15.4	1.3
May	16.3	14.9	14.4	16.1	15.4	0.9
June	14.4	15.1	14.6	14.3	14.6	0.4
July	15.2	14.2	15.6	15.0	15.0	0.6
August	14.1	17.4	17.7	17.0	16.6	1.7
September	15.1	14.6	14.2	15.4	14.8	0.5
October	16.5	15.3	14.6	15.2	15.4	0.8
November	15.0	14.1	14.7	15.0	14.7	0.4
December	16.1	14.6	15.0	11.4	14.3	2.0

Table 33: Percentage of butyrate detected on the alpha position as calculated by analysis of each carbon atom in butyrate. Carbon 1 (172.2 ppm), carbon 2 (36.6 ppm), carbon 3 (17.9 ppm) and carbon 4 (13.2ppm).

Butyrate is present on the alpha-position at an average of 14.97 % in milk fat from Aber Ogwen farm with a standard deviation of 0.65. This indicates that the variation in butyrate concentration in milk from Aber Ogwen farm is very low. This butyric acid is found solely on the alpha-position.

4.4.4 Interpretation of Fingerprint Region

The fingerprint region (30 – 28 ppm) of ^{13}C NMR spectra of triglycerides contains a large amount of information on the acyl chains of fatty acids. From this region it is possible to identify which medium and long chain saturated fats are present in the triglyceride and which position they occupy on the glycerol backbone. Quantification of components is also possible if signals are well resolved and of sufficient size to be integrated accurately. Identification of palmitate and stearate in olive oil was carried out with relative ease, as it was known they were present only on the alpha-position as the sn-2 position was exclusively occupied with unsaturated fats. Milk fat, however, contains a larger number of fatty acids. These are distributed over both the alpha- and beta-positions, which complicates identification of individual fatty acids due to the larger number of signals present in the fingerprint region.

Identification of palmitate and stearate on the alpha-position was carried out using the same characteristic peaks identified in the study of olive oil (29.016 ppm for alpha-palmitate and 29.174 ppm for alpha-stearate). Myristic acid on the alpha-position was identified by a signal at 28.702 ppm.

The proportions of laurate, myristate, palmitate and stearate detected on the alpha and beta positions of milk triglycerides from Aber Ogwen Farm are shown in Table 34.

	Stearate		Palmitate		Myristate		Laurate	
	α	β	α	β	α	β	α	β
Jan	48.0	25.0	41.3	13.3	ND	9.0	ND	8.2
Feb	44.1	18.9	41.8	11.6	ND	11.6	ND	7.4
Mar	49.7	24.2	38.2	12.5	ND	8.6	ND	6.9
Apr	43.4	23.9	40.7	32.3	ND	10.8	ND	2.3
May	48.1	35.1	39.5	25.2	ND	10.4	ND	4.1
Jun	47.5	31.9	41.6	24.8	ND	18.4	ND	4.8
Jul	48.5	34.2	46.9	23.6	ND	12.4	ND	4.6
Aug	49.5	36.1	50.0	23.2	ND	8.6	ND	4.5
Sept	44.7	40.5	42.0	21.5	ND	5.0	ND	4.9
Oct	43.3	22.0	44.3	20.6	ND	11.6	ND	6.4
Nov	43.0	16.3	40.8	26.2	ND	8.8	ND	8.6
Dec	48.2	22.5	41.9	21.6	ND	5.8	ND	10.2

Table 34: Percentages of the alpha- and beta-positions occupied by stearate, palmitate, myristate and laurate as determined by analysis of the fingerprint region of ^{13}C NMR of milk triglycerides. ND signifies none detected.

Table 34 shows the distribution of medium and long chain fatty acids on the glycerol backbone of milk triglycerides as determined by quantitative ^{13}C NMR analysis.

		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
	α	48.0	44.1	49.7	43.4	48.1	47.5	48.5	49.5	44.7	43.3	43.0	48.2
C18:0	β	25.0	18.9	24.2	23.9	35.1	31.9	34.2	36.1	40.5	22.0	16.3	22.5
	α+β	40.3	35.7	41.2	36.9	43.8	42.3	43.7	45.0	43.3	36.2	34.1	39.6
	α	41.3	41.8	38.2	40.7	39.5	41.6	46.9	50.0	42.0	44.3	40.8	41.9
C16:0	β	13.3	11.6	12.5	32.3	25.2	24.8	23.6	23.2	21.5	20.6	26.2	21.6
	α+β	32.0	31.7	29.6	37.9	34.7	36.0	39.1	41.1	35.2	36.4	35.9	35.1
	α	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C14:0	β	9.0	11.6	8.6	10.8	10.4	18.4	12.4	8.6	5.0	11.6	8.8	5.8
	α+β	6.0	7.7	5.7	7.2	6.9	12.3	8.3	5.7	3.3	7.7	5.9	3.9
	α	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C12:0	β	8.2	7.4	6.9	2.3	4.1	4.8	4.6	4.5	4.9	6.4	8.6	10.2
	α+β	2.7	2.5	2.3	0.8	1.4	1.6	1.5	1.5	1.6	2.1	2.9	3.4
	α	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C10:0	β	6.1	5.2	4.8		2.0	2.6	2.4	2.3	2.8	3.6	4.2	11.2
	α+β	2.0	1.7	1.6	0.0	0.7	0.9	0.8	0.8	0.9	1.2	1.4	3.7
	α	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C8:0	β	7.0	6.0	6.4	7.4	4.9	7.4	9.6	11.3	5.4	6.3	5.8	7.3
	α+β	2.3	2.0	2.1	2.5	1.6	2.5	3.2	3.8	1.8	2.1	1.9	2.4
	α	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C6:0	β	6.3	6.0	5.3	5.7	6.1	5.2	5.2	5.3	5.8	7.4	5.2	6.0
	α+β	2.1	2.0	1.8	1.9	2.0	1.7	1.7	1.8	1.9	2.5	1.7	2.0
	α	14.5	14.2	15.2	15.7	15.4	14.6	15.0	16.5	14.8	15.4	14.7	14.3
C4:0	β	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	α+β	9.7	9.4	10.1	10.5	10.3	9.7	10.0	11.0	9.9	10.2	9.8	9.5
	α	11.5	15.0	20.3	19.8	18.5	15.8	18.2	21.0	17.7	18.2	16.5	15.9
C18:1	β	12.4	15.6	13.2	20.8	16.9	17.1	15.3	14.3	13.7	13.9	16.4	12.4
	α+β	11.8	15.2	17.9	20.1	18.0	16.2	17.2	18.8	16.4	16.8	16.5	14.7
Total		**109.0**	**108.0**	**112.4**	**117.7**	**119.3**	**123.2**	**125.6**	**129.4**	**114.3**	**115.2**	**110.1**	**114.4**

Table 35: Distribution of fatty acids on the glycerol backbone of milk triglycerides.

ND - none detected.

Table 35 shows the molar percentages of each fatty acid present on each position of the triglyceride, and gives a total molar percentage of each fatty acid present in the triglyceride as a whole. The sum of these percentages is shown in the table. It can be seen that the error in these results is high; up to 30 %. This level of error is due to the difficulties in integrating peaks with poor resolution, the signal to noise ratio in the spectra and the usual errors associated with NMR. Errors of 30 % would not be acceptable in a method used for routine analysis of samples, but at this stage in the method development, when NMR is being used as a comparatively quick quantitative look at the fatty acids present in a triglyceride and their distribution on the glycerol backbone, these errors are acceptable.

The percentage of each fatty acid detected in milk fat using quantitative ^{13}C NMR is shown in Figure 57. This graph does not show what proportion of each fatty acid is on the alpha- and beta-positions but looks at the totals for each fatty acid.

At a glance, it can be seen that fatty acids fall into three distinct regions in Figure 57; stearate and palmitate are both found at concentrations above 25 %, oleate is found between 10 and 25 % and myristate, laurate, decanoate, octanoate, hexanoate and butyrate are all present at less than 10 % with only butyrate and myristate regularly reaching concentrations above 5 %.

Careful analysis of Figure 57 allows the detection of trends; it can be seen how fatty acid concentrations vary from month to month and how increases in one fatty acid are accompanied by decreases in another. Observations show that the concentrations of oleate and palmitate follow the same pattern of increases and decreases, and these are the opposite

of the trend exhibited by stearate; as the concentration of stearate increases, the concentrations of oleate and palmitate decrease.

The three shortest chain fatty acids (butyrate, hexanoate and octanoate) show the least variation in concentration, with standard deviations of 0.34, 0.78 and 0.84 respectively. Stearate and palmitate show the greatest variation in their concentrations ranging from 31.5 to 42.8 % for stearate (SD = 4.57) and 25.4 to 36.6 % for palmitate (SD = 3.71).

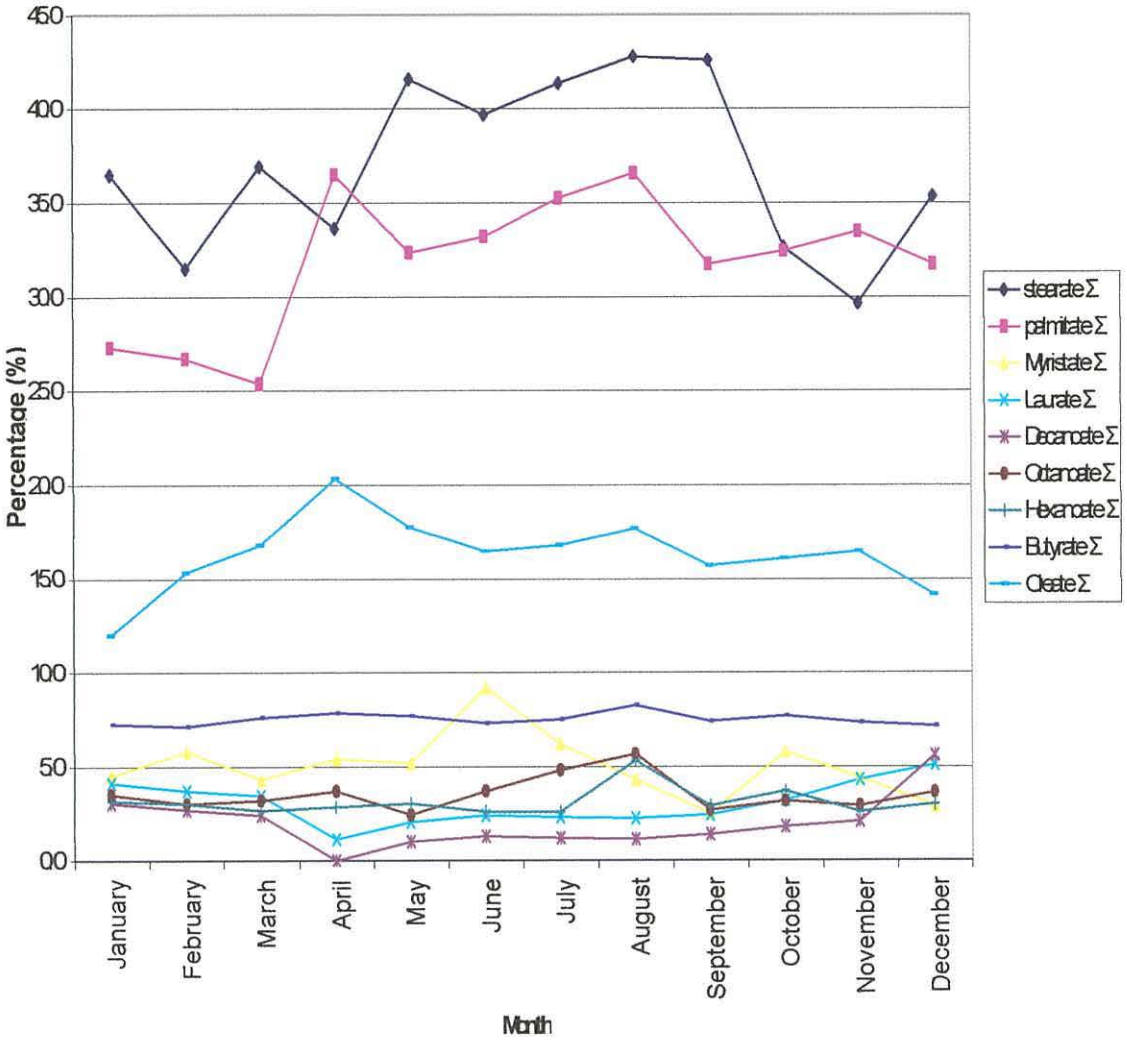

Figure 57: Monthly variation in the percentage of each fatty acid in milk triglycerides as determined by ^{13}C NMR.

The monthly variations in the fatty acid detected on the alpha- and beta-positions are shown separately in Figure 58 and Figure 59 respectively.

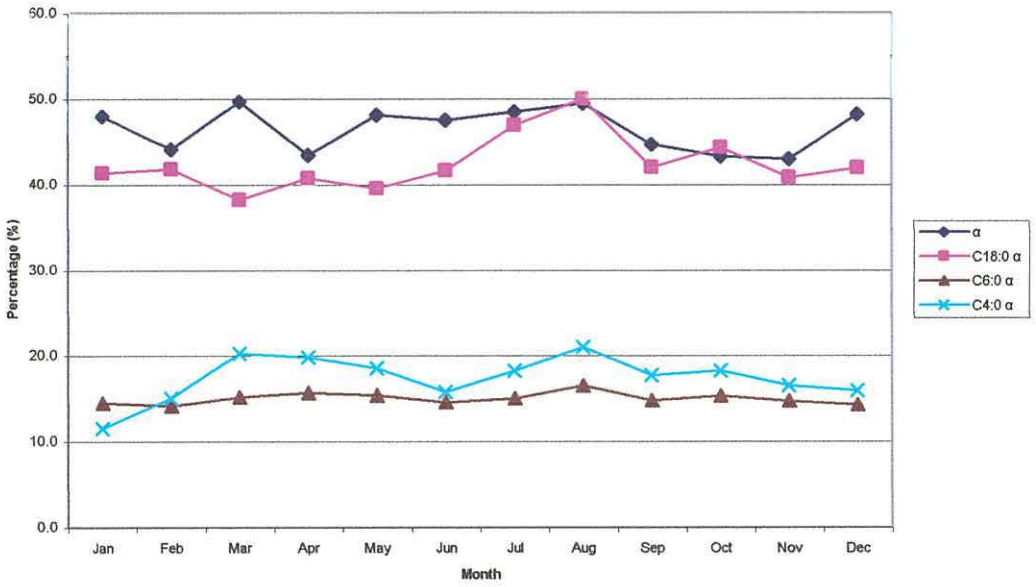

Figure 58: Monthly variation in the percentage of each fatty acid detected on the alpha-position of milk triglycerides as determined by ^{13}C NMR.

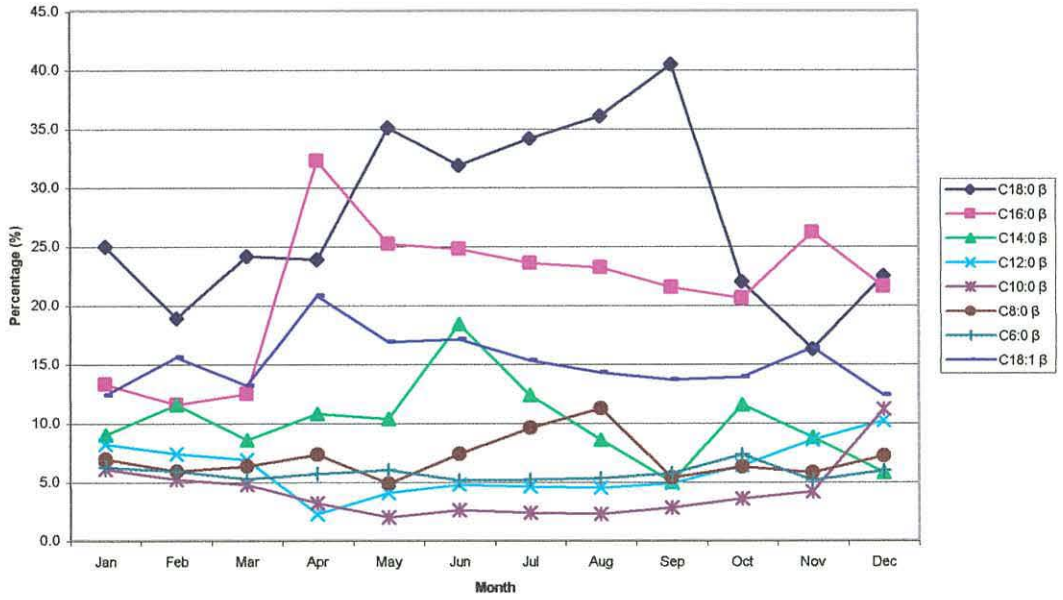

Figure 59: Monthly variation in the percentage of each fatty acid detected on the beta-position of milk triglycerides as determined by ^{13}C NMR.

It can be seen from examination of Figure 58 and Figure 59 that there is a larger number of fatty acids detected on the beta-position than on the alpha-position. Stearate, palmitate and oleate are the only fatty acids found on both positions with butyrate only found on the alpha-

position. Fatty acids with between 6 and 14 carbons in the chain (hexanoate, octanoate, decanoate, laurate and myristate) are only found on the beta-position.

The amount of stearate found on the alpha-position remains relatively constant throughout the year, but varies considerably on the beta-position. In the colder months (between October and April) the amount of stearate on the beta-position is below 25 %, but in the warmer months when the cows were out to pasture all day, the amount of stearate on the beta-position increased, even to as much as 40.5 % in September. A large reduction in the amount of beta-stearate is seen when the cows were bought in during October due to flooding. No corresponding change was noticed on the alpha-position.

When the weather improved and the cows were moved out to the fields in April, this caused an increase in the amount of beta-palmitate and beta-oleate and a decrease in the amount of beta-laurate found in the cows' milk. Again, no real change was noticed in the composition of fatty acids on the alpha-position to correspond with this change in diet and circumstance.

4.5 Comparison of results acquired using NMR with those from GCMS

NMR and GCMS are very different techniques. The results from NMR show the sample as a whole and GCMS will only show results relating to volatile components, and those that bond reversibly with the stationary phase. ^{1}H NMR of triglycerides suffers from the problem of overlapping peaks, which makes identification of individual components impossible whereas ^{13}C NMR enables not only the identification of individual components, but also their positions on the glycerol backbone. The results achievable by analysis using GCMS are very much

dependant on the stationary phase and the temperature program used for the analysis. The stationary phase used throughout this work was a CP-Select for FAME specially designed for the analysis of fatty acid esters (particularly fatty acid methyl esters), and allows good separation of different fatty acids as well as separation of positional and *cis/trans* isomers of fatty acid esters.

The preparation of butyl esters from milk triglycerides involves a transesterification using sodium butoxide and butanol as the solvent. Butanol has a high boiling point (117 °C) making it difficult to remove from the butyl esters after working up the reaction. This not only provides complications with quantifying the components of the butyl ester mixture but also interferes with the identification of butyl butyrate as butanol and butyl butyrate have overlapping retention times and similar mass spectrometry fragmentation patterns, with a large mass ion at 74. Butyl butyrate does however have a large signal with a mass of 89, which is not found in butanol.

4.6 Conclusion

Throughout this research, the analytical techniques of GCMS and ^{1}H and ^{13}C NMR have been applied to the study of milk triglycerides. The results from these three techniques have shown complementary results.

Analysis of butyl esters prepared from milk triglycerides has given an accurate and repeatable account of the fatty acids present within the milk triglyceride. Fatty acids present at low concentrations (less than 2 %) are detected and quantified by this method.

^{1}H NMR of milk triglycerides shows the ratios of fatty acid species present in the sample. This method is a quick, first glance analytical techniques as a rough over-view of the types of fatty acids present can be quickly assessed this way. ^{1}H NMR does not show which individual fatty acids are present, or which position each species occupies.

^{13}C NMR of milk triglycerides gives a detailed look at the structure of the milk fat triglycerides. The individual fatty acids present at 2 % or greater in a sample can be identified; their position on the glycerol backbone can also be assigned in a quantitative manner, however, the errors associated with this method are currently too high. This data could previously only be acquired using enzymes such as pancreatic lipase to selectively cleave fatty acids from the alpha-position to allow analysis of the fatty acids on each position separately, as described by Zamora *et al* (Zamora *et al,* 2002, p267).

5.0 Analysis of cheese triglycerides

5.1 Introduction

Cheese is a solid food made from curdled milk. Most of the cheese eaten in the UK is made from cow's milk, but is commonly made from the milk of other ruminant animals such as goats and sheep.

Britain is renowned for the large number of regional cheeses it produces, 700 – more even than France (thecheeseweb.com; cheeseboard.com). The aim of this research was to compare the triglycerides found in different regional British cheeses using ^{1}H and ^{13}C NMR. These British cheeses would be compared with a traditional Italian cheese (Parmesan) and a soft cheese made from goat's milk.

The regional British cheeses analysed in this research were from Cheshire, Wensleydale, Lancashire, Gloucester and South Wales. These cheeses all have their own unique, characteristic flavours and textures. For comparison, the triglycerides from these cheeses from Italy (parmesan) and France (chêvre, made from goats milk) were also analysed.

5.2 The history of cheese

There are seven different types of cheese; all have different manufacturing and ageing processes, the cheeses studied in this research fall into three categories – the French chêvre is a soft white cheese, Wensleydale is a fresh cheese and all the others are hard cheeses.

The seven categories of cheese are;

- *Fresh cheese:* only ripened for 10 – 15 days
- *Natural rind cheese:* usually made from goats milk
- *Soft white cheese:* very soft and runny at room temperature as it contains large amounts of whey
- *Semi-soft cheese:* edam, for example
- *Hard cheese*: contains very little whey and is often heated before being pressed into moulds to encourage a firmer texture
- *Blue cheese:* contains aerobic blue moulds which are encouraged to grow by piercing the cheese with steel rods as it matures
- *Flavoured cheeses*: are usually hard or semi-soft cheeses with fruit, nuts, herbs and spices or meat added.

The process of cheese making is an age-old tradition. Most British cheese is made from pasteurised milk to prevent harmful bacteria from growing in the cheese, the milk is then soured and rennet is added to form junket (a blancmange-like mixture of curds and whey). The junket is then cut to separate it into milk solids (curds) and liquid (whey). The whey is removed and discarded and the curds are salted and small portions are wrapped in muslin

cloth and pressed into shape in moulds. Cheeses are often dipped in hot wax after their shape has been set to give a protective coating as the cheese matures. Cheeses are left to mature, usually on wooden shelves for up to 12 months to allow the correct flavours to develop. The older the cheese, the stronger the flavour – hence mature cheddar being stronger than mild cheddar.

The cheeses analysed during this research are described below.

Cheshire Cheese: a slightly crumbly and silky texture and full-bodied fresh flavour (from the locally mined salt used in production).

Double Gloucester: A coloured cheese (usually with a vegetable dye, Annatto). This cheese has a thick rind to withstand traditional cheese-rolling ceremonies. It has a smooth buttery texture and a creamy flavour.

Lancashire Cheese: a creamy but crumbly texture and a full-bodied flavour. Lancashire cheese is widely thought of as the best British cheese for melting.

Parmesan: A traditional Italian Cheese made from un-pasteurised milk. It has a strong flavour and a hard gritty texture.

Cheddar: Originally made in Cheddar Gorge (Somerset) but now produced extensively throughout the UK. Five types of cheddar are sold in the UK; mild, medium, mature, vintage and West-Country Farmhouse cheddar; the latter having Protected Designated Origin status (PDO). The first four categories all depend on the length of time allowed for the cheddar to mature, West-Country Farmhouse cheddar has PDO status and can only be produced by

licensed manufacturers within a designated area. Mature cheddar is matured for a minimum of 12 months.

Wensleydale: A moist and crumbly cheese with a tradition going back to the 11th century. It has a sweet and creamy flavour and is traditionally eaten with fruit.

Chêvre: A French natural rind cheese milk made from goats' milk.

5.3 Analysis of cheese triglycerides

The extraction of triglycerides from cheese is complicated by the fact that the cheese is solid at room temperature. The second complication is that of the cheese protein, is not soluble in chloroform (lipids are soluble in chloroform); the last factor is the number of chemicals other than triglycerides that are in the lipid fraction (such as cholesterol, terpenes and, in some cheeses, colourings such as annatto). The process of extraction and purification of triglycerides from cheese is described in detail in Chapter 9.

Triglycerides were extracted and purified from the seven cheeses described above. These triglycerides were then analysed by ^{1}H NMR and high resolution quantitative ^{13}C NMR. ^{1}HNMR allowed the fast identification of the fatty acid species present within the triglyceride, and manipulation of the integrals of these spectra enabled the ratios of the amounts of saturated, mono-, bis- and poly-unsaturated present in the sample to be calculated. Analysis by quantitative ^{13}C NMR permitted identification of the individual fatty acids present within the cheese triglycerides, identification of the positions they occupy on the glycerol backbone and quantification of the fatty acids on each position.

As cheese is made from milk solids, the composition of triglycerides was expected to be similar to that from un-processed milk. The same method of ^{13}C NMR interpretation was used to calculate the percentages of each fatty acid present in the triglyceride as was used for the analysis of milk triglycerides. The selected integrals used for identification and calculations of molar percentage are shown in Chapter 9.

5.3.1 ^{1}H NMR

Analysis of triglycerides by ^{1}H NMR gives a quick measure of the ratios of the different fatty acid species present within the triglyceride. It gives no information on the individual fatty acids present or whether they are on the alpha- or beta-positions of the triglyceride backbone. The ratios of fatty acid species from the seven cheeses analysed in this research as determined by ^{1}H NMR are shown in Table 36.

	Sat	Mono	Bis	Poly
Wensleydale	75.46	20.58	0.22	0.03
Lancashire	78.27	18.32	0.08	0.05
Double Gloucester	78.58	18.00	0.33	0.08
Welsh Mature Cheddar	71.08	25.75	0.33	0.17
Parmesan	72.42	23.25	1.67	0.67
Goats Chêvre	75.07	20.91	1.40	0.33

Table 36: Fatty acid species ratios as determined by ^{1}H NMR of cheese triglycerides. Sat = saturated fat, mono = mono-unsaturated fat, bis = bis-unsaturated fat and poly = poly-unsaturated fat.

It can be seen from this data that cheese triglycerides contain 70 – 80 % saturated fat, 18 – 26 % mono-unsaturated fat, up to 2 % bis-unsaturated fat and less than 1 % poly-unsaturated

fat. These values are in the same range as the results from analysis of milk triglycerides from Aber Ogwen Farm as discussed in Chapter 4.

It can be seen that Parmesan cheese contains a considerably greater amount of bis- and poly-unsaturated fat than all other cheeses produced from cow's milk. Chêvre, made from goat's milk also contains more bis- and poly-unsaturated fat that cheese from cow's milk.

5.3.2 ^{13}C NMR

Analysis of the quantitative ^{13}C NMR of cheese triglycerides gives information on which individual fatty acids are present on each position on the glycerol backbone, it also allows quantification of these components.

Analysis of the carbonyl region gives information on the distribution of fatty acid species on the glycerol backbone. Groups of signals were detected for each position, alpha and beta. Saturated and mono-unsaturated fat was detected at each position. The short chain fatty acid butyrate was only detected on the alpha-position. Poly-unsaturated fat was detected on the alpha-position of one cheese (Double Gloucester), but no corresponding signals were found for poly-unsaturated fat in other regions. The distribution of fatty acid species as determined by analysis of the carbonyl region of quantitative ^{13}C NMR of cheese triglycerides is shown in Table 37.

	α-Sat	α-Mono	α-Poly	α-C4:0	β-Sat	β-Mono	α-Total	β-Total
Cheshire							0	0
Wendsleydale	66.45	19.9	0	13.9	80.5	16.8	100.25	97.3
Lancashire	67.05	19.5	0	14	80.5	17.5	100.55	98
Double Gloucester	69.65	17.35	1.75	10.55	80.5	11.3	99.3	91.8
Welsh Mature Cheddar	68.7	22.6	0	16.4	84.1	20.9	107.7	105
Parmesan	67.5	25.35	0	13.7	84.5	17.4	106.55	101.9
Goats Chêvre	74.1	24	0	6.25	80.4	19	104.35	99.4

Table 37: Interpretation of the carbonyl region of ^{13}C NMR of cheese triglycerides.

It can be seen from Table 37 that the triglycerides in goats' cheese contain a higher proportion of alpha-saturated fat and a lower concentration of butyrate than the triglycerides from cheese made from cows' milk.

Analysis of the olefinic region of quantitative ^{13}C NMR spectra of cheese triglycerides shows the presence of unsaturated fatty acids. Oleate was detected in all samples of cheese. This was to be expected as analysis of milk triglycerides shows that oleate makes up approximately 20 % of the total fatty acids present in milk fat. This is confirmed by ^{1}H NMR analysis of the cheese triglycerides showing mono-unsaturated fat to be present at approximately 20 %.

Unlike ^{1}H NMR, ^{13}C NMR allows the identification of individual fatty acids and allocates their positions on the glycerol backbone. Oleate was detected on both positions in each cheese. In all cases, the percentage of the alpha-position occupied by oleate was greater than or equal to the percentage of the beta-position esterified to oleate.

Linoleate was only present at levels high enough to be detected in one sample of cheese, the Parmesan. This again was expected as analysis of the ^{1}H NMR shows this cheese to have the highest levels of bis-unsaturated fat found in this study. Linoleate was detected on both the alpha – and beta-positions in Parmesan cheese. The distribution of oleate and linoleate on the glycerol backbone of each cheese triglyceride is shown in Table 38.

	α-Oleate	β-Oleate	α-Linoleate	β-Linoleate
Cheshire			0	0
Wensleydale	16.1	14.0		
Lancashire	14.8	14.3	0	0
Double Gloucester	16.0	9.7	0	0
Welsh Mature Cheddar	20.4	17.6	0	0
Parmesan	18.1	18.1	1.5	2.0
Goats Chêvre	17.1	15.3	0	0

Table 38: Interpretation of the olefinic region of ^{13}C NMR spectra of cheese triglycerides.

Interpretation of the fingerprint region of the ^{13}C NMR of triglycerides allows identification of the individual saturated fats present in a sample. It is also possible to quantify the saturated fats on each position. Characterisation of the fatty acids present at levels detectable and quantifiable by ^{13}C NMR is shown in Table 39.

Fatty Acid		Cheshire	Wendsley-dale	Lancashire	Double Gloucester	Welsh Mature Cheddar	Parmesan	Goats Chêvre
stearate	α	43.6	43.6	44.9	42.1	44.6	43.4	37.2
stearate	β	20.6	20.6	22.4	19.2	21.2	27.8	38.2
palmitate	α	39.9	39.9	38.9	40.1	39.1	38.9	36.7
palmitate	β	12.2	12.2	13.3	15.4	16.6	11.3	25.3
Myristate	α	ND	ND	ND	ND	ND	ND	ND
Myristate	β	8.7	8.7	8.7	7.9	11.1	6.7	6.8
Laurate	α	ND	ND	ND	ND	ND	ND	ND
Laurate	β		4.9	6.3	9.4	4.1	6.2	1.2
Decanoate	α	ND	ND	ND	ND	ND	ND	ND
Decanoate	β		6.0	8.2	5.1	5.3	4.7	2.8
Octanoate	α	ND	ND	ND	ND	ND	ND	ND
Octanoate	β	12.2	12.1	12.6	12.3	12.9	12.3	11.7
Hexanoate	α	ND	ND	ND	ND	ND	ND	ND
Hexanoate	β	11.4	11.3	14.4	12.4	13.9	12.5	13.3
Butyrate	α	13.3	13.6	13.6	9.7	16.0	12.9	5.8
Butyrate	β	ND	ND	ND	ND	ND	ND	ND

Table 39: Distribution of saturated fats on the glycerol backbone of cheese triglycerides as determined by ^{13}C NMR. Lines marked α show the percentage of the alpha-positions occupied by each fatty acids, lines marked β show the percentage of beta-positions occupied by each fatty acids. ND denoted none detected.

When looked at as a whole, the fatty acid composition of cheese is similar to that of milk. The overall fatty acid composition of cheese triglycerides is shown in Table 40.

Fatty Acid		Cheshire	Wendsley-dale	Lancashire	Double Gloucester	Welsh Mature Cheddar	Parmesan	Goats Chêvre
stearate	α	43.6	43.6	44.9	42.1	44.6	43.4	37.2
stearate	β	20.6	20.6	22.4	19.2	21.2	27.8	38.2
stearate	Σ	61.7	61.7	61.4	61.2	61.4	60.6	55.3
palmitate	α	39.9	39.9	38.9	40.1	39.1	38.9	36.7
palmitate	β	12.2	12.2	13.3	15.4	16.6	11.3	25.3
palmitate	Σ	32.2	32.2	32.8	35.5	36.2	30.8	43.7
Myristate	α	ND	ND	ND	ND	ND	ND	ND
Myristate	β	8.7	8.7	8.7	7.9	11.1	6.7	6.8
Myristate	Σ	2.9	2.9	2.9	2.6	3.7	2.2	2.3
Laurate	α	ND	ND	ND	ND	ND	ND	ND
Laurate	β		4.9	6.3	9.4	4.1	6.2	1.2
Laurate	Σ	0.0	1.6	2.1	3.1	1.4	2.1	0.4
Decanoate	α	ND	ND	ND	ND	ND	ND	ND
Decanoate	β		6.0	8.2	5.1	5.3	4.7	2.8
Decanoate	Σ	0.0	2.0	2.7	1.7	1.8	1.6	0.9
Octanoate	α	ND	ND	ND	ND	ND	ND	ND
Octanoate	β	12.2	12.1	12.6	12.3	12.9	12.3	11.7
Octanoate	Σ	4.1	4.0	4.2	4.1	4.3	4.1	3.9
Hexanoate	α	ND	ND	ND	ND	ND	ND	ND
Hexanoate	β	11.4	11.3	14.4	12.4	13.9	12.5	13.3
Hexanoate	Σ	3.8	3.8	4.8	4.1	4.6	4.2	4.4
Butyrate	α	13.3	13.6	13.6	9.7	16.0	12.9	5.8
Butyrate	β	ND	ND	ND	ND	ND	ND	ND
Butyrate	Σ	6.6	6.8	6.8	4.9	8.0	6.5	2.9
Oleate	α	16.1	16.1	14.8	16.0	20.4	18.1	17.1
Oleate	β	14.0	14.0	14.3	9.7	17.6	18.1	15.3
Oleate	Σ	22.0	22.0	21.6	17.6	27.8	27.1	23.9
Linoleate	α	ND	ND	ND	ND	ND	1.5	ND
Linoleate	β	ND	ND	ND	ND	ND	2.0	ND
Linoleate	Σ	0.0	0.0	0.0	0.0	0.0	1.1	0.0

Table 40: Distribution of saturated and unsaturated fats on the glycerol backbone of cheese triglycerides as determined by ^{13}C NMR. Lines marked α show the percentage of the alpha-positions occupied by each fatty acids, lines marked β show the percentage of beta-positions occupied by each fatty acids, lines marked Σ show the percentage of that fatty acid present when the triglyceride is examined as a whole. ND denoted none detected.

5.4 Conclusion

The position of each fatty acid on the glycerol backbone can be identified by ^{13}C NMR allowing complete characterisation of the fats present in each cheese, and the fatty acids present can be quickly identified by ^{1}H NMR analysis of the triglycerides. The composition of cheese triglycerides mirrors that of milk, which is to be expected as cheese is made from milk.

Cheese triglycerides are 70 – 80 % saturated fat, and 18 – 26 % mono-unsaturated fat. Bis-unsaturated and poly-unsaturated fats are found in small quantities, less than 2 % in some cheeses.

Small differences were noticed in the triglyceride composition of regional British cheeses, which in general, had a similar composition to the Italian cheese, Parmesan. The differences in triglyceride composition between regional cheeses are similar in magnitude to those noticed in the seasonal variation of milk triglycerides as discussed in Chapter 4.

The triglyceride composition of British cheeses was similar to that of milk from British farms, which was to be expected, as this is what is published in literature.

This method of analysis of cheese triglycerides by quantitative ^{1}H and ^{13}C NMR is a relatively fast method of analysis, ^{1}H NMR giving a fast overview of the fatty acid species present in the triglycerides and ^{13}C NMR giving a detailed quantitative picture of the individual fatty acids present on each position of the glycerol backbone. This could previously only be done using several-step enzymatically controlled reactions followed by chromatography of multiple samples.

This work could be continued by investigating more of Britain's regional cheeses; a project could be carried out studying the different fatty acid composition of triglycerides from regional cheddars as so many are produced in the UK. Comparison of British cheddars with those from other countries could also be carried out, as cheddar is such a popular cheese, and is produced in many countries including Canada and America.

6 Analysis of lipids from Lamb meat and fat

6.1 Introduction

Lamb is part of the varied diet of the western world. It is farmed extensively in the UK and forms a large part of the diet of many people. As with any red meat, the amount and type of fat present can cause health concerns for some.

Several studies have been carried out in the past investigating how the diet of lambs affects the type and amount of fat present in the meat. Particular interest is taken in increasing the amounts of ω-3 fatty acids as they have well documented health benefits.

The School of Agriculture and Forestry Science at the University of Wales, Bangor, carried out a study to investigate changes in the ω-3 fatty acid content of lamb muscle and fat when fed different diets. Groups of lambs were fed diets of different seeds, including linseed, camelina, linseed cake and a food supplement called Valomega™, which is designed to increase the ω-3 and ω-6 fatty acid content of the meat.

These diets were compared to that of the standard food concentrate lambs are usually fed. Lambs were fed these diets for two different time periods, four and seven weeks, and then slaughtered. Samples of meat were taken from the back of the lamb between the sixth and eighth rib and were then stored at -18°C until analysed. Animal handling was carried out by the team at Henfaes Farm, Abergwyngregyn.

The analysis of lipids from lamb has been carried out in previous studies chromatographic analysis of methyl esters prepared by the hydrolysis and esterification of triglycerides (Cooper *et al*, 2004). This is a two-step process that uses chemicals which are known carcinogens (diazomethane), pose toxic and explosive hazards and are now difficult to source.

The purpose of this work was to extract the lipids from lamb meat and fat using the same procedures outlined in the work by Cooper *et al* (2004). and to compare two different methods of methyl ester preparation; the two step preparation by hydrolysis and esterification, and a safer, one-step process of transesterification using sodium methoxide.

6.2 Procedure

Each lamb sample was analysed to investigate the lipid content of the subcutaneous adipose tissue (fat) and the lean muscle tissues (meat). The lipid samples from the muscle tissue were extracted using a Folch-type extraction and separated into polar (phospholipids) and non-polar (triglyceride) lipid fractions. Each fraction was then analysed separately. Lipid fractions were separated by solid phase extraction using silicic acid solid phase extraction (SPE) columns (Waters). The separation of phospholipids and neutral lipids was optimised and its performance tested by ^{31}P NMR analysis of the fractions. ^{31}P NMR analysis of the crude lipid samples showed either one or two signals; after separation, the neutral lipid fraction showed no phosphorus signals, and the polar lipid fraction contained the same signals as detected in the crude lipid sample. The lipids extracted from the adipose tissue were analysed using ^{31}P NMR but no phosphorus signals were detected indicating that the phospholipids content was negligible.

6.3 Validation of one-step transesterification method

Preparation of methyl esters from lamb triglycerides and phospholipids was carried out by Cooper *et al.* using a two-step, hydrolysis/esterification method. Validation of a one-step transesterification method was carried with the aim of acquiring the same results as those achieved by the two-step method.

Analysis of lamb muscle triglycerides and phospholipids was carried out using the method of hydrolysis followed by esterification with diazomethane as described by Cooper *et al.* and these results were compared with those using the transesterification method using methanolic sodium methoxide. This method comparison was carried out on three lamb samples. Each sample was divided into two and both preparative methods were carried out twice using each sample.

Analysis of the methyl esters produced by each method was carried out using GCMS analysis under the same run conditions. Analysis of each sample was carried out in duplicate.

6.3.1 Triglyceride analysis

Triglycerides and phospholipids were extracted from three samples of lamb muscle tissue. From these lipid fractions, methyl esters were produced by the two-step method (hydrolysis followed by esterification) and by the one-step transesterification method. Extraction and analysis were carried out in duplicate.

6.3.2 Comparison of results

Table 41 and Figure 60 show the results of GCMS analysis of methyl esters produced from lamb muscle triglycerides by both methods.

	Lamb 1		Lamb 2		Lamb 3	
Compound	**One-step**	**Two-step**	**One-step**	**Two-step**	**One-step**	**Two-step**
C12:0	0.33	0.28	0.10	0.12	0.03	0.28
C14:0	3.14	3.34	2.23	2.23	5.51	5.12
C14:1	0	0	0.10	0.14	0.24	0.16
C15:0	0.24	0.49	0.17	0.23	0.39	0.25
C15:1	0.49	0	0.43	0.53	0.79	0.07
C16:0	23.39	22.11	22.13	24.67	28.66	28.94
C16:1	0	0.98	0.78	1.01	1.20	1.27
C17:0	1.28	1.47	1.26	1.05	1.21	1.18
C17:1	0	0.46	0.56	0.46	0.55	0.55
C18:0	22.72	21.79	26.25	22.45	21.94	21.20
C18:1 vaccenic	3.26	3.90	6.12	4.61	4.90	5.74
C18:1 isomer	7.20	7.51	0	0	0	0
C18:1 oleic	34.30	34.04	36.33	38.96	27.84	29.05
C18:2	0.96	1.21	1.48	1.14	3.10	2.79
C18:3	0.75	0	0.50	0.92	1.31	1.49
C18:2	1.94	2.14	1.56	1.29	1.13	1.38
C20:4	0	0	0	0.20	0	0
C20:5	0	0.28	0	0	0.27	0
C22:5	0	0	0	0	0.26	0

Table 41: Fatty acid methyl ester composition of lamb muscle triglycerides as determined by GCMS analysis. One-step method: direct transesterification of triglycerides. Two-step method: hydrolysis of triglycerides followed by esterification of free fatty acids. Results quoted are a mean of duplicate analyses.

Variation between results is small, with the variation between individual lamb samples higher than the variation between derivatisation methods. Careful analysis of the results show that the one-step transesterification method does not consistently produce results higher or lower than those produced by the two-step method.

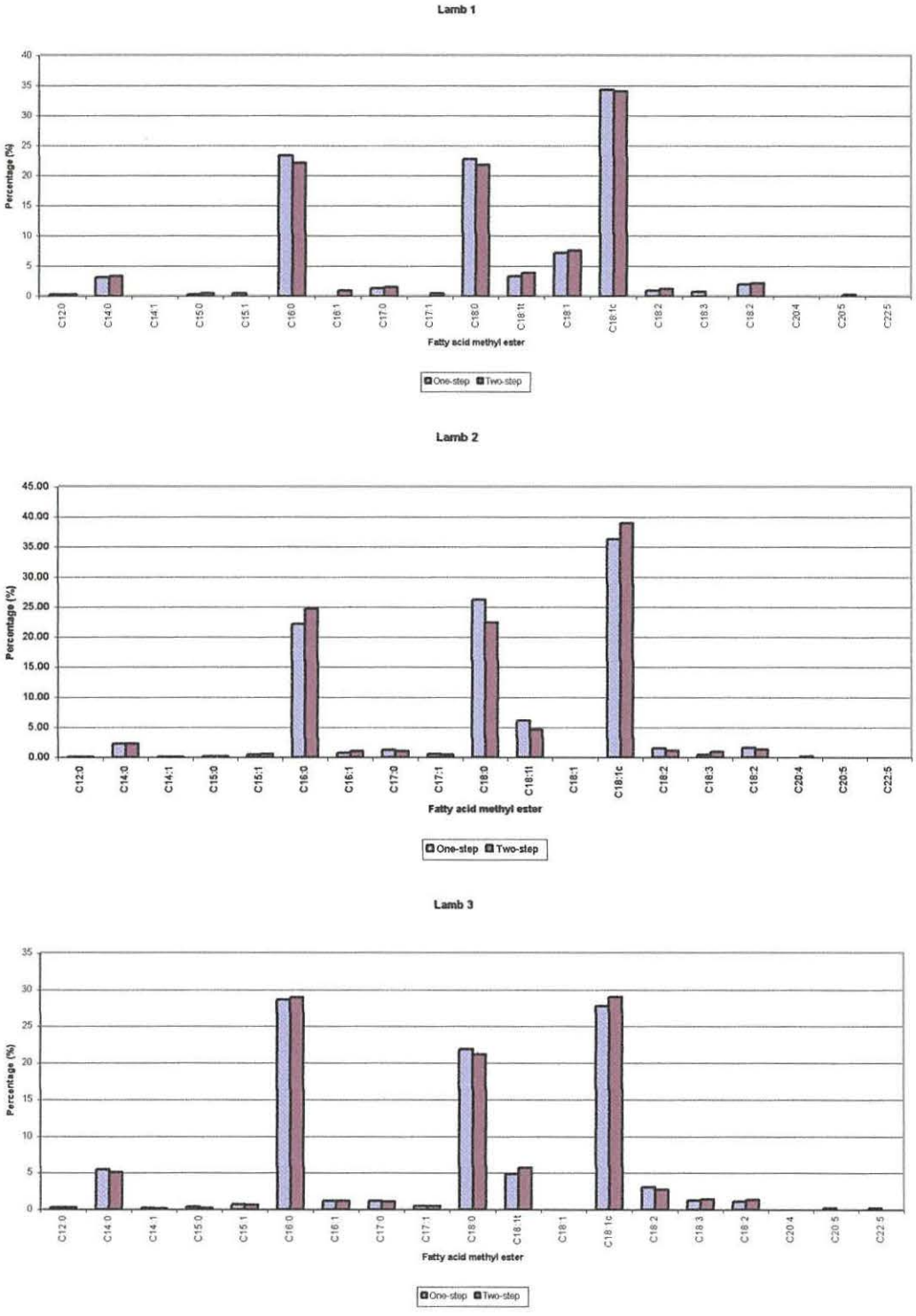

Figure 60: Fatty acid methyl ester composition of lamb muscle triglycerides as determined by GCMS analysis. One-step method: direct transesterification of triglycerides. Two-step method: hydrolysis of triglycerides followed by esterification of free fatty acids. Results quoted are a mean of duplicate analyses

6.3.3 Phospholipid Analysis

Phospholipids extracted from lamb muscle were esterified by both esterification methods. GCMS analysis of these results showed the results acquired by each method are comparable. Table 42 and Figure 61 show the GCMS results achieved by each method.

	Lamb 1		Lamb 2		Lamb 3	
Compound	**One-step**	**Two-step**	**One-step**	**Two-step**	**One-step**	**Two-step**
C10:0	5.60	5.03	0	0	0	0
C12:0	2.44	2.02	0.45	0.4	0.92	0.62
C14:0	6.24	8.00	5.77	5.44	8.99	7.66
C14:1	0.00	0	0.22	0.21	0.26	0.26
C15:0	0.50	0	0.37	0.44	0.72	0.66
C15:1	0.51	0	0.65	0.66	0.79	0.81
C16:0	20.70	23.92	28.66	28.78	27.41	27.38
C16:1	0.00	0	0.63	0.26	1.24	1.43
C17:0	1.08	1.34	1.11	0.91	0.91	0.78
C17:1	0.00	0.53	0.47	0	0.91	0.51
C18:0	23.31	21.46	20.13	20.6	14.54	15.01
C18:1 vaccenic	4.22	3.28	7.09	7.1	4.14	4.33
C18:1 oleic	26.24	24.18	31.11	32.33	31.62	33.11
C18:2	4.31	4.91	0.85	0.76	3.42	3.36
C18:3	0.76	0.88	0.72	0.42	1.49	1.47
C18:2	0.83	1.13	1.76	1.7	1.5	1.52
C20:4	1.41	2.02	0	0	0.63	0.67
C20:5	0.46	0.50	0	0	0.23	0.28
C22:5	1.38	0.72	0	0	0.29	0.14
C22:6	0	0.08	0	0	0	0

Table 42: Fatty acid methyl ester composition of lamb muscle phospholipids as determined by GCMS analysis. One-step method: direct transesterification of phospholipids. Two-step method: hydrolysis of phospholipids followed by esterification of free fatty acids.

Results acquired by both derivatisation methods show similar results. The variation between lamb samples are larger than those found between derivatisation methods. Neither method consistently produces higher or lower results.

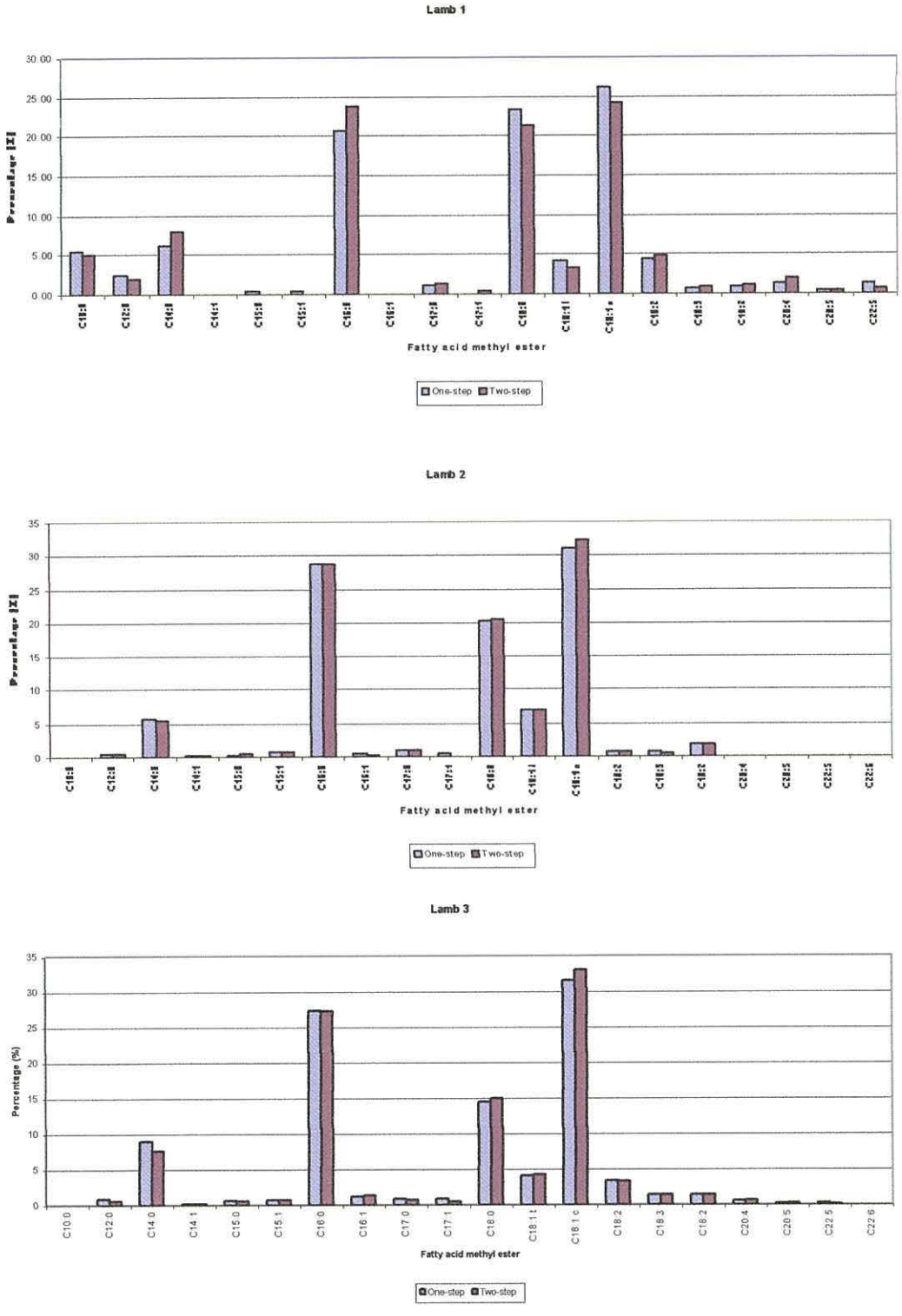

Figure 61: Fatty acid methyl ester composition of lamb muscle phospholipids as determined by GCMS analysis. One-step method: direct transesterification of phospholipids. Two-step method: hydrolysis of phospholipids followed by esterification of free fatty acids.

Analysis of the results produced by both methods indicates the one-step transesterification method is a robust and repeatable method of preparation of methyl esters from both triglycerides and phospholipids for analysis by GCMS.

6.4 Results of diet comparison

The purpose of the present work was to validate a method of analysis that could be applied to meat samples. The method was applied by others to an extensive diet comparison study; the results and interpretation of this will be published in full elsewhere. A brief account will be presented herein, simply to show the application of the method developed in this current work. These results show a sample of the data produced by transesterification of triglycerides and phospholipids from lipids extracted from lamb adipose and muscle tissues.

From 10 g of muscle tissue, the crude lipid was extracted and separated into triglyceride and phospholipids fractions. The average mass of triglyceride and phospholipid extracted were 0.31 g and 0.03 g respectively. This would indicate that the lamb muscle contains 3 % triglyceride and 0.3 % phospholipid and that the total lipid content is made up of 8.3 % phospholipid.

Comparison of the lipids from each source showed some interesting trends. Excluding the dietary effects, the adipose tissue contained a similar fatty acid composition to the muscle triglycerides. The muscle phospholipids contained more polyunsaturated fat; in particular, the amounts on n-3 and n-6 fatty acids were higher in the polar lipid fraction than the non-polar fraction.

The proportion of phospholipids in the muscle tissue was very low – from 10 g of muscle, 10 – 18 mg of phospholipids were extracted, so the n-3 and n-6 fatty acids present in the phospholipids make up a very small proportion of the total fat present in the lamb.

GCMS was used as the analytical method for determination of fatty acid composition of lamb lipid samples. An internal standard (methyl heneicosanoate, C21:0) was added to allow for accurate quantitation of components as heneicosanoic acid is not naturally occurring in lamb fat. This internal standard was added as the sample was made up into solution for GCMS analysis. Samples were prepared to contain between 6 and 12 mg of lipid/ ml of methanol and 0.1 mg of methyl heneicosanoate.

The fatty acid composition of lamb muscle and adipose tissue is shown in Table 43.

	Adipose			Muscle Phospholipid			Muscle Triglyceride		
compound	std	val	lin	std	val	lin	std	val	lin
C10:0	0.28	0.04	0.05	1.77	0.43	0.03	0.00	0.00	0.90
C12:0	0.28	0.30	0.22	1.22	0.79	0.52	0.28	0.52	0.12
C13:0	0	0.01	0	0	0	0	0	0	0
C14:0	2.66	3.96	3.22	6.16	4.95	4.54	4.12	4.03	3.02
C14:1	0.40	0.32	0.17	1.49	0.24	0.28	0.16	0.17	0.14
C15:0	0.43	0.50	0.40	1.33	0.48	0.49	0.25	0.41	0.22
C15:1	0.78	0.78	0.78	0.67	0.67	0.70	0.60	0.66	0.50
C16:0	25.13	25.08	23.54	27.75	25.26	26.31	26.94	28.51	24.02
C16:1	1.01	0.74	0.73	0.40	0.55	1.22	0.27	0.21	0.84
C17:0	1.81	1.44	1.77	1.13	1.26	1.17	1.18	1.33	1.08
C17:1	0.81	0.64	0.78	0.54	0.74	0.74	0.55	0.63	0.61
C18:0	26.73	25.00	29.68	20.33	21.32	19.53	23.20	22.94	22.68
C18:1 vaccenic	7.48	8.11	6.07	4.19	4.60	4.77	5.74	5.75	4.88
C18:1 isomer	0	0	0	0	1.89	0	0	0	0.14
C18:1 oleic	29.42	30.04	30.15	23.32	32.15	32.12	34.05	31.32	36.79
C18:2	1.10	0.78	0.48	1.81	1.91	3.19	0.79	1.19	1.72
C18:3	0.47	0.57	0.51	1.01	0.78	1.25	0.49	0.81	0.65
C18:2	1.22	1.57	1.24	1.71	1.55	1.31	1.38	1.56	1.31
C18:2	0	0.03	0.22	0.19	0	0	0	0	0
C20:4	0	0	0	2.29	0.25	0.48	0	0	0.19
C22:2	0	0	0	0	0	0	0	0	0
C20:5	0	0	0	1.39	0.08	0.34	0	0	0.19
C24:1	0	0	0	0	0	0.70	0	0	0
C22:5	0	0	0	0.79	0.09	0.32	0	0	0
C22:6	0	0	0	0.52	0	0	0	0	0
Species totals	std	val	lin	std	val	lin	std	val	lin
saturated	57.31	56.34	58.87	59.69	54.49	52.59	55.97	57.72	52.05
mono cis	32.42	32.52	32.60	26.43	34.36	35.05	35.63	32.98	38.88
mono trans	7.48	8.11	6.07	4.19	6.49	4.77	5.74	5.75	5.02
poly	2.79	2.94	2.45	9.69	4.66	6.90	2.66	3.55	4.05
ω-3	0.47	0.57	0.51	3.71	0.95	1.91	0.49	0.81	0.84
ω-6	1.10	0.78	0.48	4.09	2.16	3.67	0.79	1.19	1.91

Table 43: Fatty acid composition of lamb adipose tissue and muscle tissue phospholipids and triglycerides as determined by GCMS of fatty acid methyl esters. Results acquired from four lambs, each sample prepared in duplicate and GCMS analysis carried out in duplicate. Std, Lin and Val are the diets fed to each lamb, being the standard diet, Linseed and Valomega respectively.

The fatty acids present in the lamb have been broken down into 6 categories, saturated, mono-unsaturated (*cis*), mono-unsaturated (*trans*) and poly-unsaturated. The ω-3 and ω-6 poly-unsaturated fats are then shown separately. Graphs showing the proportions of each fatty acid species in adipose tissue, and muscle phospholipids and triglyceride are shown in Figures 61 to 63. It should be noted that the sum of ω-3 and ω-6 fatty acids does not add up to the total poly-unsaturated fats as other poly-unsaturated fatty acids, such as conjugated linoleic acid, alpha-linolenic and linoleic acid are present, but do not fit into either ω-3 or ω-6 categories.

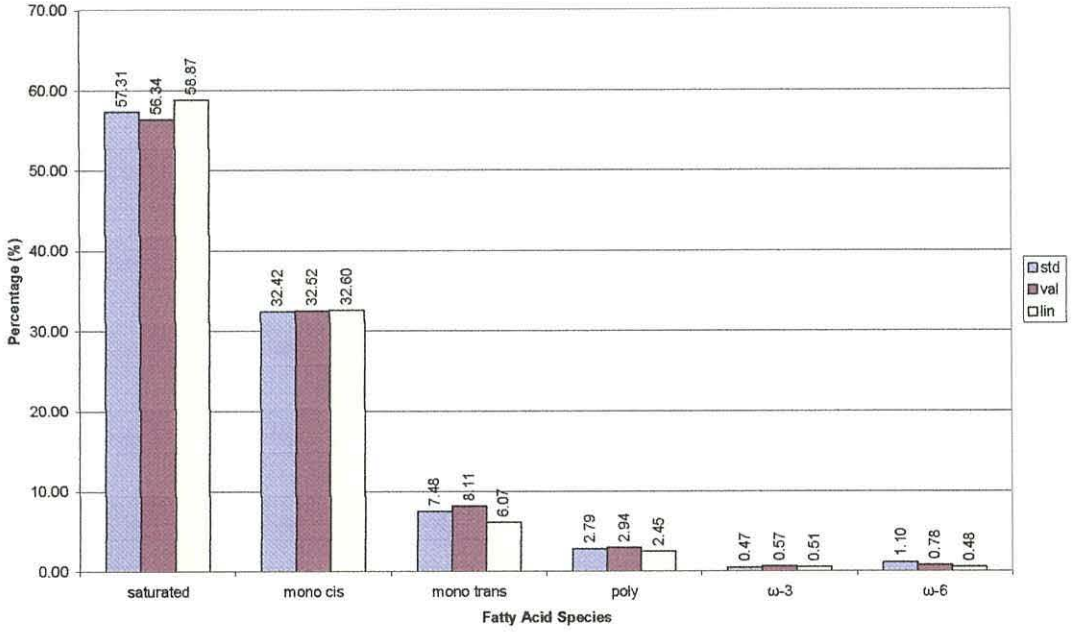

Figure 62: Fatty acid species content of lamb adipose tissue.

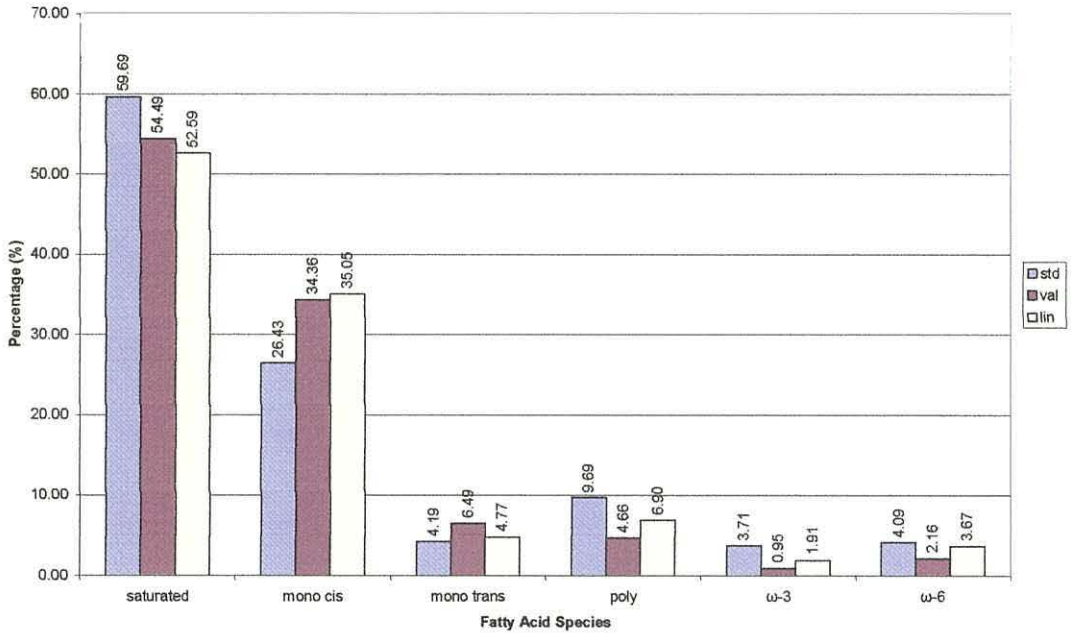

Figure 63: Fatty acid species composition of lamb muscle phospholipids.

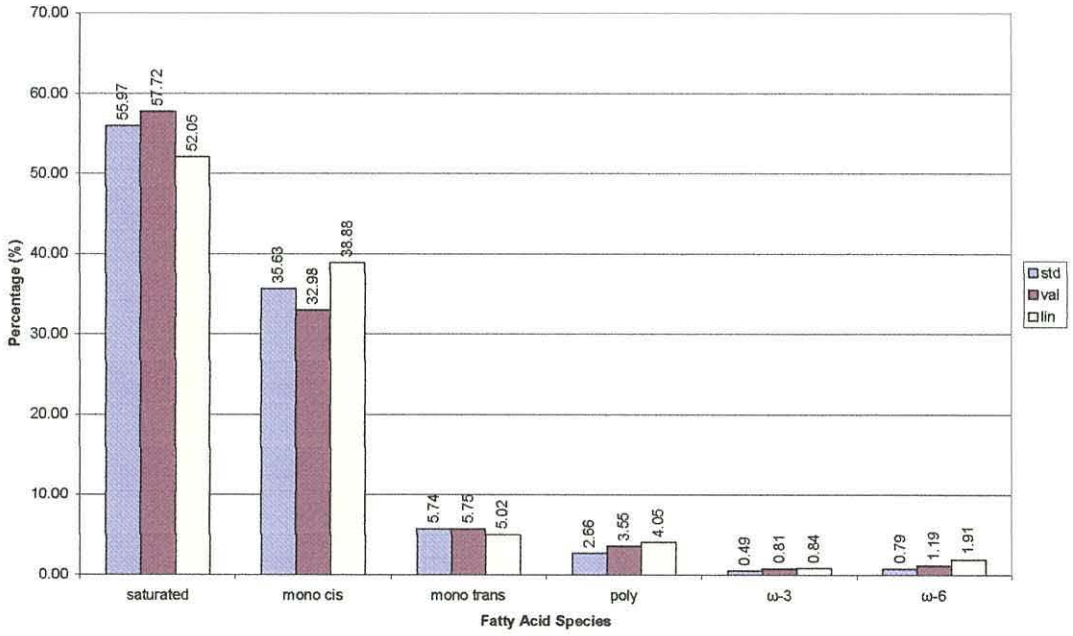

Figure 64: Fatty acid species composition of lamb muscle triglycerides.

Just comparing the fatty acid species present in the adipose tissue, it is clear to see that the diet of the lamb has very little effect on the proportions of *cis*-mono-unsaturated fat present – varying only by 0.18 %. The levels of saturated, *trans*-mono-unsaturated fat and poly-unsaturated fat are also very similar, with all measurements being within 1.5 % of each other.

Muscle triglyceride fatty acid species distribution shows more marked differences than those seen in the adipose tissue, but each diet still shows a similar response. The largest distinction is for the *cis*-mono-unsaturated fat; the linseed diet shows an increased presence of *cis*-mono-unsaturated fat when compared to the standard and valomega diets. The standard diet shows the smallest proportion of poly-unsaturated fat with levels of ω-3 and ω-6 fatty acids almost half of those shown by valomega and linseed diets.

Larger differences are noticed in the muscle phospholipid fraction. Lambs fed the 'standard' diet have higher proportion of saturated fat and a lower proportion of *cis*-mono-unsaturated fat. The standard di*et al*so gives rise to the largest proportion of poly-unsaturated fat, 38 % of which are ω-3 fatty acids and 42 % are ω-6.

Long chain fatty acids (those with more than 18 carbons in the chain) appear almost exclusively in the muscle phospholipids with the exception of arachidonic acid and eicosapentaenoic acid, each being detected at 0.19 % in the muscle triglyceride of lambs fed on linseed.

Fatty acids in animal and plant sources usually contain an even number of carbon atoms, but it can be seen that that fatty containing 13, 15 and 17 carbons, both as saturated and mono-

unsaturated fats are found in the fat of lambs. This is not unexpected as the microbes, particularly bacteria, living in the gut of ruminant animals such as sheep and cows synthesise odd chain fatty acids, which would be absorbed by the animal and incorporated into the adipose and muscle tissue. It is not uncommon for fatty acids with 13 to 19 carbons to be found at concentrations of up to 5 % in ruminant animal tissues (lipidlibrary.com). Odd chain fatty acids are found between 2.41 % and 3.83 % with an average concentration of 3.21 %. Adipose tissue contains the highest concentration of odd chain fats at 3.64 % and muscle triglycerides contain the lowest at 2.67 %.

6.5 Conclusion

Fatty acid methyl esters from triglycerides and phospholipids extracted from lamb muscle and adipose tissue were produced by two esterification methods, a one-step transesterification method and a two-step, hydrolysis/esterification method. The results acquired by GCMS analysis of these methyl esters indicate that both methods show the same recovery of methyl esters. The robustness of the transesterification method was proved and has been implemented in the study of the variation of triglyceride and phospholipids composition of lambs fed different diets.

7.0 Conclusion

Throughout this research, quantitative NMR methods have been developed for the analysis of triglycerides. These methods have been applied to triglycerides from a variety of sources; olive oil, milk and cheese.

The use of ^{1}H NMR to investigate the fatty acid species present in a triglyceride sample has been proven to be successful and to give results that match those from recognised techniques for the analysis of lipids (i.e. GC of fatty acid methyl esters).

The use of ^{1}H NMR provides a quick overview of the ratios of saturated, mono-, bis-, and poly-unsaturated fats present in a sample. It can also be used to calculate the ratios of *cis* and *trans* double bonds in a sample by comparing the ratios of integrals of *cis* and *trans* signals. This can be achieved in minutes as a standard ^{1}H NMR spectrum takes around 4 minutes to acquire. Integration of the signals present in a spectrum can be carried out automatically by NMR software and the manipulation of integrals from these spectra can be carried out using a spreadsheet. The number of samples that can be processed in a day becomes huge when compared to the more standard method of derivatisation and chromatographic analysis.

The use of ^{13}C NMR to quantitatively analyse complex mixtures is not common in organic chemistry, but it has been applied to the study of natural triglycerides throughout this research. Investigations were carried out to optimise a method for quantitative ^{13}C analysis of

triglycerides. An appropriate delay between pulses was determined by inversion recovery studies and the optimum concentration of $Cr(acac)_3$ to facilitate complete relaxation but minimise chemical shift change was calculated. These factors were combined with an increased number of scans (2000+, equivalent to approximately 2 hours) and resolution enhancement techniques (zero filling and line broadening) to achieve the best possible ^{13}C NMR spectrum of triglyceride mixtures.

Analysis of ^{13}C NMR spectra of triglycerides allowed identification of the individual fatty acids present in each triglyceride and led to their positional assignment on the glycerol backbone. As this ^{13}C NMR method was quantitative, analysis of the integrals of each of these signals gives a figure for the percentage of each fatty acid present on each position on the triglyceride.

These two quantitative NMR methods were applied to the analysis of extra virgin olive oil, to the study of seasonal variation of triglyceride composition of cows' milk and to the comparison of triglyceride composition of regional British cheeses.

Looking at the positional distribution of fatty acids on the triglyceride backbone of olive oil triglycerides showed that saturated fats were only found on the alpha-position meaning the beta-position was occupied exclusively by unsaturated fatty acids.

The results obtained by quantitative NMR analysis of olive oil triglycerides were compared with GCMS analysis of fatty acid methyl esters prepared from the olive oils. These results indicated that ^{1}H NMR shows an accurate representation of the fatty acid species present

within an olive oil and ^{13}C NMR identified the individual fatty acids present in olive oil at levels of 2 % or greater. Not only did ^{13}C NMR identify these fatty acids, but it assigned their position on the triglyceride backbone. The composition of olive oils as determined by this research corresponds to that stated in the literature.

Analysis of extra virgin olive oil showed trends in the triglyceride composition of olive oils from different countries. Olive oils produced from Spanish olives had a triglyceride composition that differed notably from oils produced from Italian, Greek and Australian oils. It was also found that oils produced from the same region of Spain but from different cultivars of olives had different triglyceride compositions.

Analysis of milk triglycerides was carried out by ^{1}H NMR and quantitative ^{13}C NMR. These results were compared with those obtained by GCMS analysis of fatty acid butyl esters prepared from milk triglycerides.

Again, the results from ^{1}H NMR and ^{13}C NMR showed the same fatty acid composition as the butyl esters produced from the triglycerides, which were analysed by GCMS. Fatty acids present within the triglyceride at 2 % or greater were identified and quantified by ^{13}C NMR and their position on the glycerol backbone was identified.

It was found that esters of butyric acid were only present on the alpha-position of milk triglycerides and that the fatty acids myristate and laurate were not detected on the alpha-position, only on the beta-position. Stearate and palmitate were found to be present on the

alpha-position in greater proportion than on the beta-position but oleate was evenly distributed across the two positions.

The overall, average composition of cows' milk as determined in this study is in agreement with average triglyceride compositions reported in the literature.

Analysis of the triglyceride composition of regional British cheeses was carried out using the quantitative NMR methods developed as part of this research. There was a noticeable difference in the triglyceride composition of cheeses from different areas. The cheese-making process for each different cheese analysed was different, but the ingredients of the cheese were the same – they were all produced from milk curds. In this way, the variations in triglyceride composition can be put down to the composition of the milk the cheese was produced from or the production process itself. The fatty acid species present in cheese triglycerides extracted from British regional cheeses showed similar profiles, which also fitted the composition of cows' milk reported in the literature. A significant difference was noticed in the fatty acid species composition of triglycerides from Italian cheese, which contained higher amounts of bis- and poly-unsaturated fats.

The fatty acid species composition of cheese triglycerides as determined by ^{1}H NMR matched that from ^{13}C NMR analysis. ^{13}C NMR analysis, once again, identified and quantified the individual fatty acids present at 2 % or greater and assigned them to either the alpha- or beta-position on the glycerol backbone.

As part of this research, a method to analyse the lipids present in lamb muscle and fat was developed. The School of Agriculture and Forestry Science at the University of Wales, Bangor, carried out a study with the aim to increase the proportion of ω-3 fatty acids present in lamb. Papers previously published in this field have used a two-step method to prepare samples for analysis by GC. After extraction of the lipids and separation of them into lipid classes, the lipids were hydrolysed to form free fatty acids and then esterified using diazomethane to produce methyl esters. This method used expensive, potentially dangerous and now hard to source chemicals. A method was developed as part of this research to carry out this esterification in one-step. This method reduced the likelihood of contamination and sample loss as only one step was required, and used less harmful chemicals. The results obtained from both methods of methyl ester preparation were compared and no significant differences in composition were detected.

After the establishment of the one-step transesterification method, a large number of samples of lamb were analysed to compare the triglyceride and phospholipids composition of the meat with the triglyceride composition of the fat when lambs were fed diets containing different fatty acids. The results of this study will be reported elsewhere; analysis of the data, however, showed that the method of analysis developed in the current work was robust and repeatable.

The methods developed throughout this research can be applied to any number of triglyceride sources and the position and proportion of fatty acids on the glycerol backbone can be assigned if the component of interest is present at 2 % or greater. A greater sensitivity could be achieved with a greater number of scans when acquiring data, but this adds to the analysis time.

This work could be furthered by investigating methods to further reduce the errors present in using integral data from quantitative ^{13}C NMR to gain a more accurate quantitative picture of the triglyceride mixture as a whole. A further study sing a hyphenated system of HPLC with NMR would allow separation of the individual triglycerides within a sample and allow them to be identified by NMR. This would give a more complete picture of triglyceride composition, as it would identify which fatty acids were adjacent to each other on individual molecular species of triglyceride.

8.0 Experimental

Extraction, derivatisation and analytical methods used in this research are outlined below.

8.1 Extraction Methods

8.1.1 Extraction of lipids from milk

Lipids were extracted using a Folch-style extraction. Milk (40 ml) was shaken with chloroform/methanol (2:1, 90 ml). This process was repeated until 240 ml of milk had been extracted. The organic fraction was collected and washed with brine (50 ml). The lipid-containing solution was then dried over $MgSO_4$ and the solvent was removed by rotary evaporation.

8.1.2 Extraction of lipids from cheese

Cheese (30 g) was grated using a fine grater, then cooled in liquid nitrogen and ground in a pestle and mortar to a fine powder. The cheese was allowed to warm to room temperature and was added to chloroform/methanol (2:1, 150 ml) and heated under reflux for 3 hours. The solution was allowed to cool to room temperature and the solution filtered through a grade 1 quality sinter funnel. The solution was then washed with brine (2 x 20 ml) and the organic fraction collected and dried over $MgSO_4$. The solvent was then removed by rotary evaporation. The crude triglyceride sample was then analysed by NMR, then purified by column chromatography under medium pressure using silica gel from BDH (particle size 33 -

70µm). petrol/ether (10:1) to remove the cholesterol and other simple lipids from the triglyceride fraction.

8.1.3 Extraction of lipids from lamb muscle tissue

Muscle tissue (10 g) was prepared by mincing using a Knifetec water-cooled blender. The meat sample was stirred at room temperature with chloroform/methanol (2:1, 30 ml, with BHT (butyrated hydroxy toluene) present at 100mg/l as an anti-oxidant) for 1 hour. The solution was filtered under vacuum, and then dried using $MgSO_4$. The solvent was removed by rotary evaporation. The lipid sample was then dissolved in chloroform (ca. 1 ml) and added to a conditioned SPE column (Waters, silicic acid, 2 g). The neutral lipids were extracted by washing with chloroform (2 x 10 ml) and the polar lipid fraction removed by washing with methanol (10 ml). Both fractions were analysed by ^{31}P NMR to confirm the separation of neutral (triglycerides) and polar (phospholipids) lipid fractions.

8.1.4 Extraction of lipids from lamb adipose tissue

Adipose tissue (3 g) was prepared by mincing using a Knifetec water-cooled blender. The adipose tissue sample was stirred with chloroform (20 ml) containing BHT (100 mg/L) for 1 hour. The solution was filtered under vacuum and dried over $MgSO_4$. The solvent was removed by rotary evaporation.

8.2 Preparation of derivatives

8.2.1 Preparation of methyl esters

Methyl esters were prepared from triglycerides by transesterification using sodium methoxide using the following method. A solution of sodium methoxide was prepared by dissolving sodium metal (0.12 g) in dry methanol (0.25 ml). The solution was stirred until the sodium was dissolved and the solution had cooled to room temperature. The triglyceride sample (0.5

g) was dissolved in hexane (*ca.* 1 ml) and added to the sodium methoxide solution. The solution was heated under reflux for two minutes and allowed to cool to room temperature. Water (10 ml) was added to quench the reaction. The solution was washed with hexane (2 x 15 ml) and the organic fraction collected and dried over $MgSO_4$. The solvent was removed by rotary evaporation.

8.2.2 Preparation of butyl esters

Butyl esters were prepared from triglycerides by transesterification using sodium butoxide. A solution of sodium butoxide was prepared by adding sodium metal (0.12 g) to reagent grade 1-butanol (30 ml). The solution was heated under reflux until complete dissolution of the sodium metal had occurred. The triglyceride sample (0.5 g) was added to the sodium butoxide solution. The solution was heated under reflux for 2 hours and allowed to cool to room temperature. The solution was washed with water (10 ml) and brine (2 x 15 ml). The organic fraction was collected and dried over $MgSO_4$ and the solvent removed by rotary evaporation and then under a stream of nitrogen.

8.3 Analysis methods

8.3.1 NMR

NMR analysis was carried out using a Bruker Avance 500 spectrometer with a 5 mm BBO probe. Solutions were all made up in deuterated chloroform (0.625 ml). Chemical shifts were reported in δ relative to the trace resonance of protonated chloroform for ^{1}H NMR (δ = 7.27 ppm) and deuterated chloroform for ^{13}C NMR (δ = 77.00 ppm). ^{13}C NMR was carried out with

2000 scan with a D_1 of 5 seconds and a spectral width of 200 ppm. ^1H NMR was carried out with 16 scans.

Samples were prepared by dissolving triglyceride (100 mg) in deuterated chloroform (0.625 ml) in 5 mm NMR tubes of 500 MHz quality. If quantitative ^{13}C analysis was to be carried out, $Cr(acac)_3$ (7.5 mg) was added.

Analysis of some triglyceride standards was performed in 3mm NMR tubes. This was carried out when the mass of the sample for analysis was less that 100 mg. Samples were prepared by dissolving the triglyceride (10 mg) in $CDCl_3$ (0.125 ml). If quantitative ^{13}C NMR was to be carried out, $Cr(acac)_3$ (1.5 mg) was added.

8.3.2 Analysis using GCMS

Analysis was carried out using an HP5890 Gas Chromatograph with automatic injection and an HP quadrupole mass spectrometer detector. The GC column contained a CP-Select FAME stationary phase, specially designed for the separation of methyl esters. It was 100m long with and internal diameter of 1.6mm with a phase thickness of 1.6µm. Helium was used as the carrier gas with a split/splitless ratio of 10:1.

Analysis of methyl esters was carried out using the following temperature program: Isothermal at 190°C for 20 minutes then an increase of 10°C/minute to 210°C for 8 minutes. Samples were dissolved in HPLC grade methanol at a concentration of between 1 mg/ml and 10 mg/ml. Fatty acid methyl esters prepared from lamb samples had an internal standard (methyl heneicosanoate) added at a concentration of 0.1 mg/ml.

Analysis of butyl esters was carried out using the following temperature program: Isothermal at 190°C for 10 minutes then an increase of 10°C/minute to 210°C for 20 minutes. Samples were dissolved in HPLC grade methanol at a concentration of 10 mg/ml.

9 Characterisation of NMR Spectra

Interpretation of ^{13}C NMR spectra of triglycerides is complex due to the large number of carbons present in the molecule. The complication of fatty acids having different chemical shifts when on the alpha- and beta-positions adds another degree of complexity to this problem.

The fatty acids commonly found in the lipids studied throughout this research are straight-chained fatty acids with between four and eighteen carbons and between zero and three double bonds. The ^{13}C NMR spectra of triglycerides of these fatty acids are shown in the figures below, showing the full spectrum as well as a closer look at the regions of interest. The short chain saturated fatty acids tributanoin (C4:0), trihexanoin (C6:0), trioctanoin (C8:0) and tridecanoin (C10:0) are shown in the first section Figure 65 to Figure 70. The medium to long chain saturated fatty acids trilaurin (C12:0), trimyristin (C14:0), tripalmitin (C16:0) and tristearin (C18:0) are shown in the second section (Figure 71 to Figure 76) and the 18C unsaturated triglycerides triolein (C18:1), trilinolein (C18:2) and trilinolenin (C18:3) are shown in the third section (Figure 77 to Figure 83).

A more in-depth study of the interpretation of these spectra is given for real samples of olive oil and milk triglycerides. These spectra contain a large number of fatty acids present at levels detectable by NMR and these fatty acids are distributed across all three positions on the glycerol backbone.

The calculations used to work out the proportions of each fatty acid present on each position in the triglyceride sample are also shown on these complex spectra. The quantitative ^{13}C NMR spectrum of an extra virgin olive oil sample is shown in Figure 84 to Figure 87 along with all the mathematics used to calculate the ratios of fatty acids present.

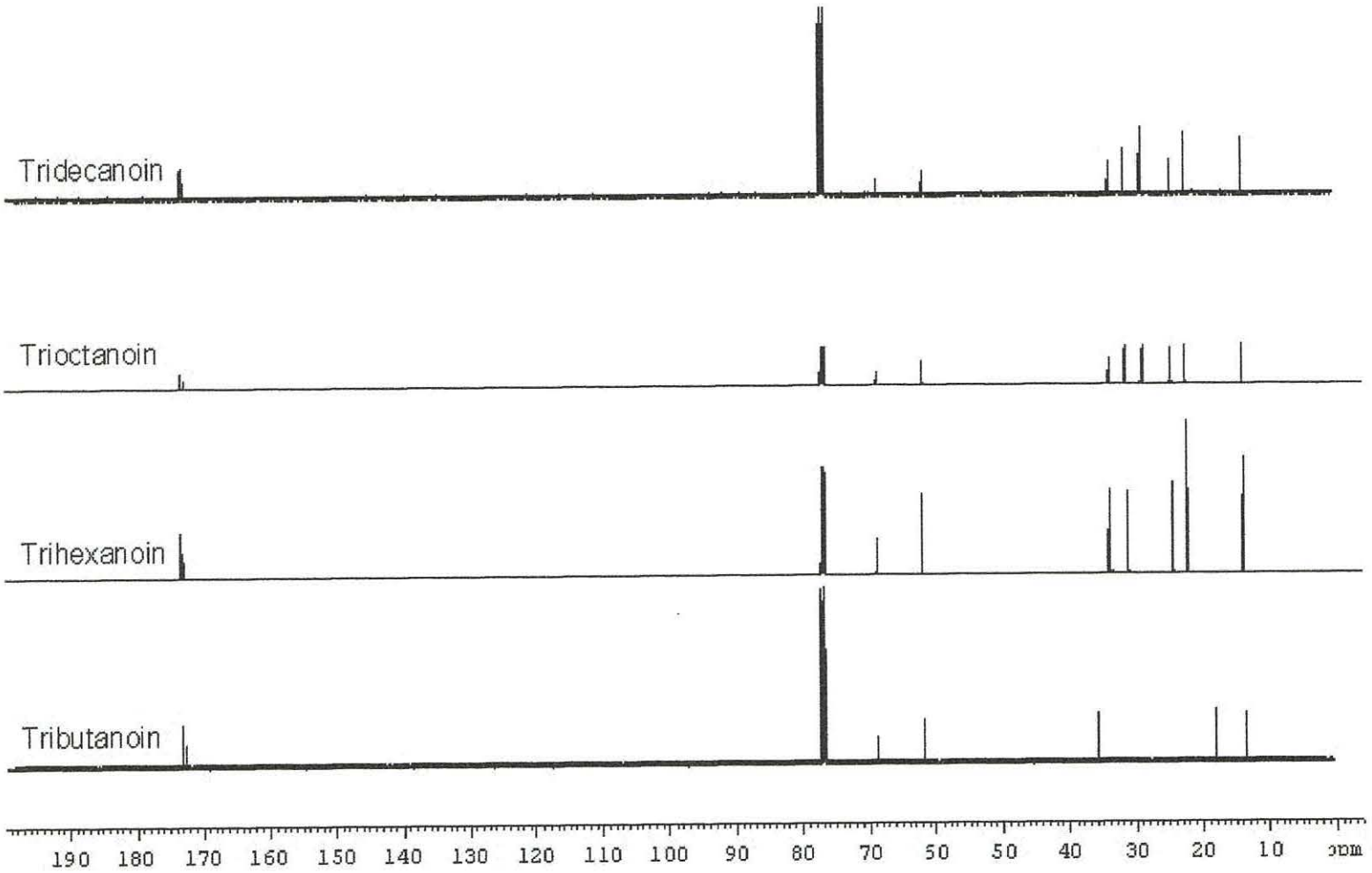

Figure 65: Quantitative ^{13}C NMR of short chain saturated triglycerides; Tributanoin, Trihexanoin, Trioctanoin and Tridecanoin.

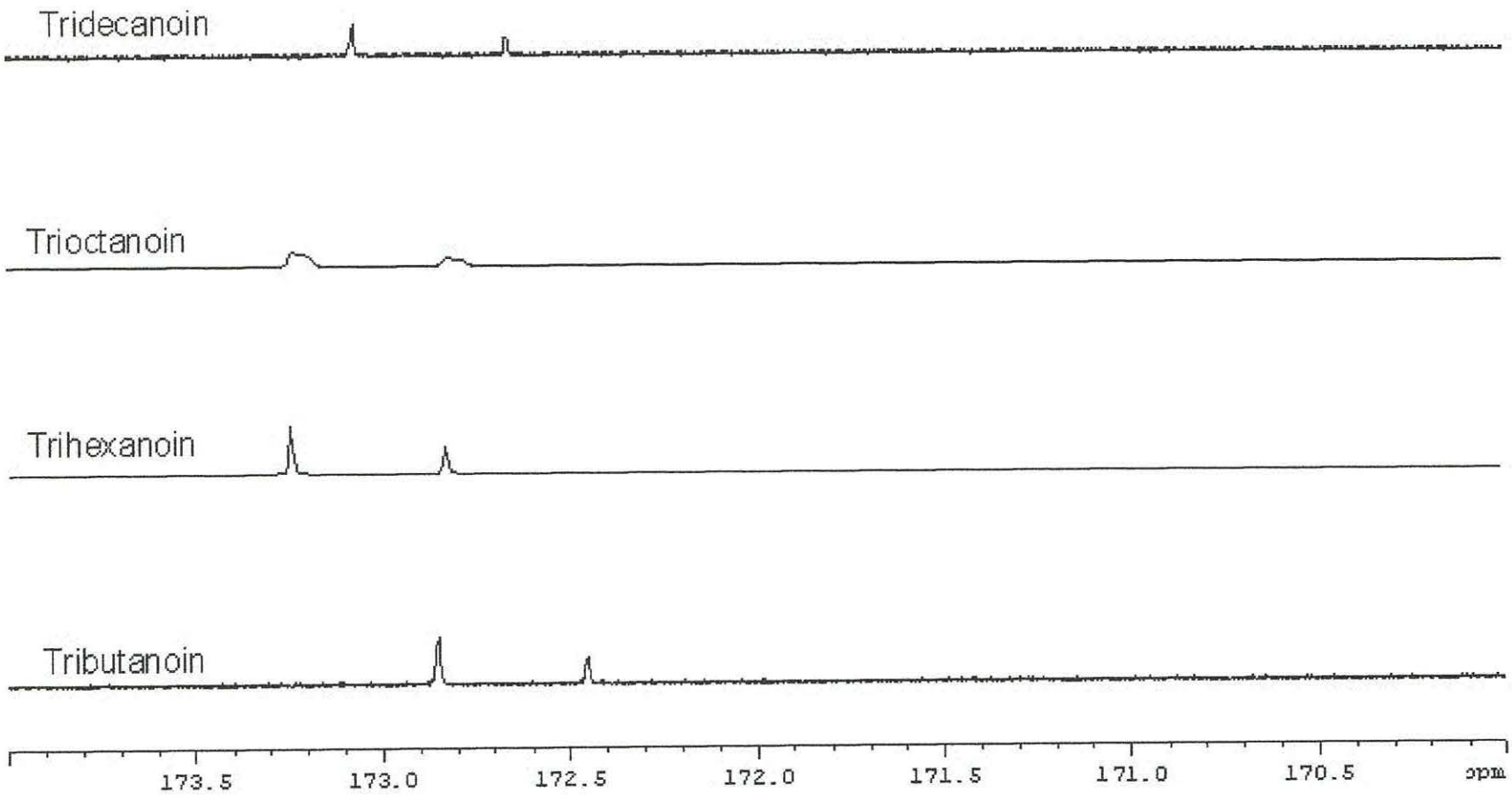

Figure 66: Carbonyl region (174 - 170 ppm) of quantitative 13C NMR spectra of short chain saturated fats.

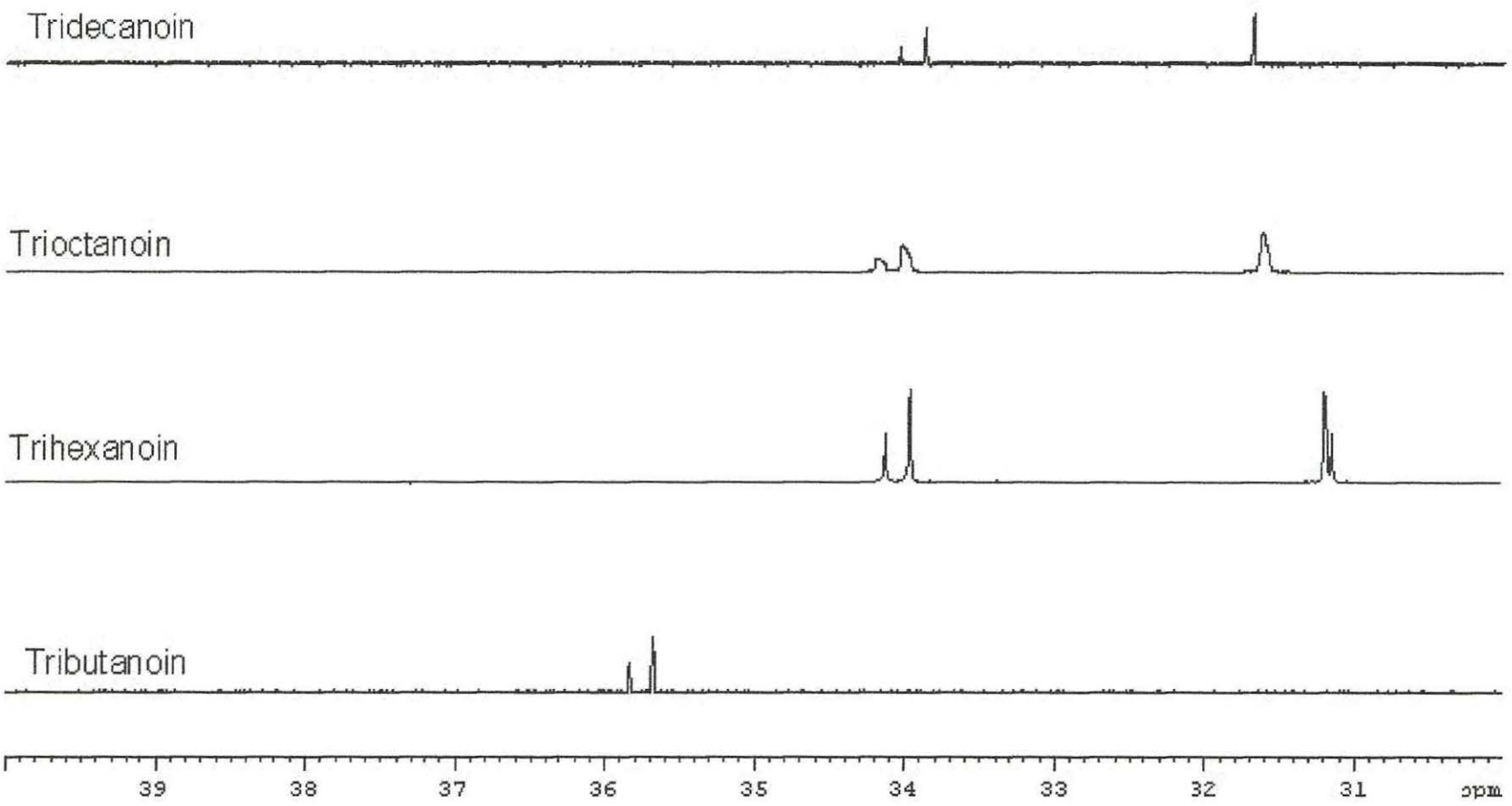

Figure 67: Acyl chain region (40 - 30 ppm) of quantitative ^{13}C NMR spectra of short chain saturated fats.

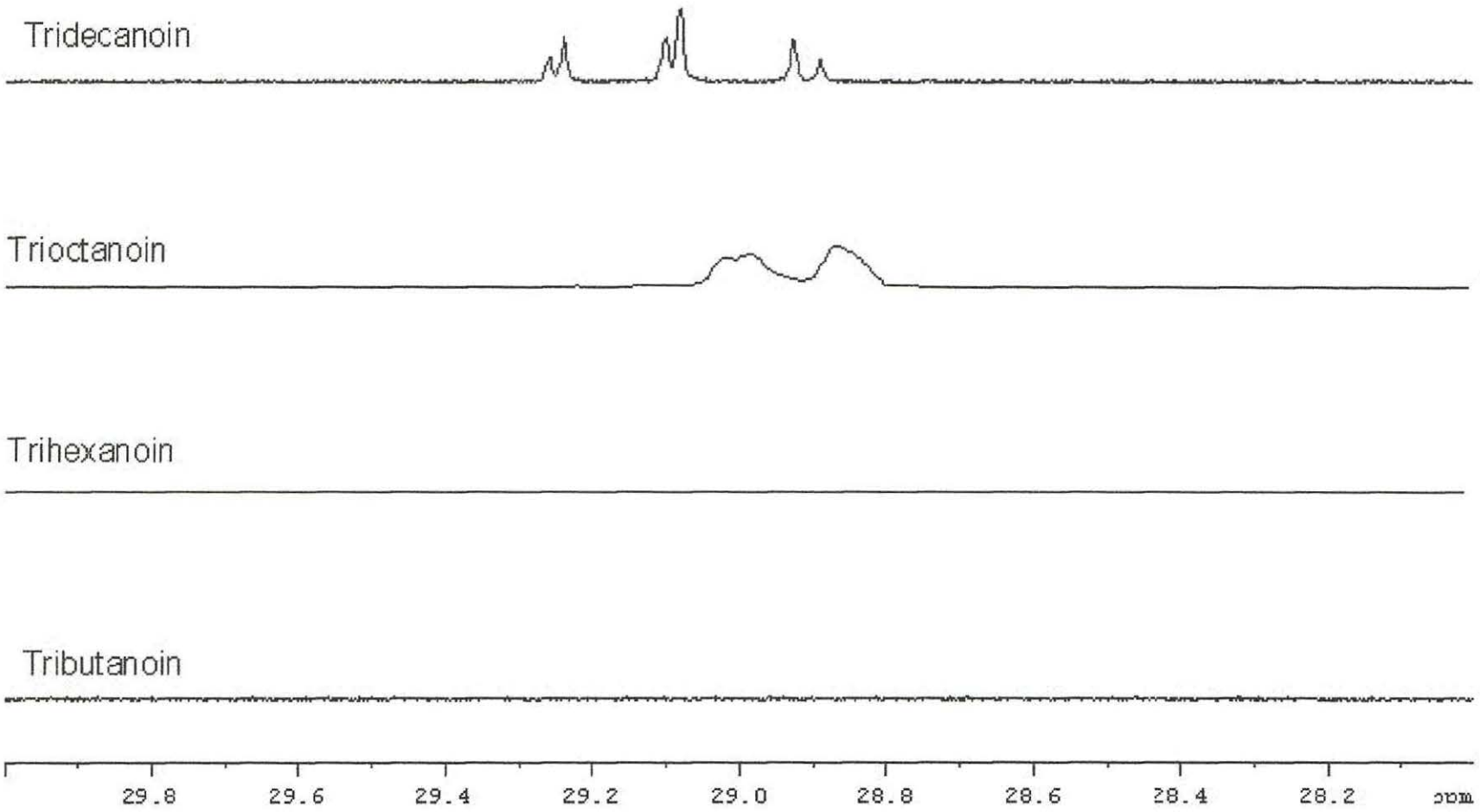

Figure 68: Fingerprint region (30 - 28 ppm) of quantitative ^{13}C NMR spectra of short chain saturated fats.

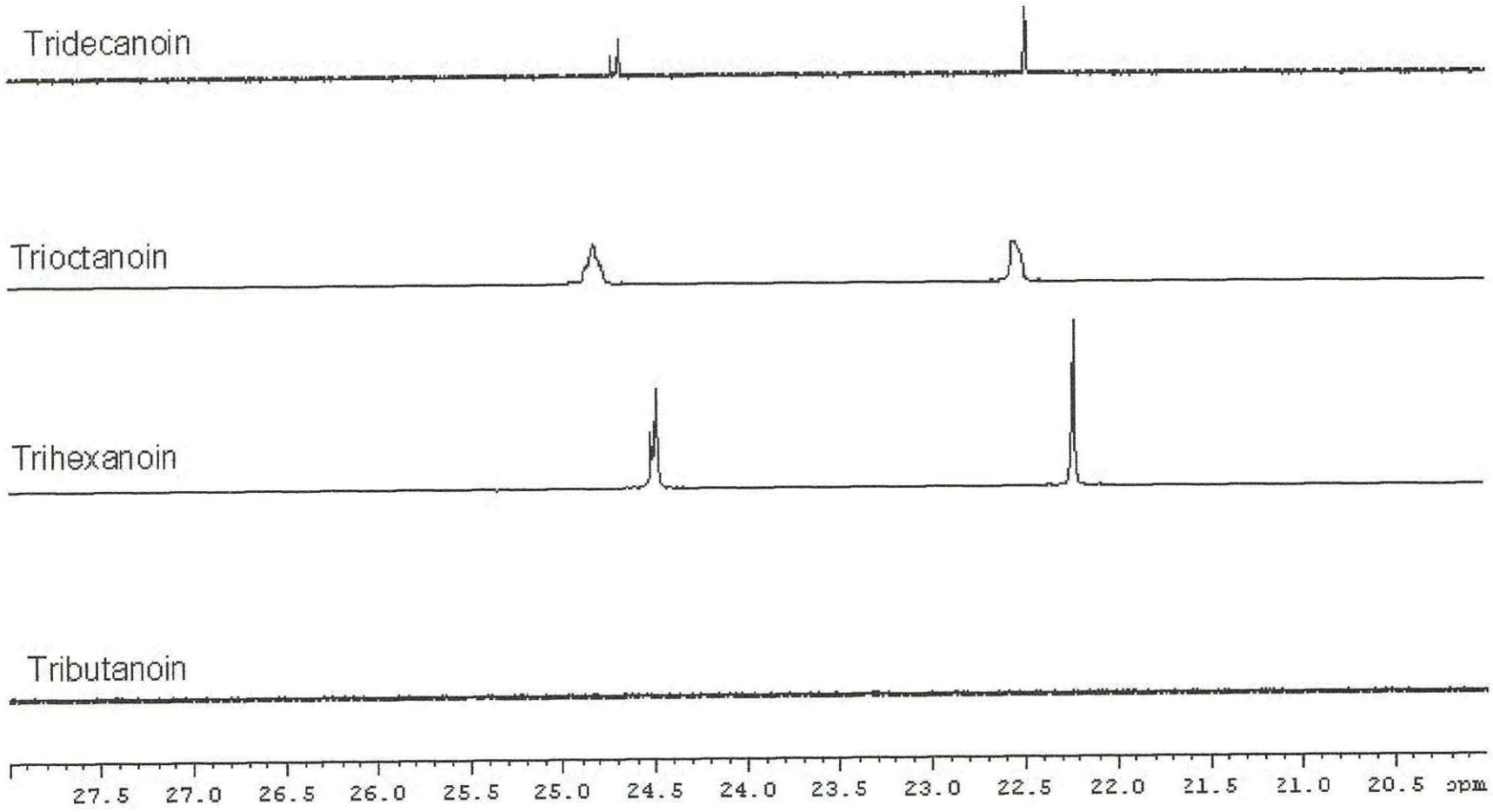

Figure 69: Acyl chain region (28 - 20 ppm) of quantitative ^{13}C NMR spectra of short chain saturated fats.

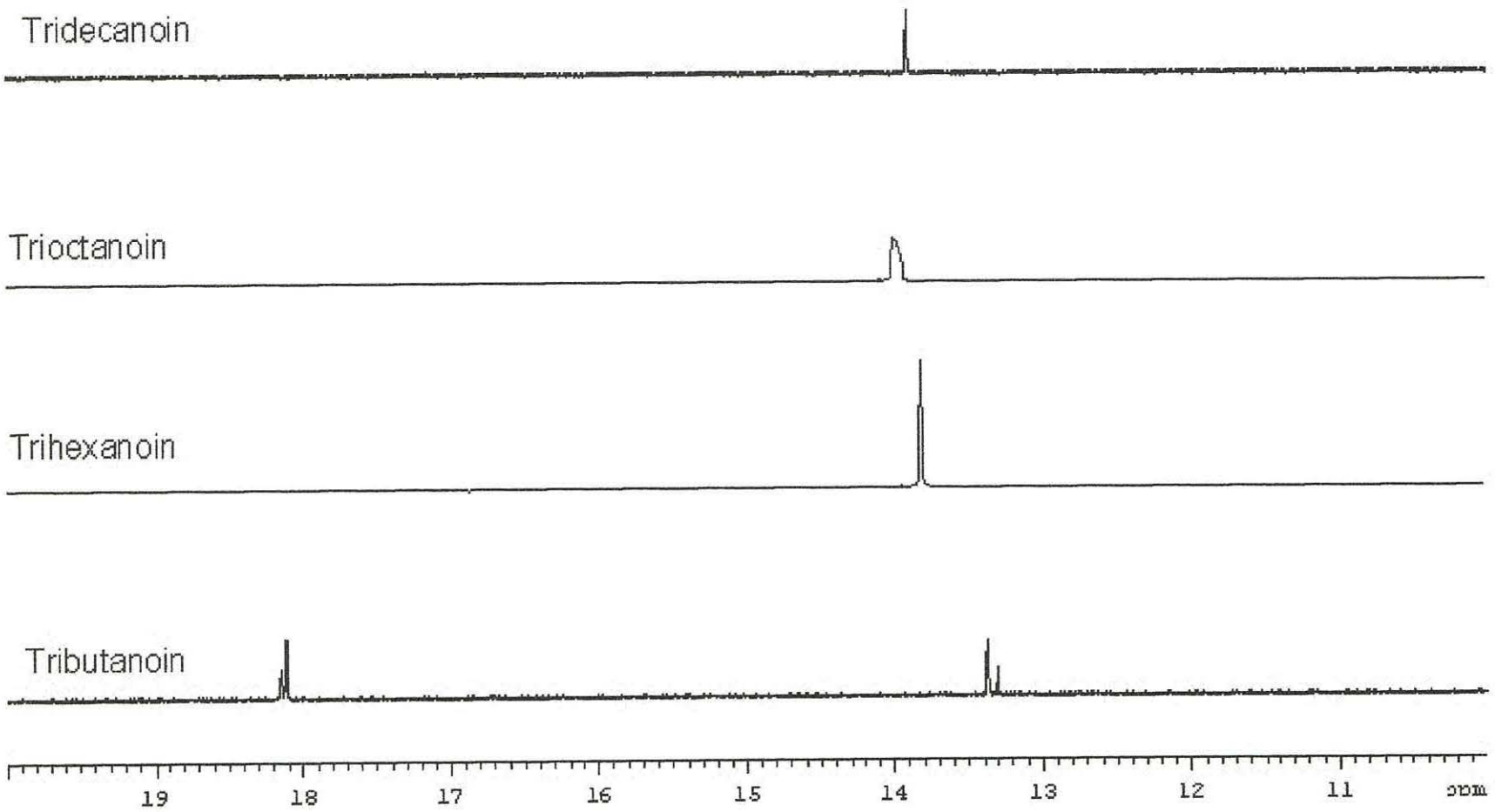

Figure 70: Terminal methyl region (20 - 10 ppm) of quantitative ^{13}C NMR spectra of short chain saturated fats.

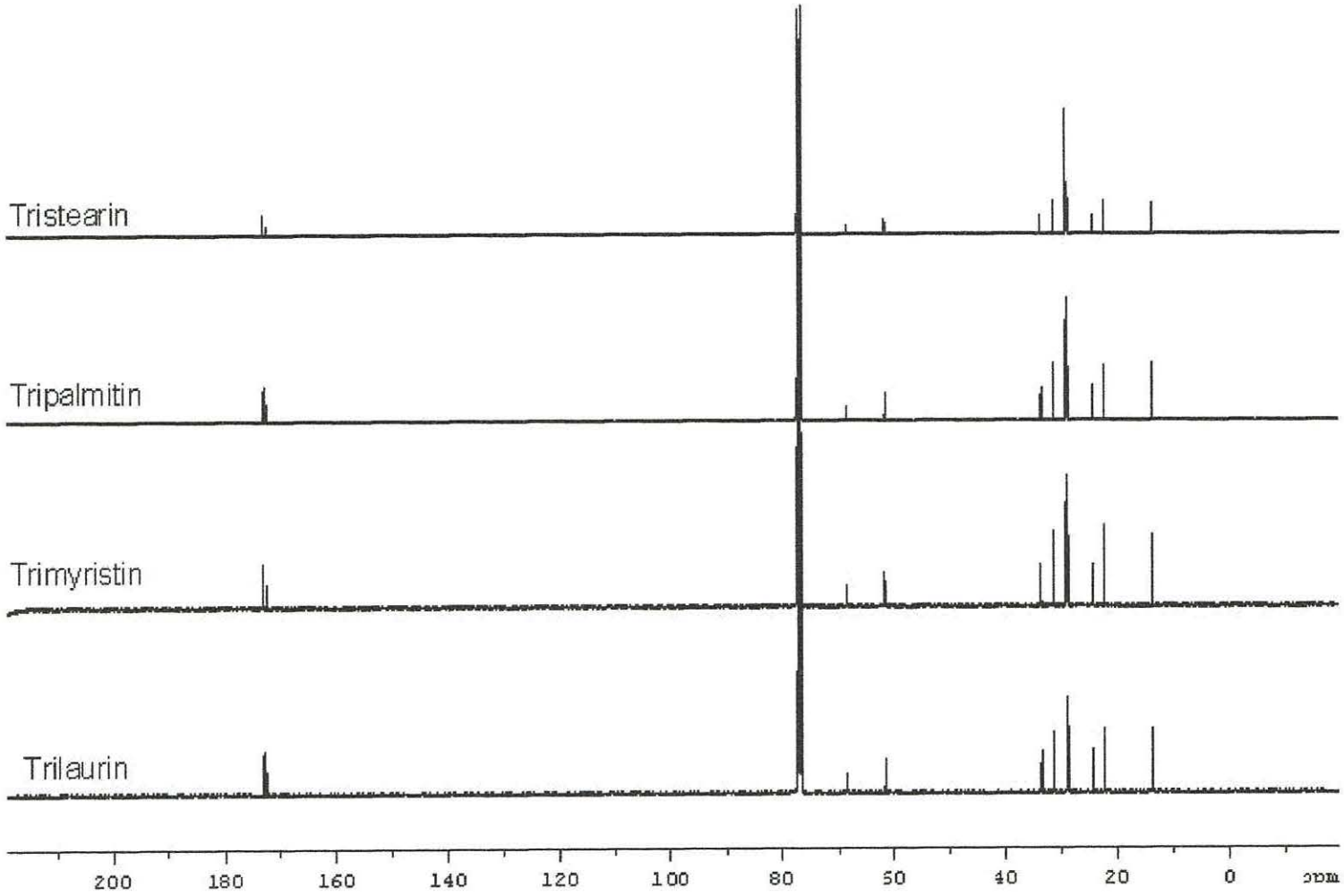

Figure 71: Quanitative ^{13}C NMR spectra of medium-long chain saturated triglycerides; trilaurin, trimyristin, tripalmitin and tristearin.

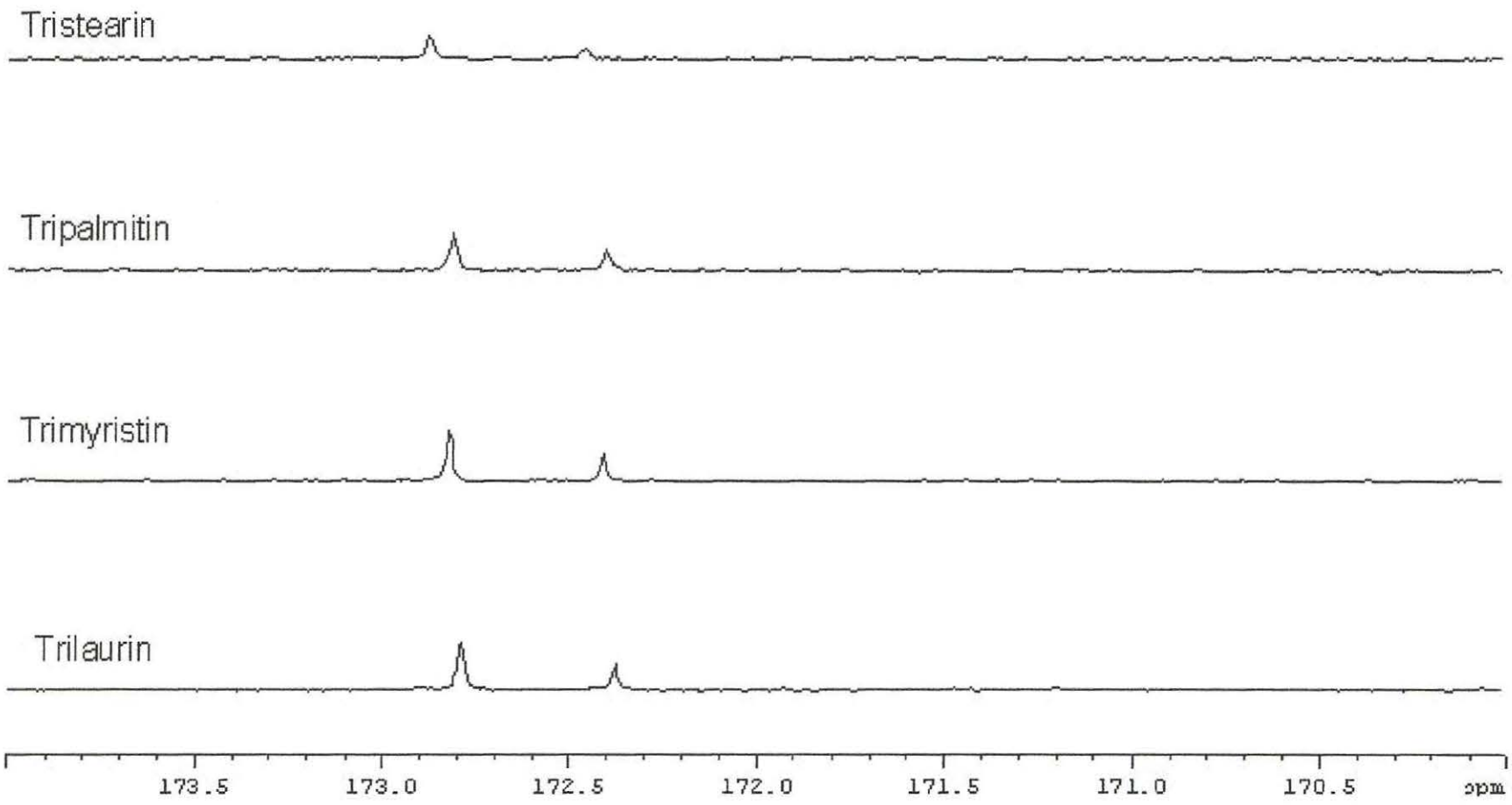

Figure 72: Carbonyl region (174 - 170 ppm) of quanitative ^{13}C NMR spectra of medium-long chain saturated triglycerides.

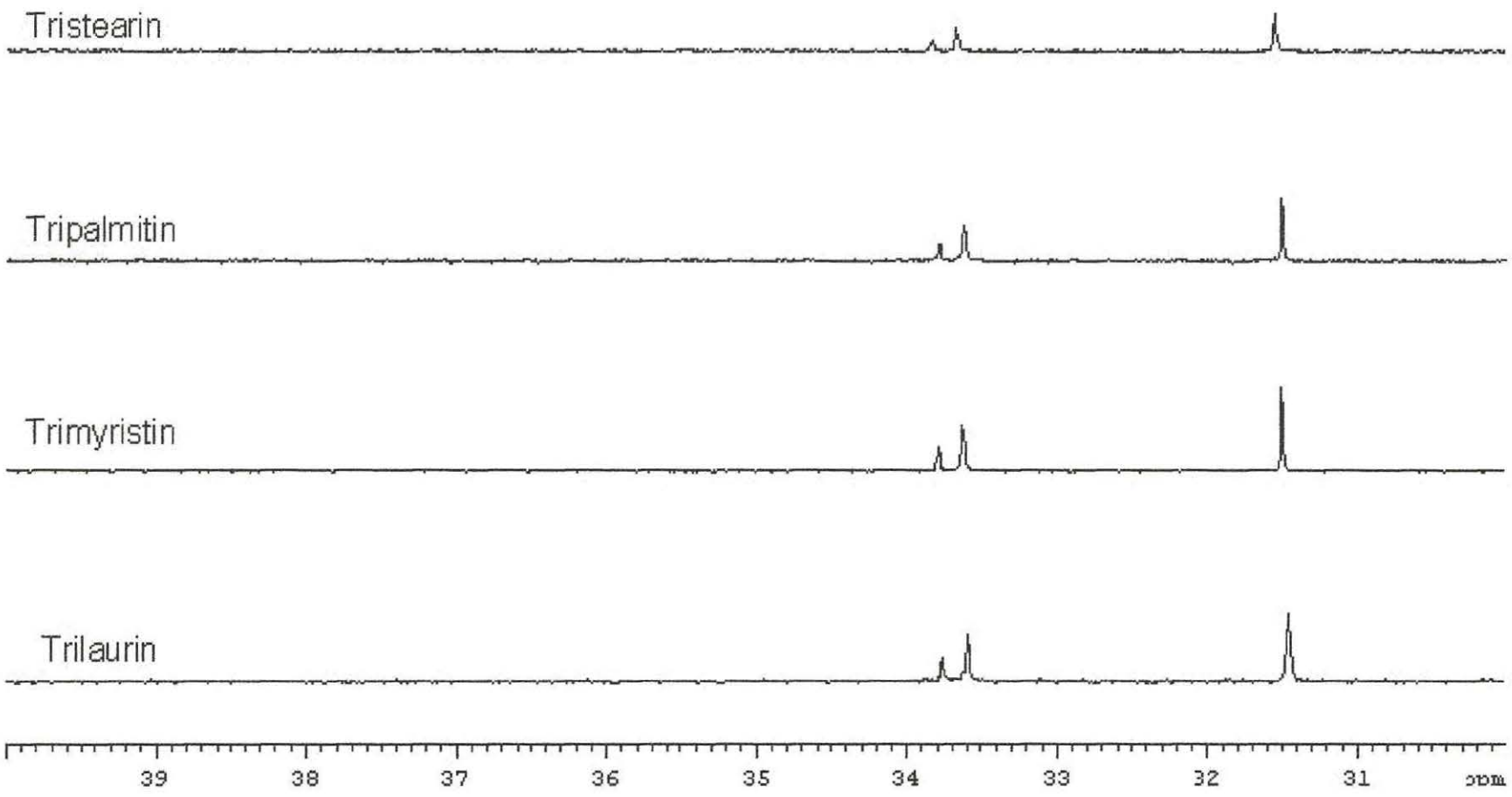

Figure 73: Acyl chain region (40 - 30 ppm) of quanitative ^{13}C NMR spectra of medium-long chain saturated triglycerides.

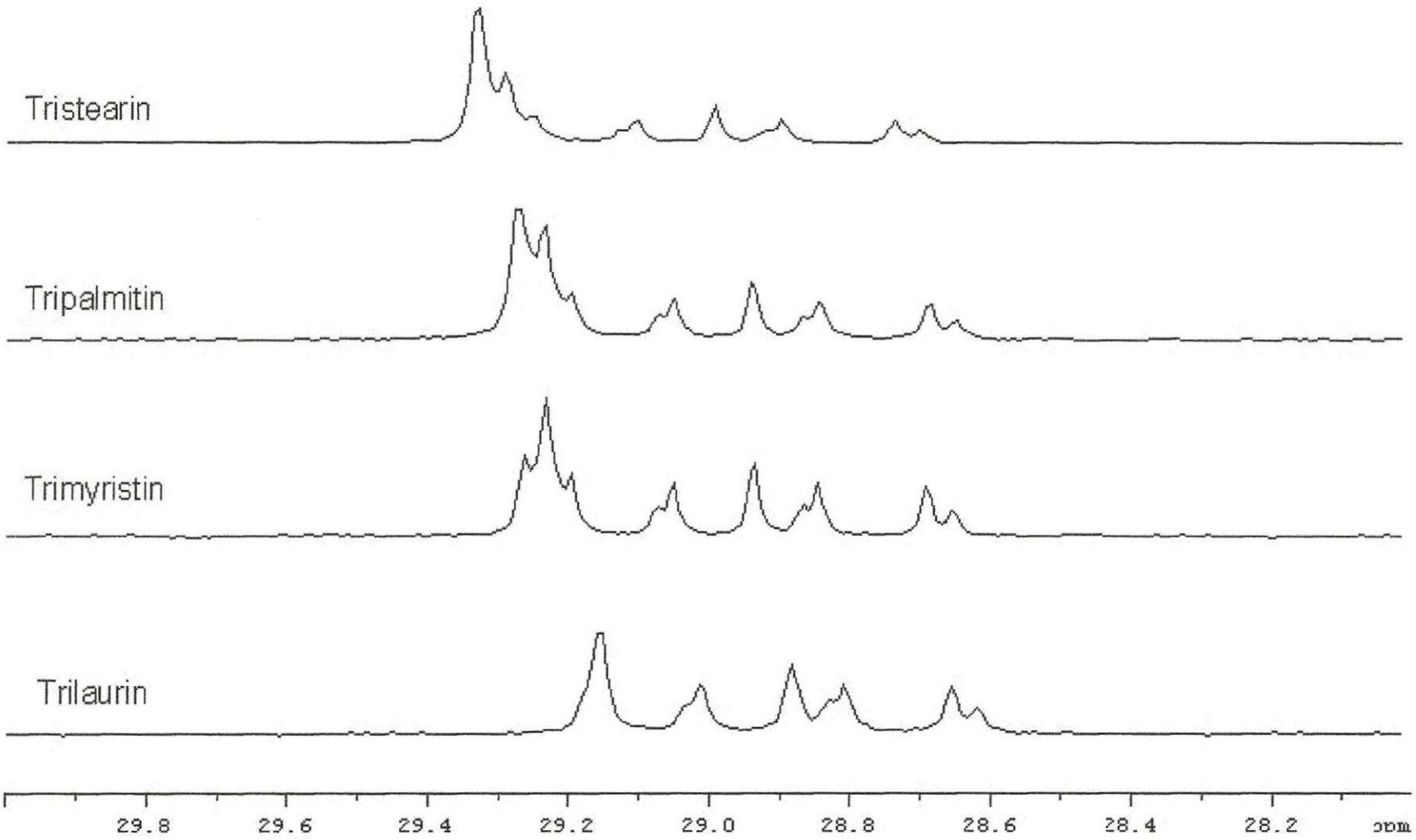

Figure 74: Fingerprint region (30 - 28ppm) of quantitative ^{13}C NMR spectra of medium-long chain saturated triglycerides.

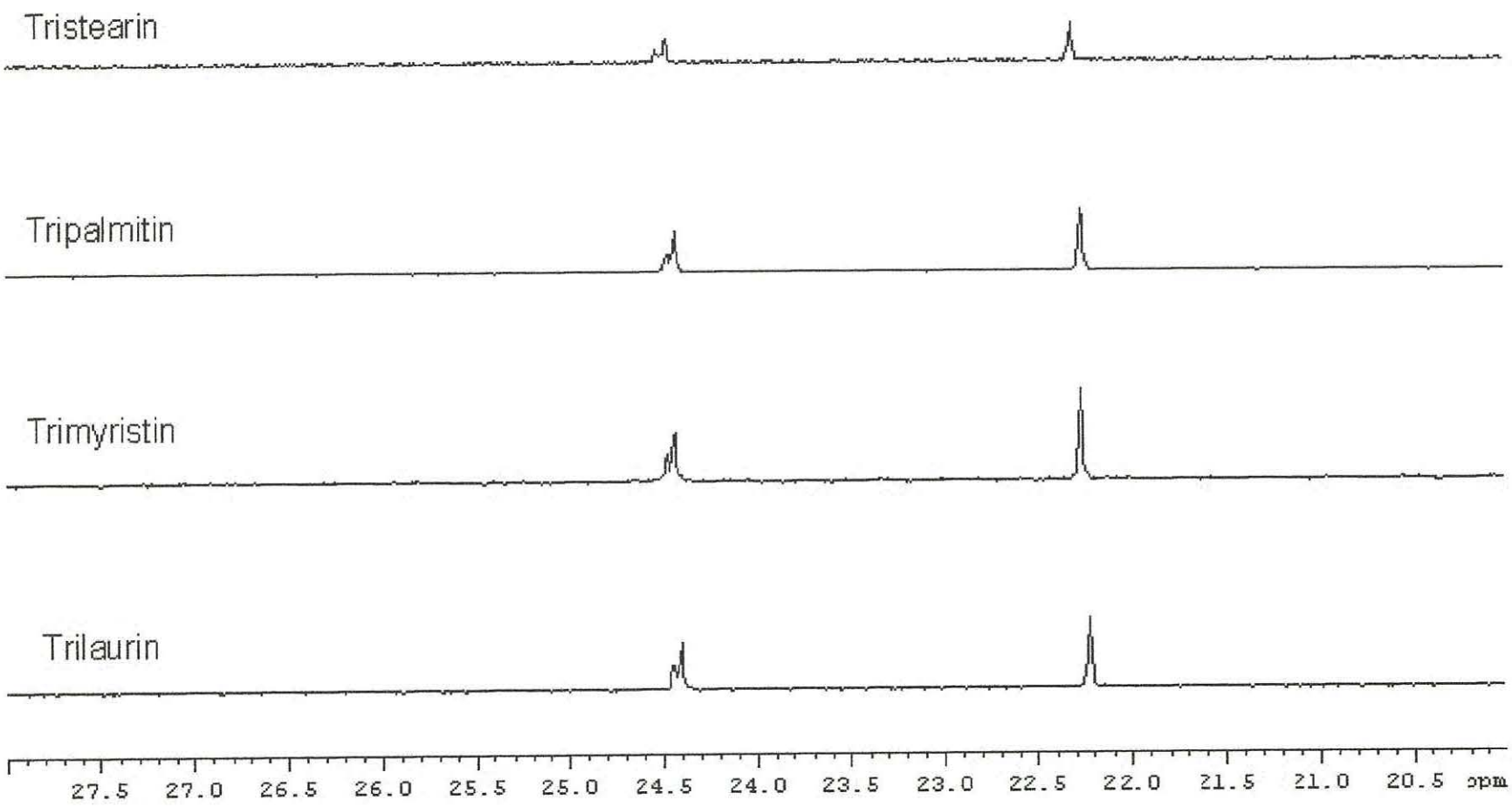

Figure 75: Acyl chain region (28 - 20 ppm) of quantitative ^{13}C NMR spectra of medium-long chain saturated triglycerides.

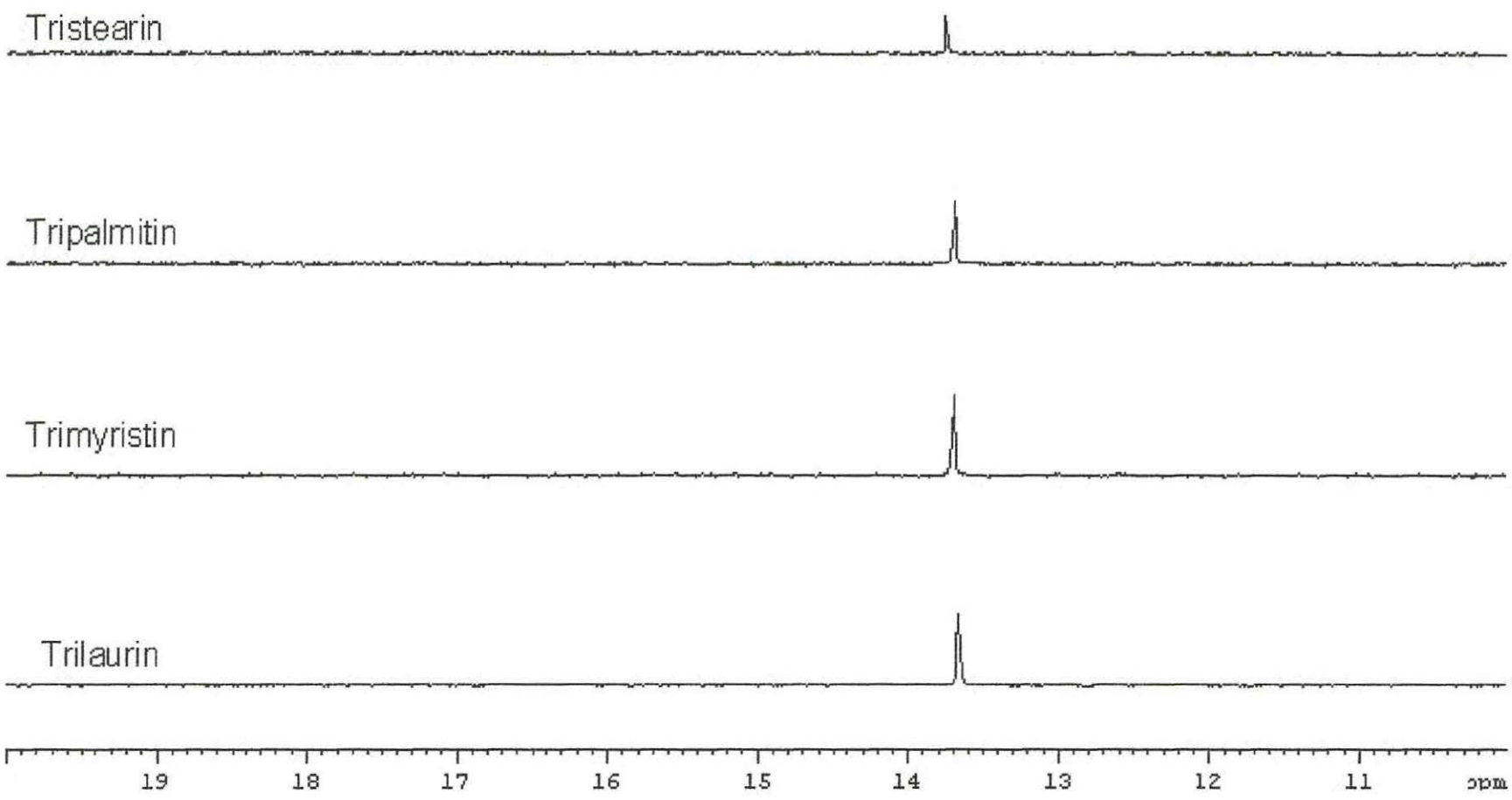

Figure 76: Terminal methyl region (20 - 10 ppm) of quantitative ^{13}C NMR spectra of medium-long chain saturated triglycerides.

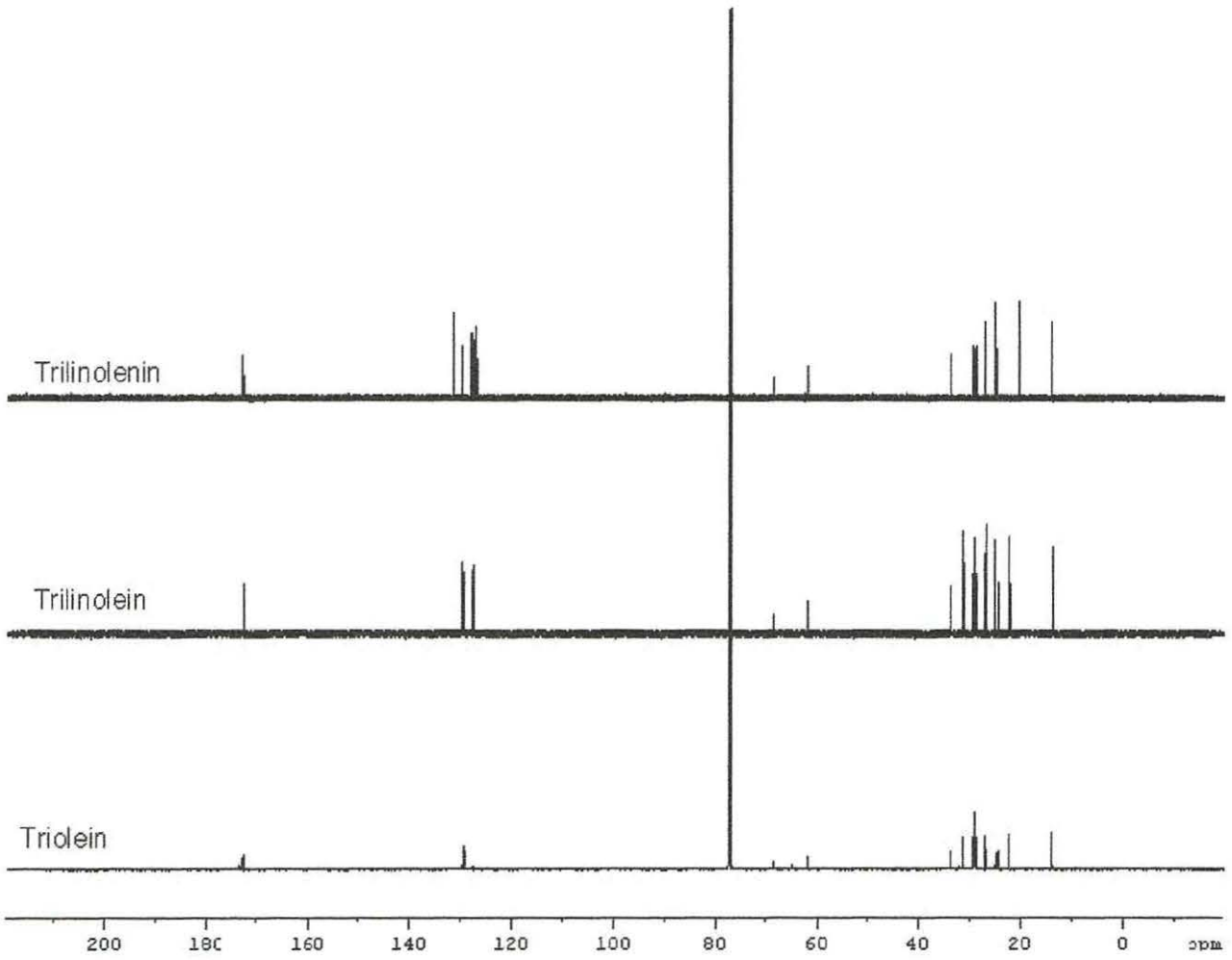

Figure 77: Quantitative ^{13}C NMR spectra of unsaturated and poly-unsaturated 18 Carbon triglycerides; triolein, trilinolein and trilinolenin.

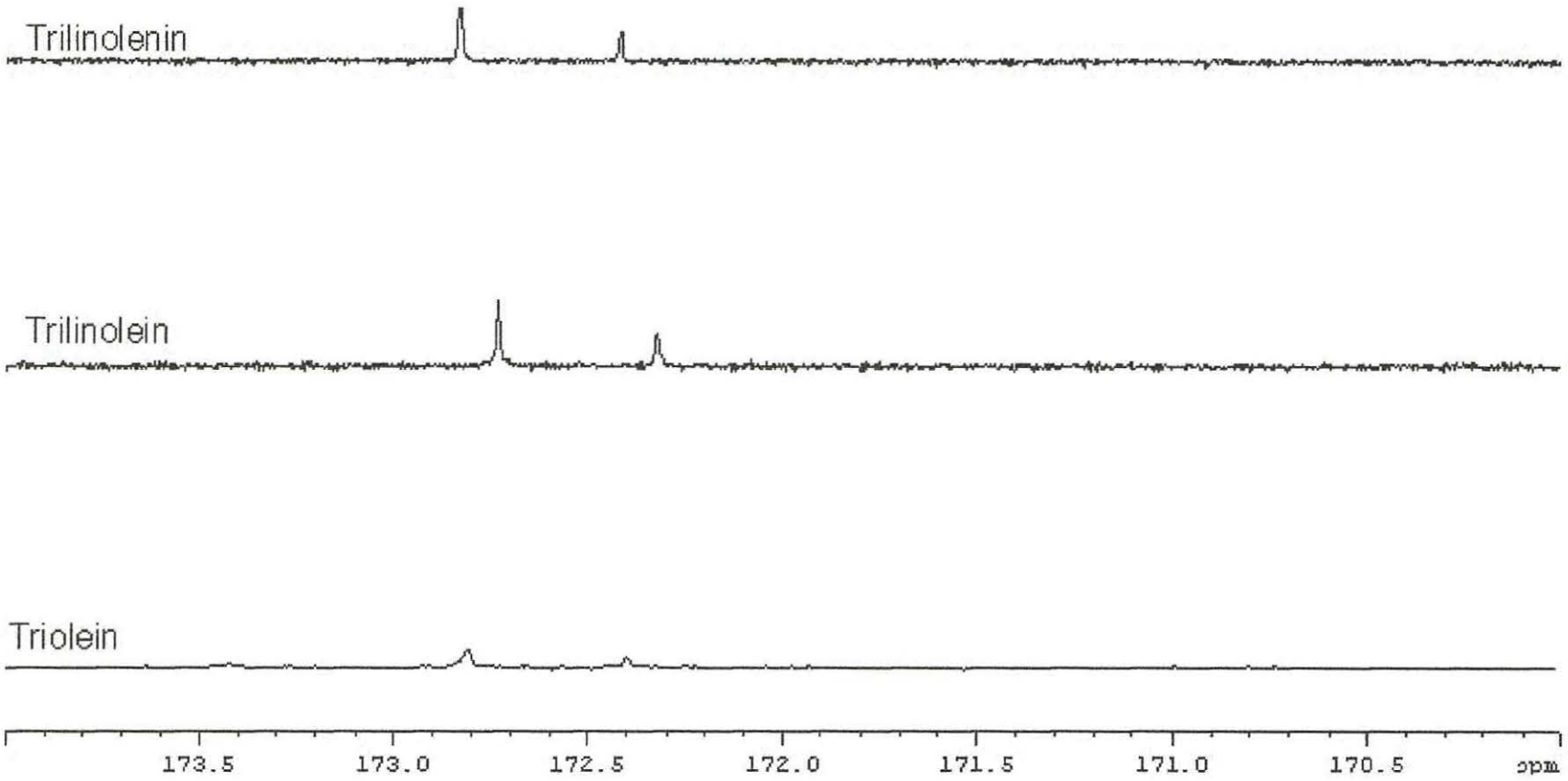

Figure 78: Carbonyl region (174 - 170 ppm) of quantitative ^{13}C NMR spectra unsaturated triglycerides.

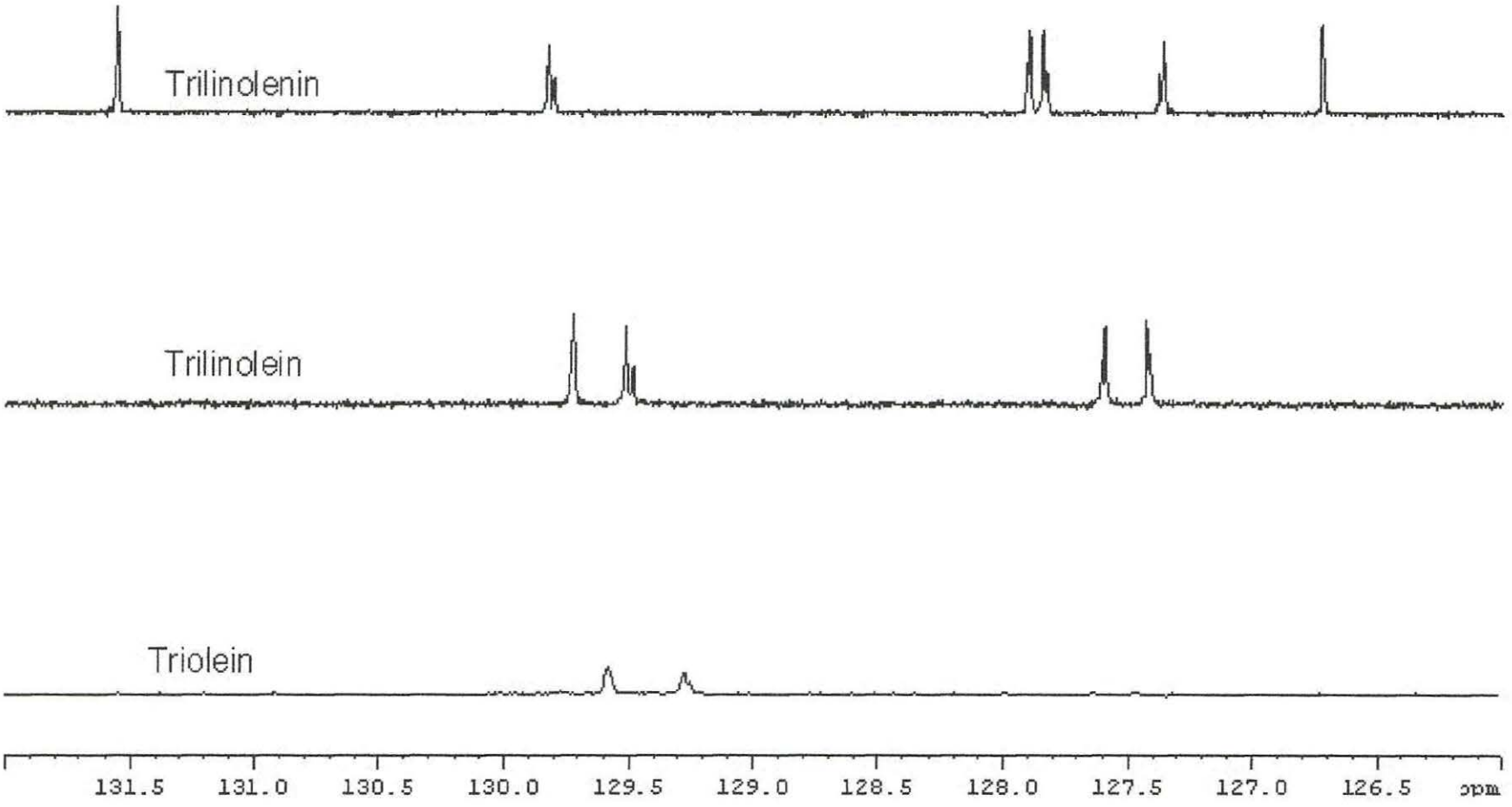

Figure 79: Olefinic region (132 - 126 ppm) of quantitative ^{13}C NMR spectra unsaturated triglycerides.

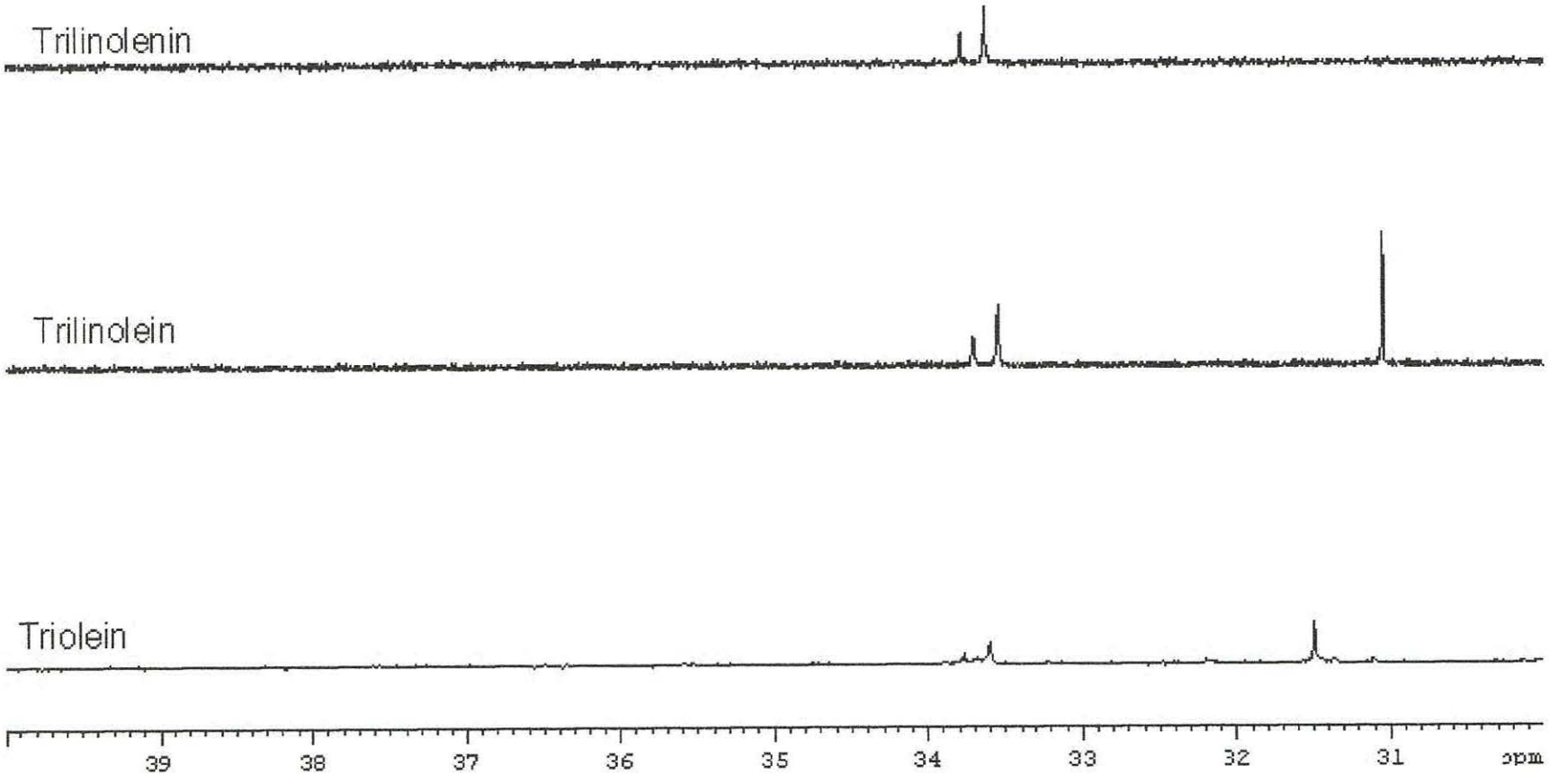

Figure 80: Acyl chain region (140 - 30 ppm) of quantitative ^{13}C NMR spectra unsaturated triglycerides.

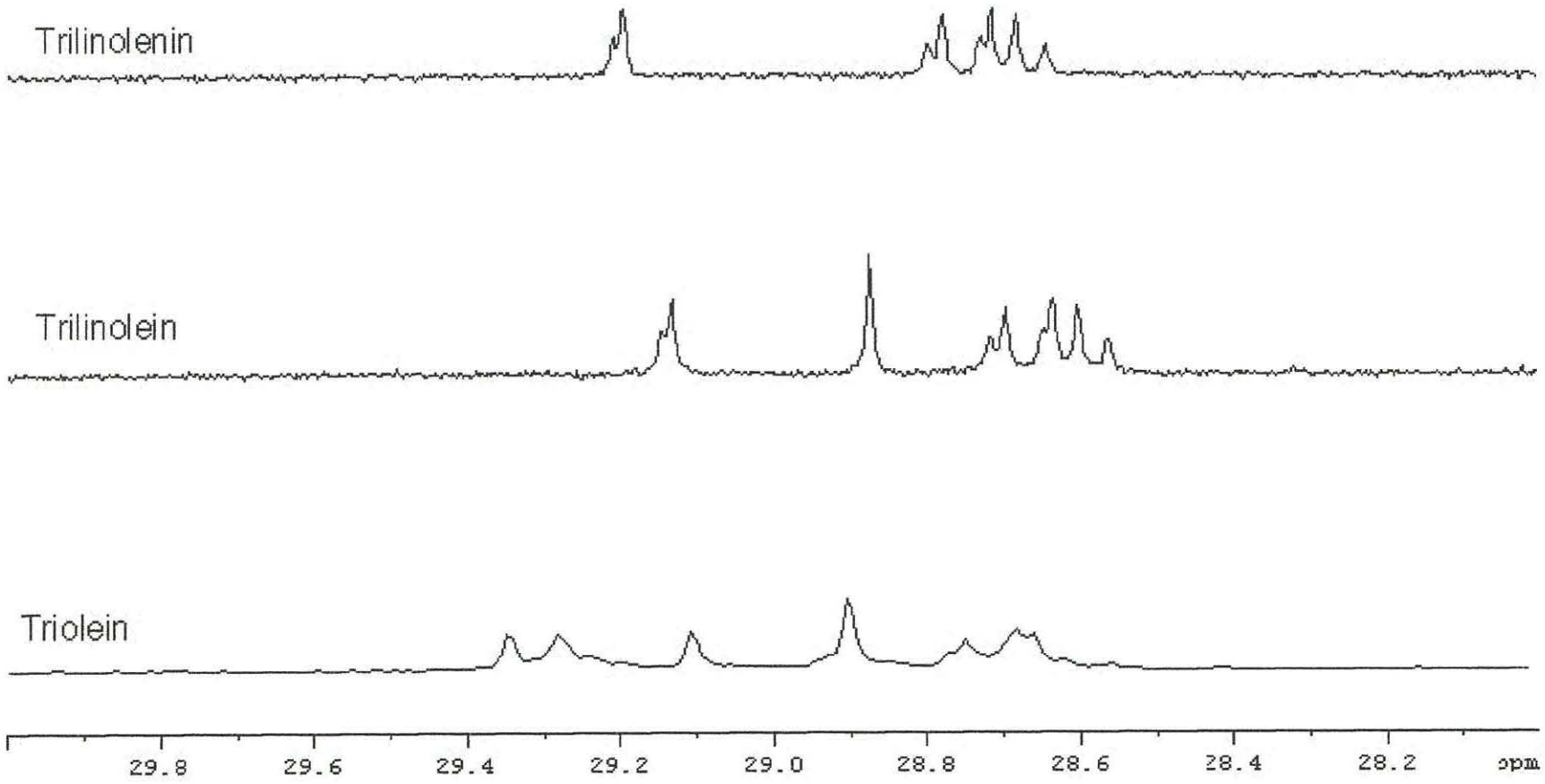

Figure 81: Fingerprint region (30 - 28 ppm) of quantitative ^{13}C NMR spectra unsaturated triglycerides.

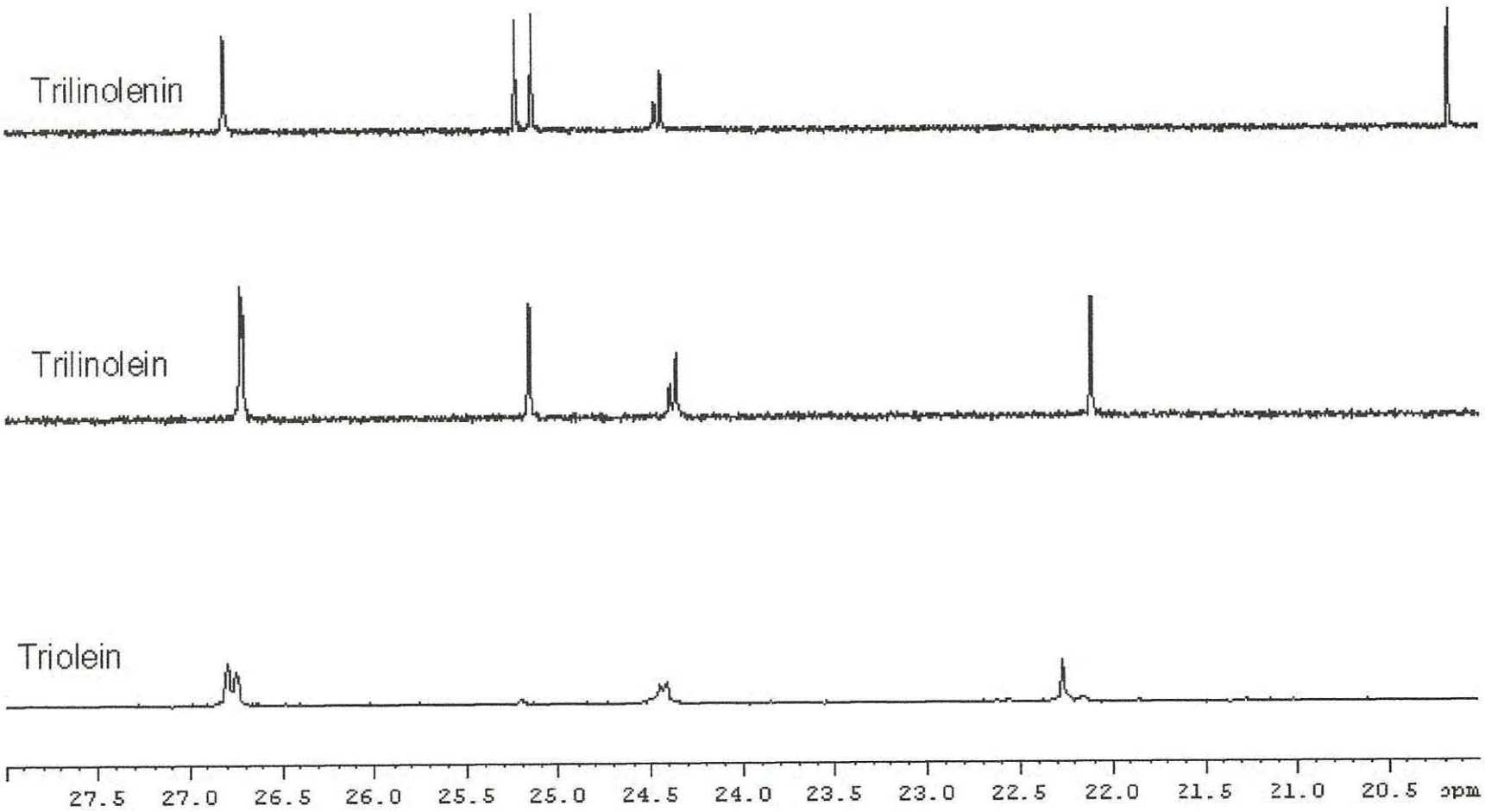

Figure 82: Acyl chain region (28 - 20 ppm) of quantitative ^{13}C NMR spectra unsaturated triglycerides.

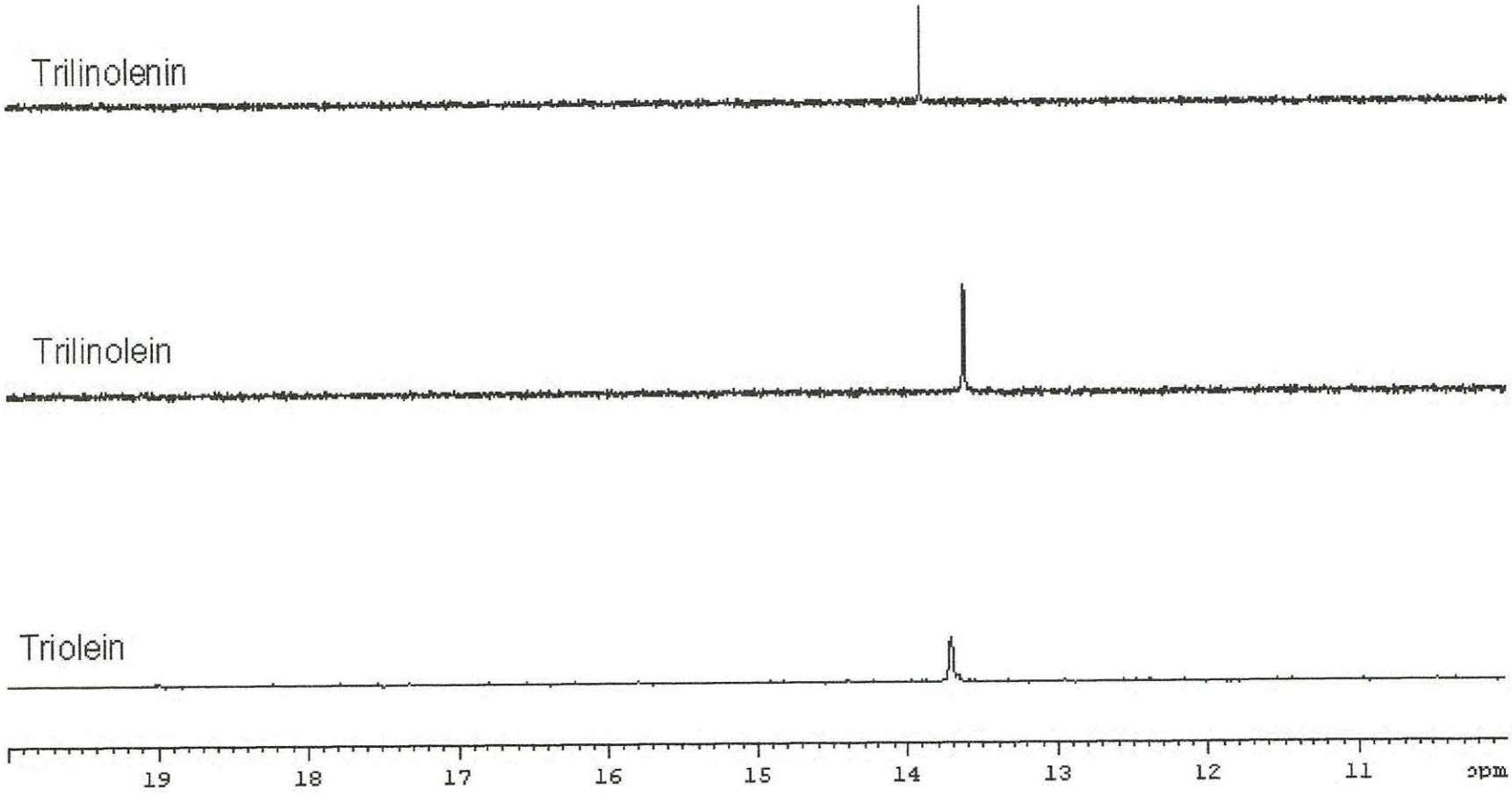

Figure 83: Terminal methyl region (20 - 10 ppm) of quantitative ^{13}C NMR spectra unsaturated triglycerides.

Figure 84: Quantitative 13C NMR of an extra virgin olive oil triglyceride.

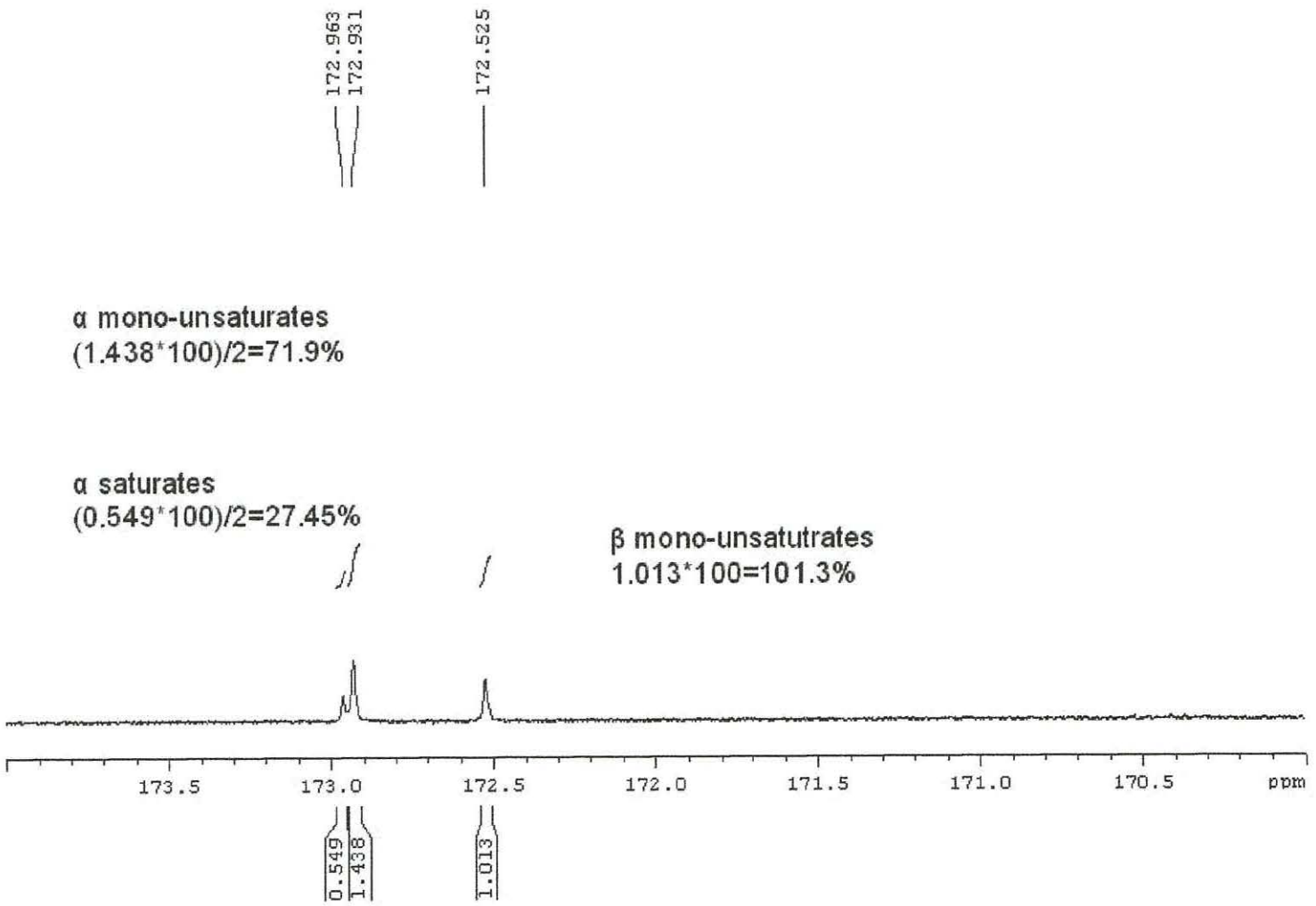

Figure 85: Carbonyl region (174 - 170 ppm) of a quantitative ^{13}C NMR spectrum of an extra virgin olive oil showing the calculations used to work out the percentage of saturated and mono-unsaturated fat present on each position of the glycerol backbone.

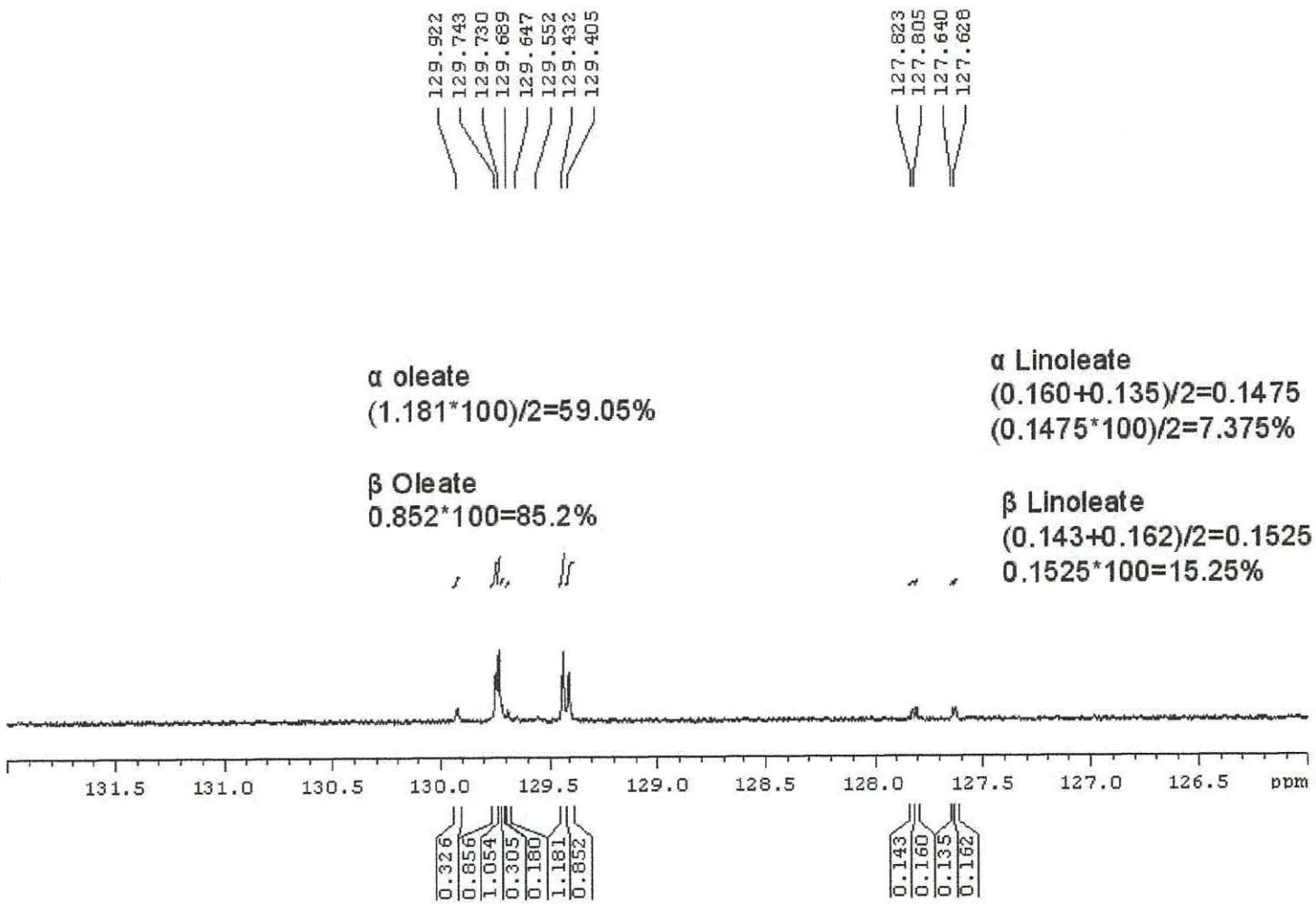

Figure 86: Olefinic region (132 - 126 ppm) of a quantitative ^{13}C NMR spectrum of and extra virgin olive oil showing the calculations used to work out the percentages of oleate and linoleate present on each position of the glycerol backbone.

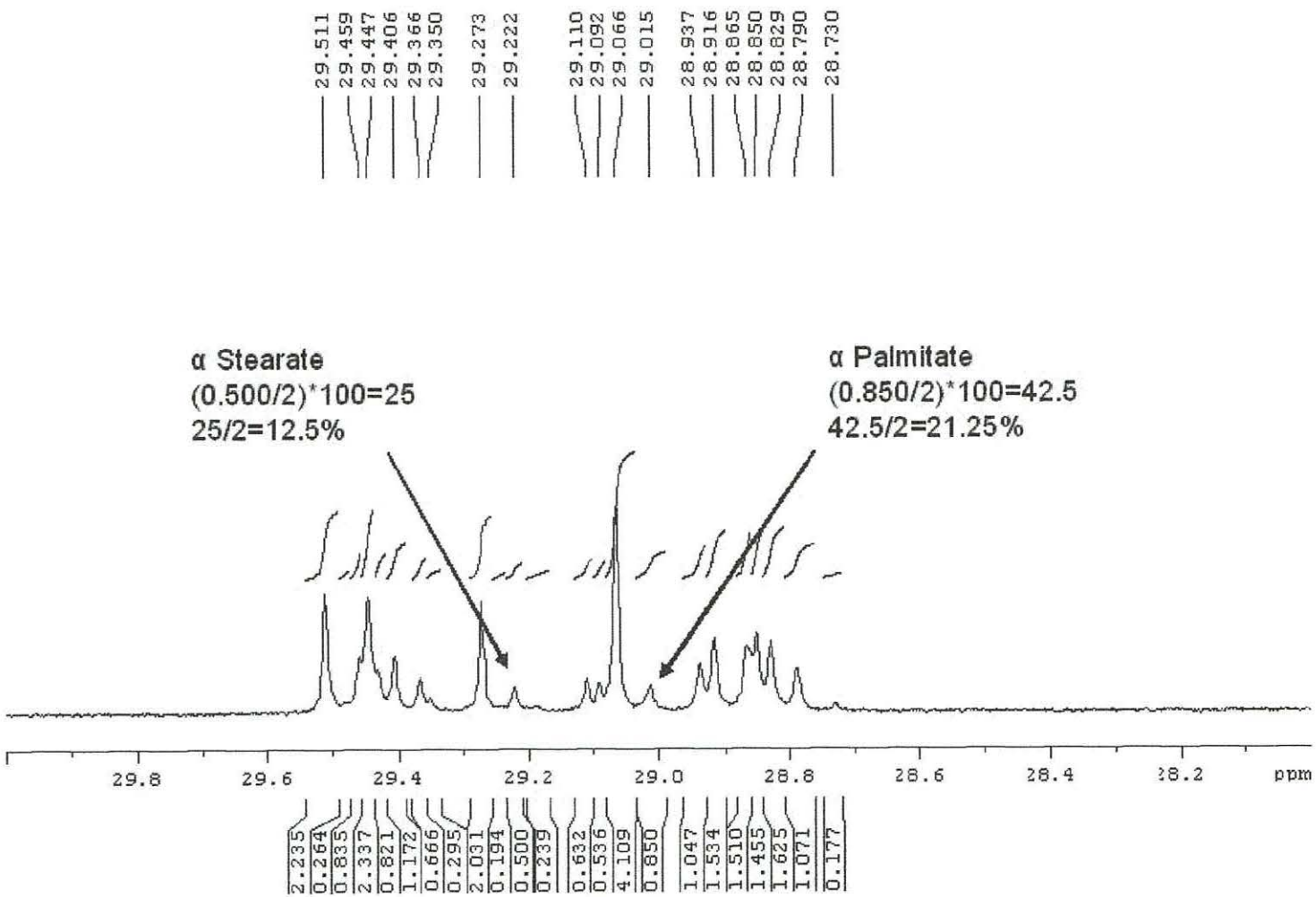

Figure 87: Fingerprint region (30 - 28 ppm) of a quantitative ^{13}C NMR spectrum on an extra virgin olive oil showing the calculations used to work out the percentages of palmitate and stearate present on the alpha-position.

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