MAJOR COMPONENT DETERMINATION OF SELECTED VEGETABLE OILS BY MEANS OF QUALITATIVE AND QUANTITATIVE ^{13}C NMR SPECTROSCOPY

By

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at Stellenbosch University

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signed: .

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Date: 7 November 2006

ABSTRACT

The aim of this study was to assign the ^{13}C Nuclear Magnetic Resonance (NMR) spectra of the major fatty acid components of six locally produced vegetable oils (apricot kernel, avocado, grapeseed, macadamia nut, mango kernel and marula oils). A first attempt at the assignment of the spectra involved the use of a literature method which had been developed for the assignment of the ^{13}C NMR spectrum of olive oil. As this method proved to be time consuming and did not allow for the complete assignment of the oils a new rapid and potentially generic graphical method for the full assignment of the ^{13}C NMR spectra of the vegetable oils was developed. The method was shown to be especially useful for regions of the ^{13}C NMR spectra where significant peak overlap occurs. The method has been validated by using it to assign the ^{13}C NMR spectrum of olive oil which has been extensively reported in the literature. Concentration studies have also proved that the method is independent of the concentration of the oil used thus contributing to its general applicability.

Quantification of the major fatty acid components of the six vegetable oils was also carried out using ^{13}C NMR spectroscopy and the results obtained were compared with those obtained from gas-liquid chromatographic studies. This study showed that ^{13}C NMR spectroscopy has use in quantifying the major fatty acid components of a variety of vegetable oils though is limited in that the minor fatty acid residues can not be detected reliably.

Finally, due to the fact that resonances were detected in the ^{13}C NMR spectra of the six vegetable oils indicating the presence of free fatty acids, a titration method was also developed for the six vegetable oils to determine the percentages of these free fatty acids. As the quantity of free fatty acids in a vegetable oil is often an indication of the quality of that oil, the development of such a technique has potential use in the quality control of the oils studied. Carrying out these titrations proved more difficult than expected, however a suitably reliable procedure has been developed and is documented here.

OPSOMMING

Die doel van hierdie studie was om ses plaaslik geproduseerde groente olies (perske neut, avokado, druiwesaad, macadamia neut, mango neut and maroela olies) deur middel van ^{13}C Kern Magnetiese Resonansie (KMR) spektroskopie te ontleed met betrekking tot hul hoof vetsuur komponente. Die eerste poging om hierdie olies te ontleed was gebaseer op 'n metode wat in die literatuur beskryf gebruik word vir die ^{13}C KMR spektra van olyf olies. Die metode was egter tydrowend en oneffektief vir die identifikasie van elke piek in die ^{13}C KMR spektra en gevolglik is 'n nuwe vinnige en generies grafiese metode ontwikkel vir die volledige identifikase van die ^{13}C KMR spektrum van groente olies. Die metode is veral effektief vir areas in die ^{13}C KMR spektrum waar piek oorvleueling voorkom. Die metode is geldig gemaak deur dit toe te pas op die ^{13}C KMR spectrum van olyf olie wat reeds volledig beskryf is in die literatuur. Konsentrasie studies het ook aangedui dat die metode onafhanklik is van die konsentrasie van die olie wat dus bydrae tot sy algemene toepaslikheid.

Kwantifikasie van die hoof vetsuur komponente teenwoordig in die ses olies is ook gedoen met behulp van ^{13}C KMR spektroskopie waarvan die resultate vergelyk is met die van gas-vloeistof chomatografie studies. Daar word gewys dat ^{13}C KMR spektroskopie gebruik kan word vir die kwantifikasie van die hoof vetsuur komponente vir 'n verskeidenheid van groente olies, maar dit is egter beperk in die sin dat dit nie doeltreffend tussen ander vetsuur komponente, wat teenwoordig is in minder hoeveelhede, kan vasstel nie.

Laastens, as gevolg van die feit dat daar pieke opgetel is wat die teenwoordigheid van vrye vetsure in die ^{13}C KMR spektra van die ses groente olies aandui, is 'n titrasie metode ontwikkel om die persentasies van hierdie vrye vetsure in die ses groente olies te bepaal. Die hoeveelheid vrye vetsure in groente olies is gewoonlik 'n aanduiding van die kwaliteit van die olie en het die ontwikkeling van so 'n tegniek gevolglik groot potensiaal in die kwaliteitsbeheer van die olies wat bestudeer is. Alhoewel die uitvoer van hierdie titrasies moeiliker was as verwag, is 'n gepaste betroubare prosedure ontwikkel en word hierin opgeteken.

CONTENTS

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Prof Klaus Koch, for his assistance and enthusiasm during this project. I would like to thank him especially for his sympathetic ear during troublesome times and his words of encouragement that always kept me going.

A special thanks to my co-supervisor, Jean McKenzie for her support and guidance during this project. Not only did she help me in times when I got stuck but greatly guided me in developing my scientific writing and presentation skills. I wish to thank her also for her patience with my constant questioning and the endless time she always seemed to have available to help me even though I knew she was very busy.

Lastly to my friends and parents, although they rarely understood the theory involved in my project, I wish to thank them for their constant interest and support. Their eagerness to try and understand and willingness to listen to newly discovered ideas, aided me greatly in getting my mind around difficult problems. For that I thank them deeply.

ABBREVIATIONS

TAG	Triacylglycerol
DAG	Diacylglycerol
MAG	Monoacylglycerol
GLC	Gas Liquid Chromatography
GLC-MS	Gas Liquid Chromatography-Mass Spectrometry
NMR	Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
FTIR	Fourier Transform Infrared Spectroscopy
TLC	Thin Layer Chromatography
ECD	Electron Capture Detector
SFE	Supercritical Fluid Extraction
UV	Ultra Violet Spectroscopy
O	Olein
P	Palmitin
S	Stearin
L	Linolein
Pa	Palmitolein
V/E	Vaccenin/Eicosenoin
FFA	Free Fatty Acid
FAME	Fatty Acid Methyl Ester

CONFERENCE PROCEDINGS

The following oral presentation was made on the linear graph method of this project:

Retief, L., McKenzie, J.M. and Koch, K.R., *A Novel Method for the Assignment of ^{13}C Nuclear Magnetic Resonance Spectra of Vegetable Oils,* Varian NMR Users Meeting, Stellenbosch, October, 2005.

The following poster presentation will be made on the linear graph method of this project:

Retief, L., McKenzie, J.M. and Koch, K.R., *A Novel Method for the Assignment of ^{13}C-Nuclear Magnetic Resonance Spectra of Vegetable Oils,* 38 th Convention of the South African Chemical Institute, Durban, December, 2006.

PUBLICATIONS

The following publications are being prepared containing results from this project:

1) Publication to be submitted to The Journal of Agricultural and Food Chemistry in 2006: Retief, L., McKenzie, J.M. and Koch, K.R., *A Novel Method for the Assignment of ^{13}C Nuclear Magnetic Resonance Spectra of Vegetable Oils.*

2) Publication to be submitted to The Journal of the Amercian Oil Chemist's Society: Retief, L., McKenzie, J.M. and Koch, K.R., *Assignment of ^{13}C Nuclear Magnetic Resonance Spectra of apricot, grapeseed, mango kernel and marula oils using a graphical technique.*

CHAPTER 1
INTRODUCTION

Vegetable oils are interesting research subjects due to their wide application in the industrial, pharmaceutical, cosmetic and nutritional markets. This chapter looks at the compounds present in vegetable oils with emphasis on the major components, namely triacylglycerols, with discussion on their structure as well as their individual fatty acid residue chains. Several analytical techniques have been applied in the past to vegetable oils for characterization, but of specific interest is ^{13}C NMR spectroscopy. A literature study is described in this chapter looking at the analysis of these components in olive oil by NMR spectroscopy since olive oil is the most studied vegetable oil by this technique to date. Further literature investigation into six locally produced vegetable oils of interest, namely apricot kernel, avocado pear, grapeseed, marula, mango kernel and macadamia nut oils, indicated that no NMR studies have been carried out on these oils making them the topic of interest for this thesis.

1.1 AN INTRODUCTION TO VEGETABLE OILS

Vegetable oils find wide use in pharmaceutical, industrial, nutritional and cosmetic products. These include products such as cooking oils, margarine, salad dressings, food coatings, paints, plasticizers, lubricants, hydraulic fluids, glycerol, synthetic fibres, lecithin, printing inks, medicines, face masks, hand creams, shower gels, soaps, detergents, and still more too numerous to mention here. "Vegetable" is the term given to any oil from a plant source so this will include olive oil, sunflower oil, and many others.

Our interest currently lies mainly in cold-pressed, i.e. unadulterated, vegetable oils that can be used in food and have nutritional value. According to market research from Euromonitor, a global market information database, vegetable oils, seed oils and olive oils together comprise over half of the edible oils and fats market (figure 1.1).[1]

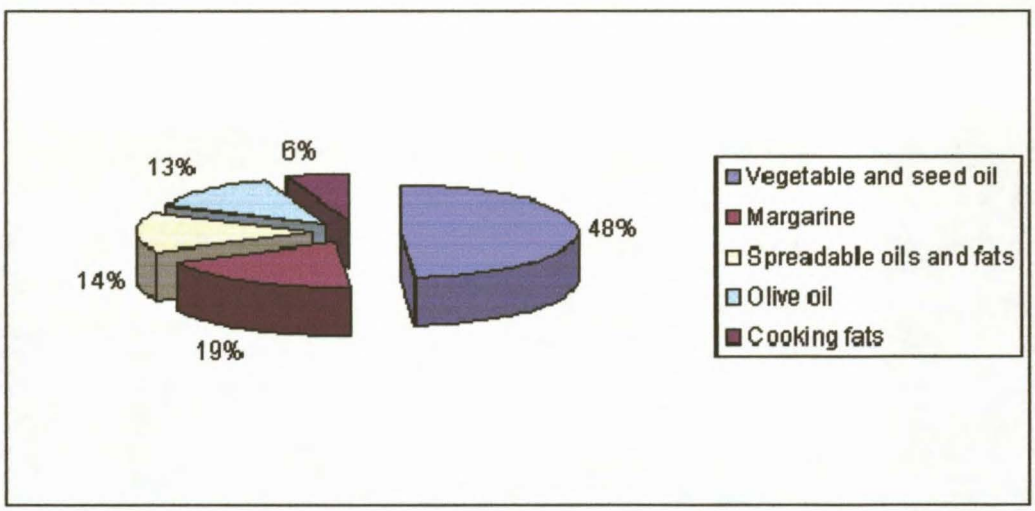


Figure 1.1: Global sales of edible oils and fats in 2000 by type.

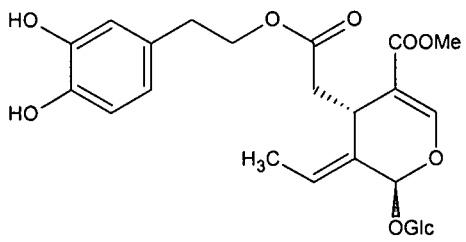
A market outlook for 2005/2006 as well as a long-term forecast through to 2013 for edible fats and oils in the USA was distributed by Global Information, Inc. and published by Business Trend.[2] The report stated that in 2003 the fats and oils sales totalled $8.8 billion in the USA and it is estimated that the sales of fats and

oils should increase at 1.5 % each year to a value of $10.2 billion by 2006. They expect the sales and consumption of vegetable oils to be affected by certain factors, such as the increased use of biotech crops, research into food production alternatives, and the use of oils as biofuels. The market for animal fats and oils will be affected by changes in supply and popularity of diets such as the Atkins or South Beach diet. The report from Business Trend agrees with Euromonitor that vegetable oils make up the majority of the edible fats and oils industry, as indicated by figure 1.1. For the period 2003 to 2013, no serious market share fluctuation is expected, since the growth rates of vegetable and animal fats and oils are expected to stay unchanged. It is projected that in 2013, vegetable oils sales will reach $5.8 billion (56,7% of the industry total).[2] Therefore it is quite clear to see that vegetable oils are commercially and industrially of great value and will continue to be so in the future.

Vegetable oils are complex mixtures of which triacylglycerols (TAGs) are the major components while the minor components comprise of among others polyphenols, aldehydes, sterols and volatile compounds. The major components are of importance for their nutritional value and also can be used for differentiating between different types of oil. Some of the minor components mentioned above are responsible for other health benefits associated with the use of vegetable oils such as their anti-oxidant properties. Several studies have been carried out on the health aspects of vegetable oils, especially olive oil. Those carried out on olive oil are discussed briefly in the next few paragraphs.

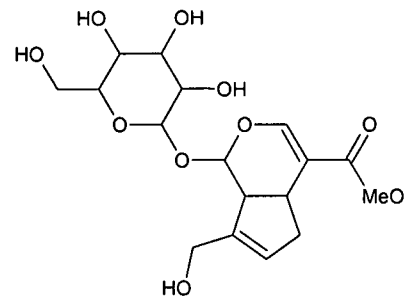
Studies were carried out by Lipworth *et. al.* to determine if olive oil gave the same oxidative problems that occurred during the use of animal fats.[3] These studies were concerned with several cancers, including breast, colon, ovarian, endometrial, prostate, pancreas and esophagus cancer. Data confirmed that olive oil does not have cancer promoting properties as some of the other fats appear to do. These authors also suggested that olive oil could even be a promising dietary tool in the prevention of certain types of cancers.

Antioxidants are important compounds that play a protective role in human health, contributing to a decrease in the occurrence of diseases like cancer, arteriosclerosis, bowel syndromes, etc.[4] Such antioxidants such as vitamins and polyphenols are found as minor components in olive oil. Low-density lipoproteins (LDLs) are found in all fats, especially those with high polyunsaturated content, and the unsaturated fatty acids can be oxidized which can be potentially harmful to the human health, leading to diseases. Antioxidants work to inhibit or decrease the formation of the oxidized LDLs.[4] Visioli *et. al.* made the observation that the occurrence of coronary heart disease and certain cancers were lower in the Mediterranean areas. This lead to their hypothesis that since olive oil, amongst other compounds, which is consumed in such large quantities, played an important part in a healthy diet. They did studies using hydroxytyrosol, oleuropein, luteolin and luteolin aglycon (refer to figure 1.2), which are all found in olive oil and found that these compounds had protective properties against the oxidations of LDLs. These authors concluded that substances with a high phenol content such as olive oil were beneficial to any human diet. Manna *et. al.* used rats to also investigate this antioxidant property of phenols in olive oil.[5] They found that rats that were fed olive oil as part of their daily diet had a higher content of antioxidants in their serum and therefore increased resistance to oxidation of LDLs. On the other hand, rats that were fed a synthetic diet containing the same fatty acids as olive oil as well as vitamin E did not produce the same results. This led Manna *et al.* to conclude that the minor components present in olive oil such as the polyphenols are important to the human diet. Giovanni *et. al.* also did studies on the human colon to determine the antioxidative effect that the phenol, tyrosol has.[6] They found that a diet rich in biophenols, such as tyrosol that is found in olive oil, can lead to the lowering of the risk of inflammatory bowel disease as well as cardiovascular diseases.

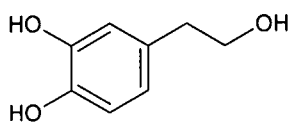


oleuropein

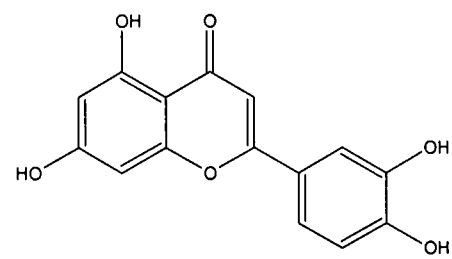
Glc=Geniposide



Geniposide



hydroxytyrosol



luteolin

Figure 1.2: Structures of oleuropein, hydroxytyrosol and luteolin.

Therefore it can be seen that not only are vegetable oils expected to increase in value from an economic point of view as shown by the previously mentioned market studies, but these oils also have important health implications. As there is continuous interest in these oils it is thus relevant to rather characterise them by modern instrumental methods. The better we understand these mixtures, the more effective they can be applied to products that are of use to the community. In addition we have found that although the major and minor components of olive oil have been extensively studied[7-19] there are many other vegetable oils which have only been examined superficially. This is where our interest lies, so that in this study we have investigated the major components present in vegetable oils by high resolution ^{13}C NMR spectroscopy, which we believe to be complementary to the currently used GLC techniques.

1.2 MAJOR COMPONENTS OF VEGETABLE OILS: TRIACYLGLYCEROLS

Triacylglycerols are comprised of fatty acids which belong to a class of compounds known as lipids. Thus in order to understand the structure of a TAG one must first begin by looking at lipids and fatty acids.

1.2.1 Lipids

Lipids are organic compounds which are found in living organisms and are soluble in nonpolar organic solvents. They play an important part in the metabolism of all biological systems. Their solubility in nonpolar organic solvents and their hydrophobic or amphiphatic nature results from a significant hydrophobic hydrocarbon component. This gives them the "oily" texture which is usually associated with this class of compounds. As they are classed according to their physical properties rather than a particular structural feature it is difficult to define them precisely, since this class of compounds has a variety of structures and functions.[20,21,22]

Lipids can however be grouped into two basic types, one containing fats, oils and waxes, the other containing terpenes, lipid vitamins and steroids (figure 1.3). The first group of compounds have ester linkages and can be hydrolyzed while the latter group do not have ester linkages and no hydrolysis can occur. The hydrolysis potential of the former class of lipids is important since its leads to the formation of fatty acids, which will be discussed later.[20,21,22]

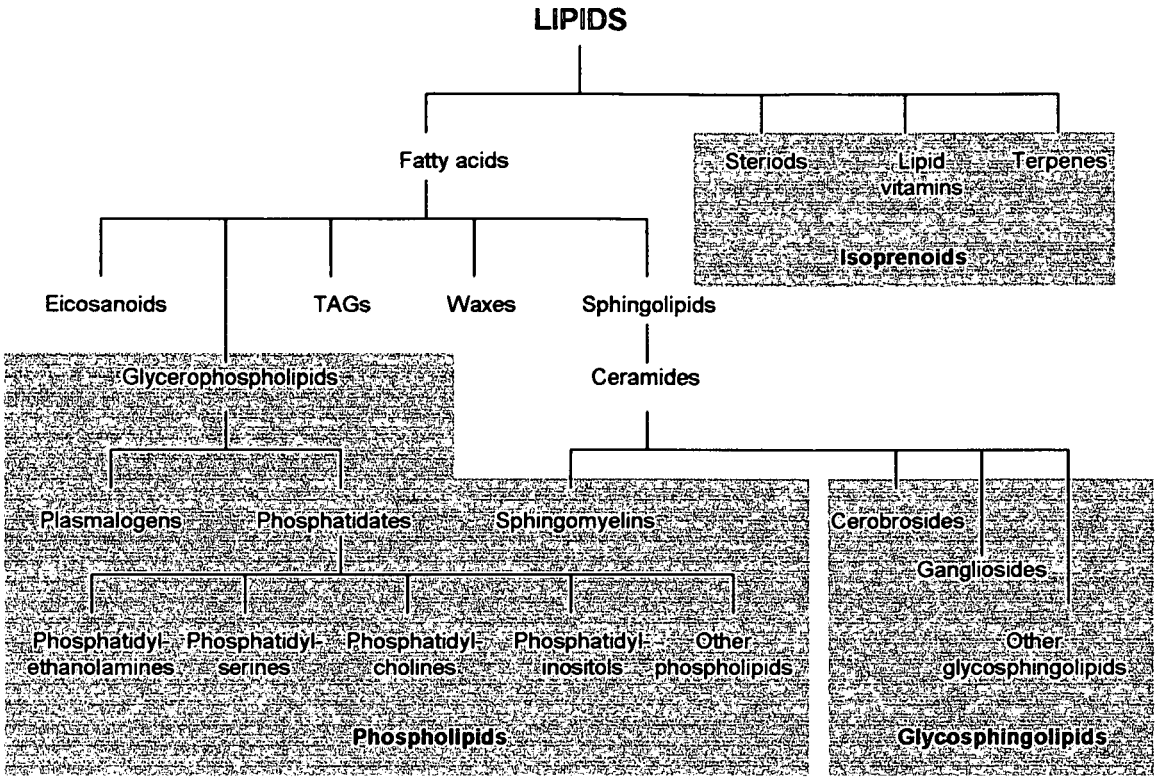


Figure 1.3: Major classes of lipids.[20]

Each minor group of lipid contains compounds with a specific functional group which gives the group its characteristic name. For instance phospholipids are lipids containing phosphate moieties; glycosphingolipids contain sphingosine and carbohydrate groups; while isoprenoids include steroids, lipid vitamins and terpenes which are all related to the five carbon molecule isoprene.[20] Figure 1.4 shows the structures of some of these lipids.

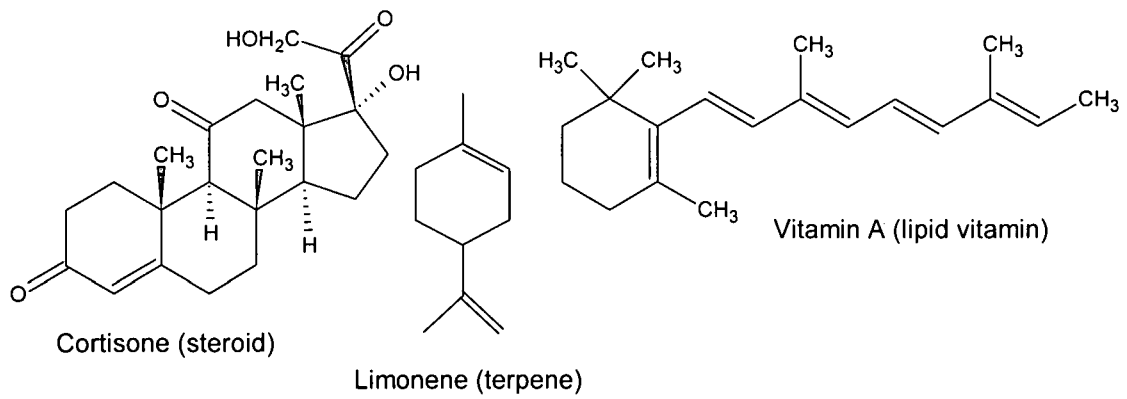


Figure 1.4: Structures of some common non-triacylglycerol lipids.

Looking at the fats, oils and waxes group, it is evident from figure 1.3 that they fall under the fatty acid category, as all three of these compounds contain fatty acids. Natural waxes are esters containing a combination of long chain carboxylic acids and long chain alcohols. These are simple structures which are nonpolar and saponifiable, meaning they can undergo hydrolysis by means of a base. Fats and oils on the other hand consist mainly of TAGs which are triesters of glycerol with three long carbon chain acids attached,[23] as discussed below.

1.2.2 Fatty acids

Fatty acids differ from one another in the number of carbon atoms in the hydrocarbon chain, the degree of unsaturation (the number of C=C double bonds), and the relative positions of these double bonds in the various chains. The basic structure of a saturated fatty acid is shown in figure 1.5.

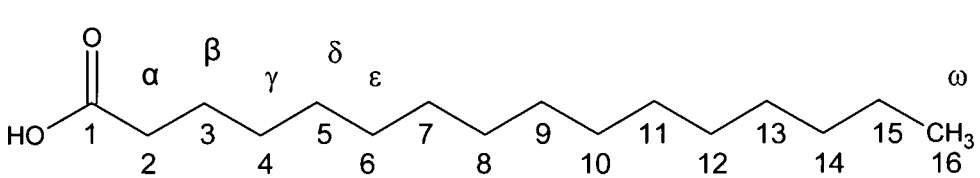


Figure 1.5: Structure and numbering of a basic fatty acid.

There are a number of different opinions about what can be considered the average length of fatty acids, but overall fatty acid chains are considered to have a length of between 12 and 20 carbons. The degree of saturation of fatty acids plays an important part in their definition. Fatty acids without any C=C double bonds are referred to as *saturated*, while those with at least one double bond are called *unsaturated*. Unsaturated fatty acids can be grouped further into *monounsaturated* (only one double bond is present) and *polyunsaturated* (with two or more double bonds). If unsaturation is present in these compounds, they are found to have a *cis* conformation at the double bond if from a natural source, and the double bonds are usually separated by a methylene group. Fatty acids with *trans* double bonds have usually undergone some form of chemical modification. Generally for fatty acids in vegetable oils, the first double bond is usually found between carbons 9 and 10, the second double bond between carbons 12 and 13 and the third double bond between carbons 15 and 16, although exceptions to these double bond positions do occur. Exceptions to these commonly found fatty acids described above, are those with an odd number of carbon atoms, branched or cyclic chains, those with a double bond *trans* conformation and conjugated unsaturation.[21, 23, 24,25,26]

Table 1.1 shows some common fatty acids with their IUPAC nomenclature given. However as their common names are mostly used when referring to these compounds, these are also shown. According to IUPAC nomenclature the carboxyl carbon is labeled as C1 and thereafter the remaining carbon atoms are then numbered sequentially (figure 1.5). There is a shorthand notation for the description of fatty acids which makes use of two numbers separated by a colon. For instance C18:1 Δ^9 can be used to represent oleic acid. The first number represents the number of carbons in the unbranched hydrocarbon chain; the second number gives the number of double bonds present in the chain; while the symbol Δ^n refers to the starting position(s) of the double bonds. In this case for oleic acid the double bond is between C9 and C10. In a fatty acid, in which there

are more than one double bonds present, the double bond positions are separated by a comma, for instance: C18:2 $\Delta^{9,12}$ or C18:2 n9,12. This shorthand notation can be used to represent linoleic acid which has two double bonds, one between C9 and C10 and the other between C12 and C13.* Another commonly used notation is the use of Greek letters to identify carbon atoms. The carbon directly adjacent to the carboxyl carbon is designated by the Greek letter α. Accordingly the following carbons are sequentially numbered β, γ, δ, ε, etc. (figure 1.5). The last carbon in the chain is numbered as ω and often the position of the first double bond from the hydrocarbon end (the ω carbon end) is referred to as for instance ω6. For this example this means that the first double bond occurs at the sixth carbon atom from the end carbon numbered ω, giving the fatty acid the common name of an ω6 fatty acid.[20,23,26] Understanding the various methods of naming, whether systematic or not, is required when undertaking research involving fatty acids and their derivatives as often vendors of nutritional products will use whichever jargon serves their marketing needs best.

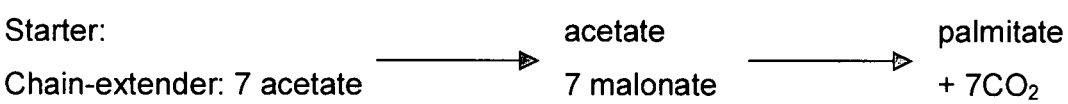
Table 1.1: Some common fatty acids with their IUPAC and trivial names.

Number of carbons	Number of double bonds	IUPAC name	Trivial name
12	0	Dodecanoic	Lauric
14	0	Tetradecoanoic	Myristic
16	0	Hexadecanoic	Palmitic
16	1	*cis*-Δ^9-Hexadecenoic	Palmitoleic
18	0	Octadecanoic	Stearic
18	1	*cis*-Δ^9-Octadecenoic	Oleic
18	1	*cis*-Δ^{11}-Octadecenoic	Vaccenic
18	2	*cis*-$\Delta^{9,12}$-Octadecadienoic	Linoleic
18	3	*cis*-$\Delta^{9,12,15}$-Octadecatrienoic	Linolenic
20	0	Eicosanoic	Arachidic
22	0	Docosanoic	Behenic
24	0	Tetracosanoic	Lignoceric

* An indication of whether the double bond is in *cis* (Z) or *trans* (E) conformation is written as follows: C18:1 n9*c* which means that the double bond between C9 and C10 of oleic is in cis conformation. The same is the case for the use of the symbols Z, E and *t (trans)*.

Biosynthesis of fatty acids of TAG type:

The biosynthesis of fatty acids occurs via several steps.[26] The first step is the *de novo* synthesis of the saturated acids. Palmitic acid is used as example. Eight molecules of acetate are involved to form one molecule of palmitic acid. While one of these acetate molecules acts as primer (starter molecule) in the following condensation reactions, the remaining seven are converted to malonate ($HOOCCH_2COOH$). However one of the carbon atoms in each malonate is lost during condensation so that all sixteen the palmitic carbon atoms can be acetate-derived. Several enzymes are involved in this condensation reaction including a *transacylase*, a condensing enzyme, two *reductases*, and a *dehydrase*. The detailed steps of the *de novo* synthesis are not discussed here.



The second step is chain elongation where the RCOOH acid is converted to its bishomologue RCH_2CH_2COOH. This occurs with saturated and unsaturated substrates. Compounds involved in the chain-extension process are acetate and/or malonate and coenzyme A derivatives. An example of the chain-extension of oleate to 20:1 and 22:1 in two elongation cycles are shown:

$$18:1(9c) \rightarrow 20:1(11c) \rightarrow 22:1(13c)$$

The extender that is used, namely acetate or malonate can be replaced by propionate or methylmalonate compounds which would lead to the formation of polymethyl branched acids:

$$20:0 \rightarrow 2\text{-methyl } 22:0 \rightarrow 2,4\text{-dimethyl } 24:0 \text{ etc.}$$

The third step in the biosynthesis of fatty acids is referred to as monoene biosynthesis where monoene acids are produced in two ways, of which the one is according to an anaerobic route and less common, mostly confined to lower

is according to an anaerobic route and less common, mostly confined to lower life forms. The more common route found in plants, animals and micro-organisms requires oxygen, NADH or NADPH. One hydrogen atom each from C9 and C10 is removed to form a double bond in a *cis* configuration. The substrate involved may be a coenzyme A ester, an ACP ester or an acyl chain that is already incorporated into a phospholipid. This desaturation process produces acids of varying lengths which can now once again be converted to monoene acids by chain-elongation.

The fourth step is the polyene biosynthesis. Plants convert monoenes to polyenes by further desaturation of the distal end of the molecules while in animals the additional double bonds are inserted in the proximal end. The distal end of the molecule is between the existing double bond and the ω-methyl group, while the proximal end is between the existing double bond and the carboxyl group. In this way the addition of *cis* unsaturation centres are introduced in a methylene interrupted way.

Distal end $CH_3CH_2CH_2CH = CHCH_2CH_2CH_2CO_2H$ Proximal end

For instance oleic acid is converted to linoleic acid and then to linolenic acid in plants, but since animals cannot insert double bonds in the distal end they produce the polyene acids from the monoene precursors directly by desaturation and chain-elongation. Notice that the linoleic and linolenic precursors cannot therefore be produced in animals and the precursors need to come from plants.

Oleic 18:1 (9*c*) ⟶ *n* – 9 polyenes

↓

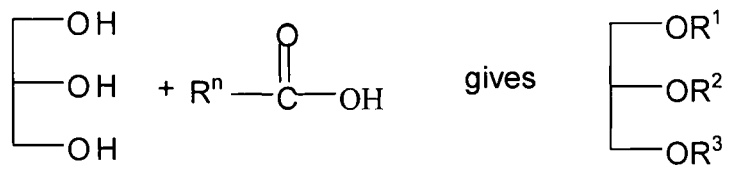
Linoleic 18:2 (9*c*, 12*c*) ⟶ *n* – 6 polyenes

↓

Linolenic 18:3 (9*c*, 12*c*, 15*c*) ⟶ *n* – 3 polyenes

1.2.3 Triacylglycerols

Triacylglycerols (TAGs) are the major constituents of vegetable oils. They are nonpolar, water insoluble substances composed of three fatty acid residues esterified to a glycerol backbone (figure 1.6) hence their hydrophobicity.



glycerol fatty acid triacylglycerol

Figure 1.6: The structure and constituents of a triacylglycerol, where R^n represents a hydrocarbon chain.

The three fatty acid residue chains of naturally occurring TAGs in vegetable oils are mostly of different type. Exceptions to this are in the case of for instance triolein found in olive oil where all three of the fatty acid residues are olein moieties and hence identical. Such compounds where all three of the fatty acid chains are identical however are rarely found in naturally occurring TAGs present in vegetable oils. This positional distribution is shown in figure 1.7 as usually different fatty acids on each of the three glycerol backbone positions in a naturally occurring TAG.

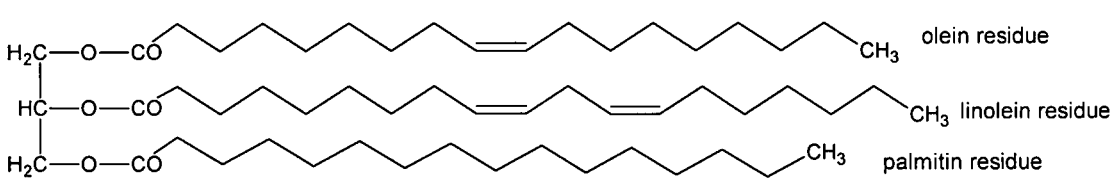


Figure 1.7: Structure indicating the position distribution of different fatty acids on the glycerol backbone of a natural occurring TAG.

The fatty acids chains on the glycerol backbone can be denoted in two ways. The first is by identifying the two outer chains as α and the inner chain as β. The other

method is by strictly numbered (sn) identification whereby each chain is identified as sn 1', sn 2' or sn 3'. Refer to figure 1.8 for the illustration of these numbering schemes.[27,28]

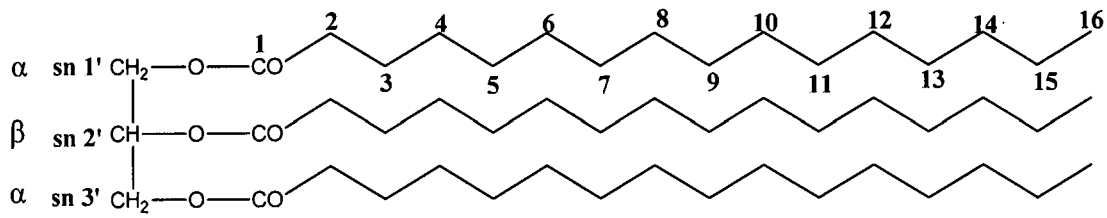
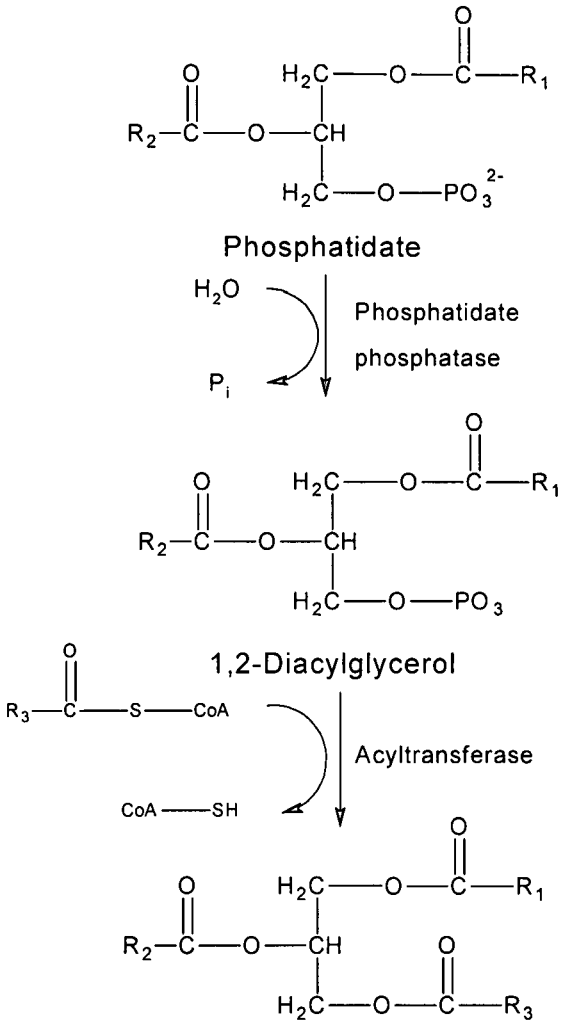


Figure 1.8: Numbering scheme for tripalmitin.

TAGs were previously referred to as triglycerides (TGs) although both names are still in use. Saturation and chain length plays an important part in the form that TAGs are found. For instance those that contain only saturated, long-chain fatty acid groups are solids while unsaturated or short chain fatty acid groups yield liquids.

Biosynthesis of TAGs:

The biosynthesis of TAGs makes use of an intermediate called phosphatidate. The synthetic pathway for phosphatidate starts with glycerol-3-phosphate which forms the backbone of the acylation reactions. Two separate *acyltransferases*, which use fatty acyl CO molecules as acyl-group donors, act as catalysts. The first *acyltransferase* has a preference for saturated fatty acid chains and catalyzes esterification at C1 of the glycerol-3-phosphate. The second *acyltransferase* has a preference for unsaturated fatty acid chains and catalyzes esterification at C2 of the mono-acylglycerol-3-phosphate. Formation of TAGs from phosphatidate begins with dephosphorylation which is catalysed by phosphatidate phosphatase. The formed product, 1,2-diacylglycerol (DAG), can directly be acylated to a TAG.[20]



1.2.4 Fatty acids in vegetable oils

TAGs are the major constituents of vegetable oils, so that vegetable oils of different origins differ from one another in these major components. Each vegetable oil has varying quantities of different fatty acid residues present as TAGs. Table 1.2 indicates the fatty acid residue content present in some of the common vegetable oils, such as linolein, olein and palmitin residues.

In vegetable oils it is unusual also to find some saturated fatty acid in the β position of the glycerol backbone for naturally occurring TAGs. The presence of saturated fatty acids in this position is in general an indication of the adulteration of the vegetable oil by chemical means.

Table 1.2: Fatty acid residue content of some common vegetable oils.[25,29,30]

Fatty acid residue	Coconut	Corn	Cottonseed	Olive	Palm	Peanut	Rapeseed	Safflower	Soybean	Sunflower
6:0	0.3-1.2									
8:0	6-15									
10:0	3.2-15									
12:0	41-51.3				0.1					
14:0	13-23	0.1	0.5-2	0.1-1.2	1-1.2	0.1	0.1	0.1	0.1	
16:0	7.5-18	0.2	17-29	7-16.9	44.3-46.8	6-15.5	1.5-4.5	2-10	6.8-10.6	5.2-7.2
16:1	1-4.7		0.5-1.5	1.8	0.15	0.1	0.1	0.1-0.5	0.1	
18:0	4.3-12	2-2.2	1-4	1-3	3.8-4.6	1.3-6.5	0.7-1.5	1-10	3.2-4.4	2.7-6.5
18:1	1-5	25.4-26.6	13-44	65-85	37.6-38.7	33.3-72	12.1-61.7	7-42	11.5-60	14.3-50.9
18:2	2.5	58.7-59.6	33-58	4-15	10-10.5	12-47.5	11.4-22.1	55-81	25-54.3	37.9-72.5
18:3		0.7-1.2	0.1-2.1	0.6	0.3	0.02-0.04	5.3-12.5	1	2.3-12.1	0.01-0.3
20:0		0.2-0.4		0.1-0.3	0.2-0.3	0.17-2.7	0.9	0.4-0.5	0.2-0.7	0-1
20:1 (n9)				0.1		0.5-2.1		0.1		
20:1 (n11)					0.3		0.9-10.9	0.5	0.3-0.9	
22:0		0.1		0.2		10.7-4.8	0.7	0.2-0.5		0.7
22:1							0.2-58.6			
24:0						0.46-2.8	0.2		0.1	

FATTY ACID RESIDUE CONTENT %#

The percentage values were taken from the literature and it is unknown whether these values reflect weight, mole, volume or peak integration percentages.

As can be seen in table 1.2, most of the oils contain fatty acid residues with carbon chains between 14 and 22 carbons in length. However there are a few exceptions such as coconut oil, which contains the TAGs with short chain caproic (C6:0), caprylic (C8:0) and capric (C10:0) acids. Although most of the vegetable oils in this table contain the same fatty acid residues, the oils differ in the quantity of TAG present as shown by the major components present in each oil. For instance in olive and palm oils, the oleic acid residue is present in the highest quantity (65-85% and 37.6-38.7% respectively) while linoleic acid is the major component for corn and safflower oils (58.7-59.6 % and 55-81% respectively). These differences in the percentages of fatty acid residues makes it possible to differentiate between different vegetable oils by analytical techniques.

Several minor components are present in vegetable oils. Monoacylglycerols (MAGs) and diacylglycerols (DAGs) are such components, but if found in significant quantities in a vegetable oil, this often gives an indication of adulteration or aging of the oil. This is due to the fact that upon hydrolysis of TAGs, the mono- and diacylglycerides form. This hydrolysis occurs naturally in two ways, either enzymatically in the vegetable or fruit, or during storage due to the presence of water and long term exposure to oxygen in air. Formation of MAGs and DAGs also leads to the presence of free fatty acids (FFAs), also a minor component found in vegetable oils. The hydrolysis can however also occur by the deliberate chemical modification of the vegetable oil content which is referred to as "adulteration" in this context.[25,31] Other minor components of vegetable oils include:[32,33]

- pigments, including chlorophyll and carotenoids
- alcohols
- sterols, including free sterols and sterol esters
- tocols, including tocopherols, tocosterols and tocotrienols
- phospholipids
- hydrocarbons, including alkanes, alkenes (squalene), polycyclic aromatic hydrocarbons and carotenes

For this particular study these minor components were not of interest to our research, but will be the subject of further study of the structure.

1.3 ANALYSIS OF VEGETABLE OILS

A number of analytical techniques have been used for the identification and classification of vegetable oils. Such techniques are used to determine unsaturation, melting point, and acidity, among other things. Titrations have commonly been used for determining average unsaturation and FFA content though more recently the most popular methods for the analysis of vegetable oils are spectroscopic and chromatographic techniques and include Gas-Liquid Chromatography (GLC), High Pressure Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Mass Spectrometry (MS), Infra-red (IR) spectroscopy (including Near-Infrared and Fourier Transform IR) and Ultra Violet-Visible (UV) spectroscopy. Each of these techniques yields information on the qualitative as well as quantitative properties of vegetable oils. Table 1.3 gives a summary of the techniques used for the measurement of vegetable oil properties.

Table 1.3: Chromatographic and other analytical techniques for the measurement of chemical properties of vegetable oils.[33,34]

Technique	Detection of property of vegetable oil
GLC / GLC-MS	Quantitative and qualitative determination of fatty acid composition Determination of tocopherol content
Head space GLC	Determination of volatile halogenated solvent content
HPLC	Determination of tocopherol content
Adsorption spectroscopy	Quantitative determination of Vitamin A
UV adsorption	Identification of fats and fatty acids with conjugated double bonds
UV adsorption	Detects state of oxidation and changes brought about by technological processes
Emission spectroscopy	Detection of metals in ash of oils
Fluorescence spectroscopy	Detection of adulterants in olive oil
FTIR spectroscopy	Measurement of *cis-trans* ratios, iodine values, saponification number, free acid content, peroxide value, anisidine value
Titrations	Determination of FFA content, iodine and peroxide values
Capillary GLC	Determination of aliphatic alcohol content
Capillary-column GLC	Determination of sterol composition and content
TLC	Determination of presence of erythrodiol and uvaol
TLC/GLC	Determination of the saturated fatty acids in position 2 of the TAG
GLC with ECD	Determination of volatile halogenated solvents of olive oil

1.4 NMR SPECTROSCOPY OF OLIVE OIL

Our interest mainly lies in the use of Nuclear Magnetic Resonance (NMR) spectroscopy which is a powerful and increasingly popular method for the analysis of vegetable oils. In general it is ideal to analyze pure compounds by NMR spectroscopy and it is not always the first technique of choice for dealing with mixtures where separation techniques such as GLC and HPLC usually find more use. Although the qualitative and quantitative analysis of vegetable oils by

NMR spectroscopy poses some challenges, in recent years the availability of high resolution and new multidimensional NMR techniques has made the analysis of complex mixtures of molecules viable. This is particularly evident in the application of NMR spectroscopy to the analysis of biological fluids in the new field of metabolomics.[35] Thus the application of high resolution ^{1}H and ^{13}C NMR for the qualitative and quantitative analysis of vegetable oils is clearly viable. This is confirmed by recent studies of olive oil. Olive oil has been the most thoroughly studied vegetable oil by ^{1}H and ^{13}C NMR spectroscopy and the work carried out has indeed demonstrated the versatility of this technique when applied to the analysis of vegetable oils. In order to appreciate this more fully a review of the literature which deals with the application of NMR spectroscopy to the analysis of olive oil is given below. It should also be noted at this point that olive oil is used throughout this thesis as a "reference" because of the significant amount of research that has been carried out on this oil by comparison to other vegetable oils, some of which have not been examined by NMR spectroscopy to our knowledge.

1.4.1 Assignment and quantification of ^{13}C and ^{1}H NMR spectra of olive oil

A significant amount of research has been carried out on the assignment and quantification of compounds present in olive oils that can be detected by various forms of NMR spectroscopy by 1D and 2D techniques.[36-42] Such compounds include the major components, TAGs, as well as some minor components such as DAGs, MAGs, phenolic compounds, aldehydes, sterols, *etc.* Full assignment of the ^{13}C NMR spectrum for the TAGs in an olive oil was attempted by Mannina *et al.* by first assigning the spectra of standard TAGs.[39] In this study they made use of an INADEQUATE (Incredible Natural Abundance Double Quantum Transfer) NMR experiment for the assignment of the resonances of standard TAGs. With the help of these assignments it was possible to assign the ^{13}C NMR spectra of olive oils, by the addition of standard TAGs to each of the oils.[39] This method is further discussed in Chapter 2. ^{1}H and ^{13}C NMR studies on some of the minor components by various groups led to the discovery of new phenolic

compounds present in olive oil, including (3,4-dihydroxyphenyl)ethanol derivatives and two (p-hydroxyphenyl)ethanol derivatives,[40] pineresinol and 1-acetoxypineresinol,[41] and 4-(acetoxyethyl)-1, 2-dihyroxybenzene.[42] Assignment of the ^{13}C NMR resonances, of all the major TAGs, in olive oils by Mannina *et al.* led to further research regarding the concentration dependence of chemical shifts of these TAGs.[43] It was found that for each fatty acid, the ^{13}C chemical shift of a certain carbon atom is dependant on the acyl positional distribution in the TAG. Moreover the chemical shifts show marked oil concentration dependence. Mavromoustakos *et al.* also used high resolution ^{13}C NMR spectroscopy for the quantitative analysis of the fatty acid residues in virgin olive oils from different areas in Greece.[44] The quantitative results for the most abundant fatty acids were compared with GLC results and found that they only differed in 1-2 mol % of the fatty acid.

Other studies have been carried out, linking oil stability to oil content using NMR spectroscopy. In one such study the olive oil composition, specifically the fatty acid residues, was determined by ^{13}C NMR spectroscopy in order to understand the effect of thermal stressing of olive oil.[45] Comparison of the two methods used, namely NMR spectroscopy and Matrix-Assisted Laser Desorption and Ionization Time-of-Flight (MALDI-TOF) MS, revealed that where MALDI-TOF detected any direct changes, NMR could then provide detailed structural information on the products present with low molecular weight. Separation of oils by chromatographic techniques and subsequent analysis by ^{13}C NMR spectroscopy has also been used for oil stability predictions and was shown to give better results than those obtained from classical chemical determinations due, to the fact that the NMR technique could take more variables into account.[46] Another study showed that the use of chromatographically separated oil fractions increases the discriminative power of NMR and therefore gives better results for the classification of components by ^{13}C NMR than using the full original oil samples.[47] This is understandable as NMR spectroscopy is typically a technique which is used to best effect when analyzing pure components or simple mixtures.

1.4.2 Use of ^{31}P NMR spectroscopy for the analysis of olive oil

A number of studies have been carried out on olive oils using ^{31}P NMR spectroscopy. Detection and consequent assignment of spectra has been a topic of research, and has included studies which classified and quantified the polyphenols, dihydroxy- and polyhydroxy phenolic compounds by ^{31}P NMR spectroscopy and 1D and 2D NMR techniques.[48-50] Where the molecules themselves did not contain any phosphorus derivatisation was first carried out to incorporate a moiety containing phosphorus to allow for the subsequent ^{31}P NMR studies. This for instance was carried out in a study by Spyros *et al.* which quantified the MAG and DAG content found in virgin olive oils by ^{31}P NMR spectroscopy.[48] In another study the DAG content of 96 samples of virgin olive oils, 15 extra virgin, and pure olive oils, and three refined pomace oils was determined. ^{31}P NMR spectra yielded information about the DAG contents in the different oils from various regions. The quality of virgin olive oils, commercial olive oils, refined and pomace olive oils can be monitored by the relationship between the 1,2 DAGs and the total DAG content. They concluded that ^{31}P NMR is a powerful technique to detect and quantify the DAG content of various olive oils.[49]

A further use of ^{31}P NMR is with the application of aging studies. In one such study the DAG isomers and free acidity of five extra virgin olive oil samples with different initial acidities as a function of storage time and conditions was determined.[50] TAGs were hydrolyzed and the 1,2-DAGs isomerized to 1,3-DAGs over a period of 18 months of storage in different light and at ambient temperature conditions. Some samples were kept in ambient temperate in the dark, some in the light and others at 5 °C in the dark. The hydroxyl (from FFAs) and carboxyl (from DAGs) groups formed were treated with 2-chloro-4,4,5,5-tetramethyl dioxaphospholane, after which these phosphorylated compounds were subjected to ^{31}P NMR spectroscopy. Results showed that this structural isomerization is dependant on the rate of hydrolysis of the TAGs, the initial free acidity as well as the storage conditions. The ratio of 1,2-DAGs to the total

amount of DAGs was found to be concentration independent of the time of TAG hydrolysis. Based on these studies a quantitative method was developed to estimate the storage time or aging of olive oil. The technique was applied to several oil samples with known and unknown storage history and these were compared with samples of known storage between 10-12 months. It was found that for longer storage periods where the isomerization of DAGs was close to equivalence to each other, the calculated values were only an indication and not exact.

1.4.3 Studies on positional distribution and structural isomerisation of fatty acid residues by ^{13}C NMR spectroscopy

Studies on the positional distribution of fatty acid residues on the glycerol backbone have been carried out by NMR spectroscopy (see figure 1.7). NMR spectroscopy has the unique advantage over chromatographic techniques for analyzing the positional distribution as this can be achieved without the selective enzymatic cleavage of the fatty acids from the glycerol backbone which is required for GLC analysis. It has been found that oleic and linoleic acids are not randomly distributed in the 2-position of the TAG[51] and that there is a preference of unsaturated fatty acids for the 2 position on the glycerol backbone.[52] It was also observed that a random distribution for unsaturated acids at the 2-position was affected by the concentration of the fatty acid residue. Scano *et al.* were able to determine the positional isomer on the glycerol backbone of 11-*cis* monoenoic fatty acid which is usually present in only small quantities in olive oils and thus in general difficult to analyse.[53]

A study carried out by Bergana *et al.* on the effect of unsaturation in the fatty acid chains on the ^{13}C NMR chemical shift found that the carbonyl chemical shifts are more dependant on the position of the double bonds than the degree of unsaturation in the chains, although the chemical shifts seemed to be influenced by the position of the first double bond.[52] Marcel and co-workers were able to show that the position of the double bonds affected the glycerol carbon chemical

shifts in the case of 22 synthetic (Z)-ethylenic and (E)-ethylenic TAGs where all three fatty acid residues were of the same type.[54]

1.4.4 Identification of regional and cultivar differences of olive oils by ^{1}H and ^{13}C NMR spectroscopy

The European Union has implemented laws which protect particular regional foods. The title PDO (Protected Designation of Origin) is given to the names of a variety of foodstuffs such as wine, beer, olives and many others which can be strongly associated with a particular region. Thus for a food to be given a particular label it has to come from that designated region, e.g. for sparkling wine to be classified as Champagne it must indeed have come from the Champagne region in France. Mediterranean olive oils are also PDO classified and so names that are given to olive oil must correspond to the geographical region in which it is produced. However as olive oils, especially extra virgin olive oil, have high commercial value there is often the threat of olive oils being sold under the incorrect regional name to increase the oil's value. As a result there is considerable interest in analytical methods to be able to identify the region from which an oil comes and its cultivar.

Several studies have proven that ^{1}H and ^{13}C NMR spectroscopy has great potential to identify and authenticate virgin olive oils by geographical origin and variety.[7-19] A ^{1}H NMR study by Sacchi *et al.* used the minor components of 55 different varieties of extra virgin olive oil samples from four Italian regions as tools to distinguish between regions.[7] Vlahov used high resolution ^{13}C NMR spectroscopy combined with statistical analysis to classify 173 samples of olive oils.[8] These were evaluated against a test set consisting of PDO olive oils from three areas in the Puglia region. He was able to assign most of the oils correctly into their respective groups, except in the case where two shared the same predominant cultivar. Studies on extra virgin olive oils from different Italian regions, and in Tuscany from three different regions, indicated that regardless of the cultivar of the olive oil, the geographical origin of the oil produced was the discriminating factor.[9]

Not only can NMR spectroscopy aid in distinguishing between olive oil geographical origin, but also between the variety of olive oils. Shaw *et al.* tried to discriminate extra virgin olive oil by both region and variety.[10] They concluded that multiple, specialized models give better results than global ones since the inclusion of certain variables negatively influence the multivariate calibration process. In another study analytical measurements, including studies on humidity, oil acidity, peroxide value and UV, and ^{1}H NMR spectroscopy combined with multivariate statistical analysis was used by Sacco *et al.* on the phenolic extracts of 28 Italian extra virgin olive oils of different cultivars and geographic origin.[11] The analytical measurements gave information on the fatty acid composition of the oils and could be used in the discrimination of olive oil variety, while the NMR data classified the oils according to geographical origin.

Other cultivar discrimination was carried out by NMR spectroscopy using ^{13}C DEPT experiments and it was shown that this was a powerful technique for discrimination between oil cultivars and grades by looking at the unsaponifiable compounds, total DAG content of the extra virgin olive oil varieties as well as the ratio of 1,2- and 1,3-DAGs.[12,13] Cultivar discrimination on the basis of fatty acid composition was proven to be effective in grouping monovarietal oils of the same cultivars.[14]

Another use of NMR spectroscopy applied to geographical origin is in determining the year of production of olive oils. In one such study Rezzi *et al.* used ^{1}H NMR spectroscopy and multivariate analysis on olive oils from various Mediterranean areas.[15] They found that ^{1}H NMR could successfully be applied for determination of year of production and that chemometric techniques such as multiple linear regressions or generalized pair-wise correlation did not yield better results.

Studies on the effect of pedoclimatic conditions on the composition of olive oils have also been undertaken. One such study was done on 16 monovarietal olive oils matching Italian and Argentina cultivars.[16] This analysis allowed the identification of cultivars which were less affected by their climatic conditions and were therefore proposed as colonizing plants in the regions (such as Argentina) where the desired Mediterranean cultivars can be grown.

1.4.5. Detection of adulteration of olive oil by NMR spectroscopy

As mentioned already the higher grade olive oil such as virgin and extra virgin olive oil has a high commercial value. As a result adulteration of these oils to try to increase the quantity of the oil sold is a concern. This occurs by the addition of other lower quality and value vegetable oils to the vegetable oil of interest, such as olive oil. Thus detection of adulteration of olive oils has been studied with a number of analytical techniques including NMR spectroscopy.

One common adulterant of olive oils are other lower value vegetable oils such as hazelnut, corn, sunflower and soybean oils. Studies using ^{31}P, ^{13}C and ^{1}H NMR spectroscopy, have shown that the presence of these contaminators oils can be detected. The studies mentioned above concerned with geographical origin and discrimination between varieties have aided in authentication of vegetable oils. Properties such as oil's acidity, iodine value, fatty acid composition and the ratio of 1,2-DAG to total DAG content can be used to detect adulteration of olive oil with seed oils. In one such study with the use of ^{1}H NMR and ^{31}P NMR spectroscopy the presence of hazelnut, corn, sunflower and soybean oil could be detected.[55] Discriminant analysis proved the detection limit for adulteration as low as 5%. Certain regions of a ^{13}C NMR spectrum such as the olefinic region are able to provide significant amounts of information. Using pyrazine as internal reference Mavromoustakos *et al.* were able to integrate the resonances representing the olefinic carbons, and upon addition of seed oils, they observed that these olefinic intensities were affected.[56] They were thus able to develop a

semi-quantitative method to detect the adulteration of virgin olive oil by other seed oils using ^{13}C NMR spectroscopy.

As hazelnut oil is extremely similar in composition to olive oil, it is difficult to identify adulteration of olive oil with hazelnut oil. In one such study mixtures of virgin olive oil samples with 5-50% hazelnut oil was successfully classified with respect to their high oleic acid and linoleic acid content.[57] Mannina *et al.* used NMR and GLC for this purpose and found that both the GLC and NMR results confirmed the presence of hazelnut oil in adulterated olive oil samples.[58] Diego *et al.* were successful in developing a method to detect the adulteration of olive oil with low concentrations of hazelnut oil using ^{1}H and ^{13}C NMR spectroscopy and the limit of detection was found to be about 8 %.[59]

Another common adulterant is soybean oil, and it has been proven that ^{13}C DEPT NMR spectra can be successfully used for detecting and quantifying the adulteration of olive oil with soybean oil.[60]

Not only have olive oils been adulterated with other vegetable oils, but also with other lower quality olive oils.[57,61,62] In one such study on three different grades of olive oil, it was shown that mixtures of extra virgin olive oil with refined olive oil and lampante olive oil (a low quality olive oil), could be detected by ^{31}P NMR spectroscopy. Discrimination analysis was applied to the data collected and the adulteration of extra virgin olive oil could be detected to amounts as low as 5%.[62]

Another interesting application of NMR spectroscopy, as discussed by Ogrinc *et al.*, is with the use of Site-specific Natural Isotopic Fractionation-NMR (SNIF-NMR) and Isotopic-Ratio MS (IRMS).[63] The combination of these techniques with chemometric methods can be used successfully for the detection of adulteration in olive oils.

1.5 A LOOK AT SIX LOCALLY PRODUCED OILS

From the above discussion it is clear that olive oil has been extensively studied by multivariate NMR spectroscopy, however little in-depth NMR analysis of other vegetable oils using this technique has been carried out. It is our intention to apply some of the NMR spectroscopic methods developed for the analysis of olive oil to other vegetable oils of interest. We have thus chosen to study six vegetable oils that are produced currently in South Africa, these being apricot kernel oil, avocado pear oil, grapeseed oil, macadamia nut oil, mango kernel oil and marula oil. The research that has been published in the literature on the analysis of each of these oils using various techniques is reviewed below. Although limited NMR spectroscopy has been carried out on two of the oils, a review of the literature shows how limited these studies have been.

1.5.1 Apricot kernel oil

Apricot kernel oil comes from the fruit's nut of the apricot tree, *Prunus armeniaca,* and is light yellow in color.

The oil composition of apricot kernels of eleven varieties found in the Ladakh region of India has been studied by Kapoor *et al.*[64] They found that the pit constitutes 7.3-19.0 weight % of the fruit, the kernels 21.9-38 % of the pit from which 27-67 % oil can be obtained. The kernels also contain 20-45 % proteins. The apricot kernel oil was found to contain 51-80 % oleic, 10-46 % linoleic and 3-11% palmitic fatty acid residues. UV, IR and NMR techniques were used for this analysis and no unusual fatty acids were found, including those with epoxy, cylcopropene, hydroxyl or other oxygenated functional groups. Although this study involved the use of NMR spectroscopy, there was no indication that any NMR analysis was done on the oil itself. Another study by El-Aal *et al.* extracted and characterized apricot kernel oil in order to evaluate its use in preparing biscuits and cakes.[65] They used hexane as the extracting solvent and also found the major fatty acid components to be oleic, linoleic and palmitic acid. Chloroform-methanol extracts yielded neutral lipids and TAGs as major fractions,

while glycol- and phospholipids made up the minor fractions, which includes acylsterylglycosides and phosphatidyl choline as major components. These studies revealed that apricot kernel oil has excellent properties for preparation of foods, and is comparable to corn oil and did not affect the flavour, colour or texture of the prepared biscuits and cakes.

1.5.2 Avocado pear oil

Three avocado tree varieties are found: *Persea Americana* Mill. var. (also known as *P. gratissima* Gaertn), *P. Americana* Mill. var. *drymifolia* Blake (also known as *P. drymifolia* Schlecht. and Cham.) and *P. mubigena* var. *guatemalensis* L. Wms. The oil which is obtained from the fruit of these trees is dark green in color.

Although no NMR studies on avocado pear oil have been documented in the scientific literature, a few other analytical studies have been done.

Moreno *et al.* studied the changes that avocado pear oil undergoes with the use of four different extraction methods.[66] These included studying physical and chemical changes, fatty acid profile, *trans* fatty acid content and the identification of volatile components. They found that the major fatty acids present in the oil were palmitic, palmitoleic, oleic, linoleic and linolenic. Their results showed that there is an effect on the physical and chemical characteristics of the avocado pear oil, which included the fatty acids present as TAGs and volatile compounds, with the use of different extraction methods. Extraction of oil using solvents instead of a microwave, results in greater deterioration of oils. In another study Hierro *et al.* were interested in using HPLC with a light scattering detector to identify the TAG composition of avocado pear oil.[67] Lipid fractions of two varieties, *Fuerte and Hass*, were used. The qualitative compositions were found to be similar, while quantitative differences were found between the two varieties. Four *Persea Americana* varieties including *Zutano, Bacon, Fuerte* and *Lula* were investigated by Lozano *et al.* for their unsaponifiable matter (UM).[68] The UM content of the oils was found to be higher in immature fruits than those for mature fruits, and using UM fractions obtained by HPLC it was found that the sterol and tocopherol content for immature fruit was higher; the tocopherol content was

different for each variety. Du Plessis studied avocados of four different cultivars obtained on a monthly basis for the 1978 season from South Africa, Pretoria.[69] The fruit mesocarp was isolated by centrifugation and analyzed for fatty acid, iodine values, phospholipids and FFA contents. Changes in these contents were investigated over time.

1.5.3 Grapeseed oil

From the seed of the grape, *Vitis vinifer*, a light green oil may be extracted. Grapeseed oil is one of the better investigated vegetable oils and the TAG composition of grapeseed oil has been studied by a number of researchers.

In one such study a method was developed for the classification of these oils using analysis of the TAG composition by HPLC followed by linear discriminant analysis.[70] A simple method was developed for the regiospecific analysis of TAGs in grapeseed oil which is the analysis of whether fatty acids are situated in the sn 1', 3' or sn 2' positions on the glycerol backbone.[71] This method made use of the partial de-acylation of the TAGs with ethylmagnesiumbromide, followed by the derivatization of the MAGs with n-butyrylchloride. The analysis of these derivatives was carried out using GLC. It was concluded that this method was simple, since it is not as time-consuming as the conventionally used TLC method, as well as being sensitive, since the quantification of low levels of derivatized MAGs could be obtained. Cao *et al.* extracted grapeseed oil by using Supercritical Fluid Extraction (SFE) and separated the FFAs by high speed counter-current chromatography. SFE was used to study the effect which certain parameters, including pressure, temperature and particle size of sample, have on the yield and composition of the extracted oil.[72] Results indicated that grapeseed oil could be successfully extracted by using supercritical CO_2 without the use of a modifier. Studies also indicated that the yield of the oil is independent of the pressure and temperature that is applied during the extraction process as well as independent of the particle size of the seeds. Andrikopoulos discussed the chromatographic, spectrophotometric and spectroscopic methods for the detection, identification and quantification of TAG composition of vegetable oils,

including grapeseed oil.[73] Methods such as HPLC, Capillary Gas Chromatography (CGC), and Flame Ionization Detector (FID) were reviewed as well as these methods in combination with MS, SFC, and TLC. It was found that a combination of these different techniques yielded a comprehensive picture of the TAG content of the oils.

Compounds other than fatty acids present in grapeseed oil have also been investigated. Dionisi *et al.* used Reversed-Phased HPLC (RP-HPLC) with amperometric detection for the analysis of tocopherols and tocotrienols in vegetable oils.[74] While none of these compounds were detected in olive oil, high levels were observed in grapeseed oil. Results indicated that this method was accurate, repeatable, sensitive, easy to perform, sample preparation was simple and it avoids any possible saponification from occurring which could lead to oxidation. The method also guaranteed high recovery. Kolarovič *et al.* used glass capillary GLC for the determination of polycyclic aromatic hydrocarbons (PAH),[75] for vegetable oils and fats of which the PAHs were complexed with caffeine in a formic acid solution and then separated on a liquid column, as well as by TLC before being injected into a GLC. Quantitative and qualitative results showed that grapeseed had the lowest amount of PAH. Watermelon seeds and grapeseeds were studied by Kamel *et al.* for their nutritional quality and oil characteristics.[76] Of specific interest was the percentage yields obtained for the different compounds present in the seeds. These compounds include the crude protein, fat and fiber, as well as minerals and fatty acids. Iodine numbers, free acid values and saponification numbers were also studied. The acid profiles of seed cakes prepared from both watermelon and grapeseed were determined and compared with the protein of hen's eggs. The conclusions showed that both grapeseed and watermelon had an overall good nutritional value. Kinsella studied the food composition of grapeseeds finding it contained 10% moisture, 9-12% fat and 8-12% protein,[77] as well as investigating the physiochemical properties of the oil such as the iodine value, acid value, etc. and the changes in fatty acid composition upon heating and frying of the oil.

Grapeseed oil was also subjected to studies similar to those done for olive oil, already discussed in sections 1.4.1 and 1.4.4, by Hidalgo *et al.*[78] *and* Zamora *et al.*[47,57]

1.5.4 Macadamia nut oil

There are two species of the macadamia nut tree with edible fruit, *Macadamia integrifolia and Macadamia tetraphylla*, while the fruit of the remaining species, *Macadamia ternifolia*, is inedible. Macadamia nut oil extracted from the fruit is light yellow in color.

In a study carried out by Holčapek *et al.* the TAG and DAG content of sixteen different plant oils, of which macadamia nut oil was one, was analyzed.[79] The data was collected using HPLC-MS with atmospheric pressure chemical ionization (APCI) and UV detection. Identification of TAGs was done by APCI-MS while the characterization of TAGs and DAGs were carried out with HPLC-APCI-MS. Otherwise no other analytical studies of this oil could be found and no NMR spectroscopy has been carried out on macadamia nut oil according to a survey of the available scientific literature.

1.5.5 Mango kernel oil

Oil from *Mangifera indica,* is extracted from the mesocarp of the fruit or the kernel and is light yellow to brown in color.

In one study Moharram *et al.* analyzed the fat from *Mangifera indica* for its chemical and physical properties, TAG, fatty acid and phospholipids contents and compared it with cotton seed oil, cocoa butter and two of its commercial substitutes, namely Croklaan Special 555 and Wesco Special E.[80] Oil content was determined at 6-12 weight %, the seed is 16-32 % of the fruit and the kernel 50% of the seed. The kernel was found to consist of 13.8 % moisture, 72.6 % carbohydrates, 13.7 % oil, 7.08 % crude protein, 1.75 % ash and 2.95 % crude fiber. TAGs with fatty acids containing 1, 2 ,3 and 4 double bonds were also found to be present, including palmitic, palmitoleic, stearic, oleic, linoleic and linolenic. Results indicated that toffee prepared from mango oil and cocoa butter

is almost identical in the sense that their characterization and composition are similar. They also found no difference in taste, texture and odour.
No published NMR spectroscopic studies have been carried out on mango kernel oil to the best of our knowledge.

1.5.6 Marula oil

The light yellow colored marula oil is extracted from the seed kernel of the marula tree, *Sclerocarya birrea* subspecies *caffra*, also referred to as *Sclerocarya caffra*. The investigation of oil and seed content has been done by several researchers. In one such study Jaenicke *et al.* carried out studies on the marula fruits and seeds from Kenya, Botswana, Namibia and South Africa.[81] They studied the fruit from two Kenyan districts, namely Kibwezi and Kitui, to determine if they were of the same quality to those found in South Africa. Contents such as Vitamin C, carbohydrates, crude fiber, crude fat and protein and minerals were analyzed. They found that the marula fruit and seeds from Kenya have the same contents and are of equal quality to those found in South Africa. This led to the suggestion that the wide variety of natural marula plants in countries other than South Africa have the potential for further improvement of the species and therefore the possibility to compete with commercial production of high quality South African marula oil. In another study Glew *et al.* investigated the contents of twenty four plants of which marula fruit was one.[82] Components such as lipids, amino acids, proteins, minerals and fatty acids were determined. It was concluded that a few of the plants comprised of most of the essential nutrients, and although not one single plant contains all the above mentioned nutrients, the combined results of these 24 plants could play an essential part in a healthy human diet. Burger *et al.* studied the oil and protein composition of marula seed kernels obtained in South Africa and Namibia.[83] The major fatty acids in marula oil included palmitic, oleic, stearic and linoleic. The sterol and tocopherol content was also studied and found that the oil contains no natural anti-oxidants with the exception of γ-tocopherol. Investigation into the stability of the oils obtained from the kernels was also done. These studies revealed that the high oxidative stability of marula

oil makes it useful as a content in frying oils or as a dried fruit coating. They also suggested that marula oil could replace high-oleic safflower oils for use in baby food formulas. The fatty acid composition of the oil of different wild fruits and plants were determined by Engelter *et al.* with the use of GLC.[84] Marula oil was one of these samples studied, showing that for marula oil, oleic acid made up 70% of the total fatty acid content, the rest of the major fatty acid components were stearic, palmitic, linoleic, and linolenic acids.

As for avocado pear, macadamia nut and mango kernel oils no NMR spectroscopic investigation of these oils appear to have been undertaken previously.

1.6 AIMS OF THIS PROJECT

The aim of this project is the full ^{13}C NMR spectroscopic assignment and quantification of the major TAGs present in the six above mentioned vegetable oils (figure 1.9) produced in South Africa. This type of study has not been previously carried out for these oils.

Figure 1.9: The seven vegetable oils used in this study: 1 apricot kernel, 2 avocado pear, 3 grapeseed, 4 macadamia nut, 5 mango kernel, 6 marula, 7 olive.

In the following chapters it will be explained how this assignment and quantification was accomplished. Chapter 2 discusses the ^{13}C NMR spectra of standard TAGs as well as their application in the newly developed graphical method for the assignment of the ^{13}C NMR spectra of vegetable oils. The methodology of the development of this new method is discussed as well proof of its validity and concentration independence. Chapter 2 also discusses the results obtained for the assignments of the ^{13}C NMR spectral data for the standard tripalmitolein and the six vegetable oils that was assigned using the graphical method developed. Chapter 3 looks at the GLC and GLC-MS analysis that was needed to gain information regarding the fatty acid composition of the vegetable oils. Chapter 3 also compares the quantification data that were obtained for the six vegetable oils by ^{13}C NMR spectroscopy and GLC-MS. During the ^{13}C NMR studies, some evidence of the presence of FFAs was found in the oils, and consequently this was verified by a titration technique and was used to estimate the percentage of the FFAs in each oil. The development of this titration technique as well as the percentage of FFAs determined for each of the six oils are discussed in chapter 4. Chapter 5 discusses the possible future work that can be carried out as a continuation of this study on these and other vegetable oils.

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CHAPTER 2

THE USE OF A NOVEL "LINEAR GRAPH METHOD" FOR THE ASSIGNMENT OF ^{13}C NMR SPECTRA OF VEGETABLE OILS

A rapid and potentially generic method for the full assignment of the ^{13}C NMR spectra of the major fatty acid components of six vegetable oils (apricot kernel, avocado, grapeseed, macadamia nut, mango kernel and marula oils) has been developed. The method is shown to be especially useful for regions of the ^{13}C NMR spectra where significant peak overlap occurs. Assignments of the ^{13}C NMR spectra of standard TAGs aided in proving that the developed method was more accurate than a method used where the oil is spiked with these standard TAGs. The method was validated by means of the assignment of the ^{13}C NMR spectrum of olive oil which has been extensively reported in the literature. Concentration studies also proved that this method was independent of the concentration of the oil used. This resulted in the facile full and unambiguous assignment of all the ^{13}C NMR peaks in the ^{13}C spectra of the vegetable oils, which have not previously been examined or assigned.

2.1 INTRODUCTION

The aim of this project has been to use ^{13}C NMR spectroscopy as a technique for the analysis of apricot kernel, avocado pear, grapeseed, macadamia nut, mango kernel and marula vegetable oils. The main area of interest was in the analysis of the major fatty acids present as TAGs in the oils by ^{13}C NMR spectroscopy. However as a starting point some information was needed about the oils and we thus chose to first analyze the oils using GLC for the identification of the major fatty acids present as TAGs in the six vegetable oils of interest. Once the fatty acids were determined, we attempted the assignment of the ^{13}C NMR spectra of the vegetable oils. Chapter 3 discusses the experimental procedure and methods used for the analysis of these vegetable oils by GLC and GLC-MS analysis. This chapter however looks at the research which led to the development of a "linear graph method" for the assignment of the ^{13}C NMR spectra of vegetable oils as well as the validation, further investigation and application of this novel method.

2.2 ASSIGNMENT OF ^{13}C NMR SPECTRA OF STANDARD TAGs

In order to fully assign the ^{13}C NMR spectrum of any vegetable oil using the standard-additions method, it was first necessary to have the complete assignments of the ^{13}C NMR spectra of the standard TAGs. Therefore ^{1}H and ^{13}C NMR spectroscopy experiments were run on several standard, commercially available TAGs known to be present in the vegetable oils as detected by GLC analysis (refer to figure 3.1 in chapter 3). These standard TAGs included triolein, trilinolein, tripalmitin, tristearin, trimyristin and tripalmitolein. This exercise also aided in developing NMR skills before embarking on the novel aspect of the project, that being the assignment of the ^{13}C NMR spectra of oils that have not been previously studied by this technique.

The assignments of the ^{13}C NMR spectra of the standard TAGs were compared to results in the literature,[1,2] while ^{1}H NMR spectroscopy yielded information

regarding the purity of the compounds. Our results for the ^{13}C NMR spectra correlated well with those of Mannina *et al.*[1] with a maximum difference of 0.056 ppm in any one chemical shift measured. Comparisons were also made with certain of the ^{1}H NMR signals obtained by Guillén *et al.*[3]

2.2.1 Experimental

NMR spectra were run on a Varian ^{Unity}*Inova* 400 NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The following acquisition parameters were used for collection of ^{13}C NMR spectra: number of points: 256 K; spectral width: 195 ppm; relaxation delay: 7 s; acquisition time: 4.5 s; pulse width π/6. 100 µl of the standard TAG was dissolved in 700 µl of $CDCl_3$ with TMS as a reference standard.

2.2.2 Comments on the ^{1}H NMR spectra of standard TAGs

Before continuing a few comments will be made about the ^{1}H NMR spectra of TAGs.

The two hydrogens present on carbons 1' and 3' of the glycerol backbone of all the standard TAGs are diastereotopic, which leads to them being non-equivalent in a ^{1}H NMR spectrum. This term is usually given to CH_2 protons in a compound that is prochiral and has the potential to be diastereomeric if one of the CH_2 hydrogens is replaced with an R-group. An example of a diastereomeric compound is given in figure 2.1.

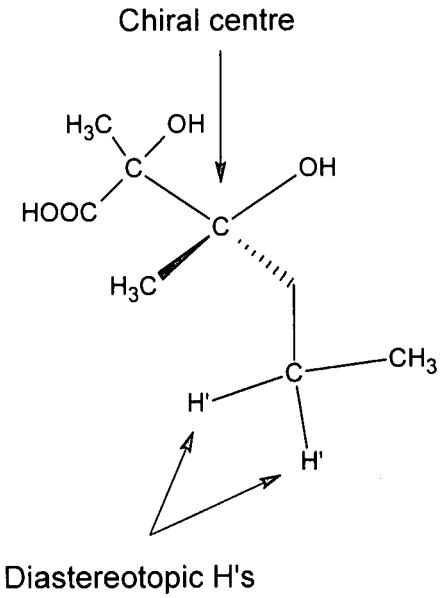


Figure 2.1: Example of chiral compound with a diasteretopic CH_2 group.

In the case of a standard TAG, the molecule is achiral overall as no chiral center is present. However if for instance H3a' is replaced by an R'-group, then the molecule will immediately have two chiral centers at C2' and C3' (see figure 2.2) making it a diastereomer. Thus the C1' and C3' protons are considered diastereotopic and the two protons (Ha and Hb) on each carbon are in different environments and have separate chemical shifts.[4,5,6] The replacement of H3a' is however not the only situation to make the molecule diastereotopic. Any of the protons from the C1' and C3' chains can be replaced by an R' group to give the same result.

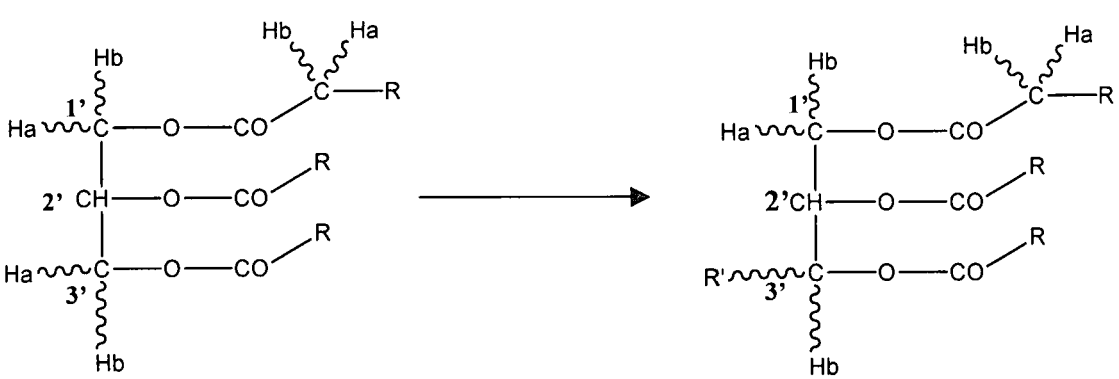


Figure 2.2: Replacement of a H by an R'-group forming a chiral compound.

Looking at the ^{1}H-NMR spectra (figure 2.3) of a standard TAG, the peak for H2' shows a complicated splitting pattern. Figure 2.4 shows the splitting diagram for this peak which appears at around 5 ppm. The two coupling constants are estimated from the separate couplings of Ha and Hb. As in indicated in figure 2.5 H2' couples to the two protons Ha and Hb (either H1' or H3') to give a triplet (strictly speaking a double of doublets however the coupling constants of the doublets are the same), then coupling with the second set of protons giving another triplet resulting in the overall splitting diagram represented in figure 2.4. The coupling constants are then extracted from this splitting diagram.

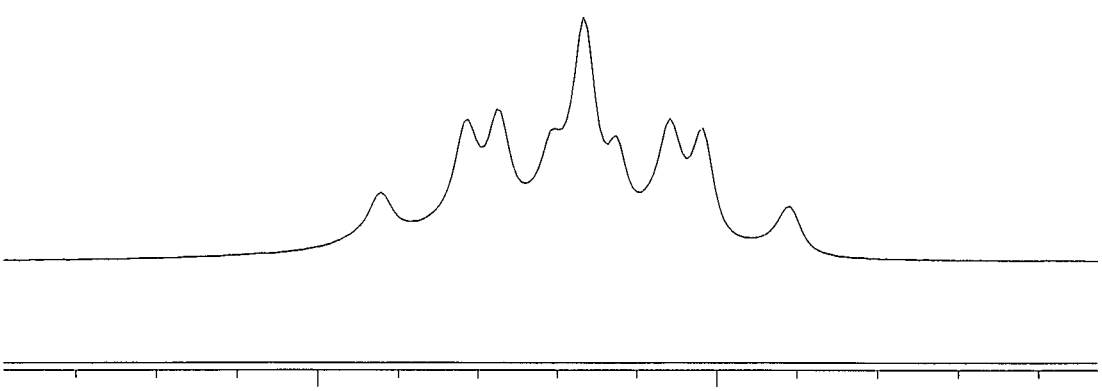


Figure 2.3: Expansion of the ^{1}H NMR spectrum of H2' for tripalmitin at 400 MHz in $CDCl_3$ at room temperature.

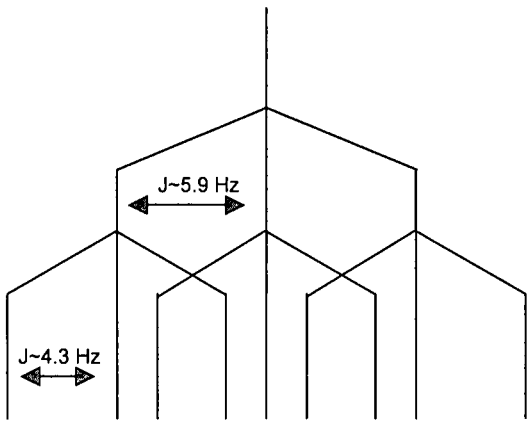


Figure 2.4: Peak splitting diagram of H2' in the ^{1}H NMR spectrum of a saturated TAG.

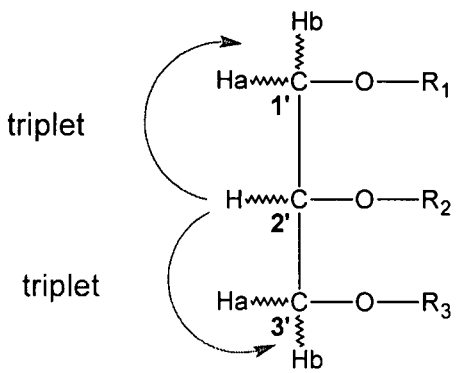


Figure 2.5: Coupling of H2' to the adjacent protons of H1' and H3'.

The splitting of H9, 10 for triolein (see figure 2.6) and H9, 10, 12, 13 for tristearin (see figure 2.7) was too complicated to draw splitting diagrams for due to the unsaturation present in the fatty acid chains. The ^{1}H NMR spectrum of triolein shows a multiplet containing 16 peaks while tristearin's multiplet has about 34. This was unexpected since according to first-order ^{1}H-^{1}H coupling rules, a simple multiplet containing a doublet of doublet of doublets is expected in both cases. The complicated multiplets were in all likelihood due to second-order coupling effects, and full assignment was not attempted.

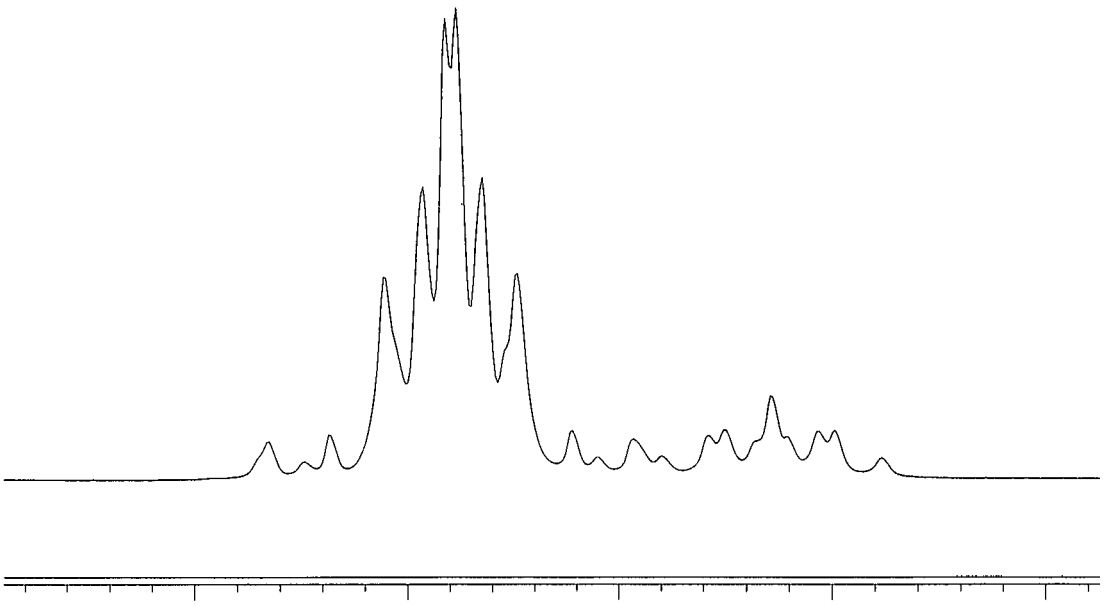


Figure 2.6: Expansion of the ^{1}H NMR spectrum of triolein showing signals due to H9, 10 and H2' at 400 MHz in $CDCl_3$ at room temperature.

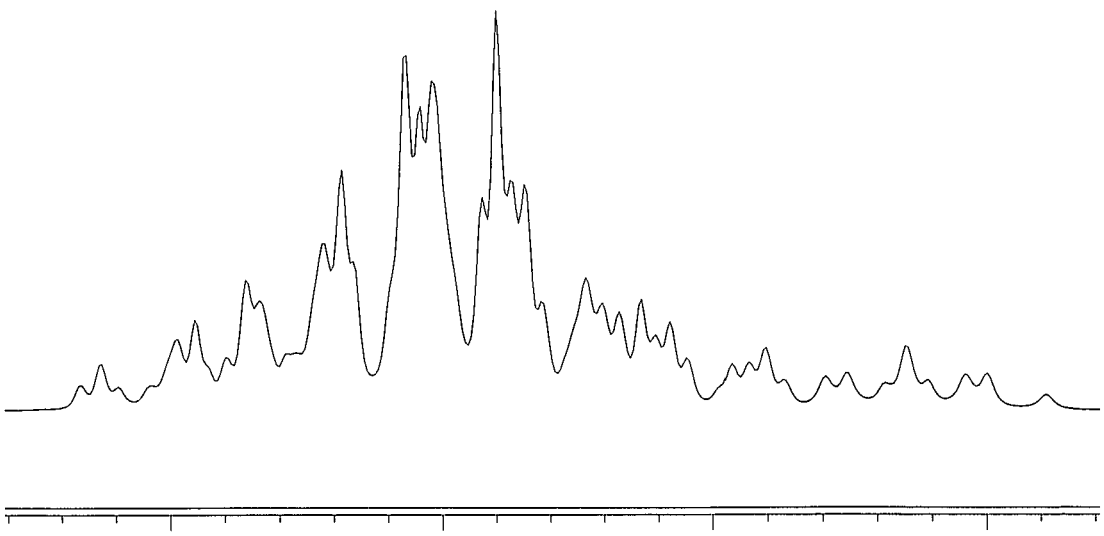


Figure 2.7: Expansion of the ^{1}H NMR spectrum of trilinolein showing signals due to H9, 10, 12, 13 and H2' at 400 MHz in $CDCl_3$ at room temperature.

Note that most natural TAGs (such as those found in vegetable oils) are chiral at C2' as there are different fatty acids on each glycerol arm. However this still means the CH_2's are diastereotopic.

2.2.3 Comment on the ^{13}C NMR spectra of standard TAGs.

The ^{13}C NMR spectrum of a standard TAG is unique in a sense that not only can the carbons of the compound be detected, but distinction between carbons can be made with respect to their position on the glycerol backbone. Therefore carbons belonging to the α positions can be separately identified from those in the β position. These resonances can also easily be differentiated by inspection. Not only are they found close to each other in a ^{13}C NMR spectrum, but due to chemical equivalence of the carbons that are present in the two α positions, the resonance representing those carbons are found to be roughly twice the size of those for the β position.

In certain regions of the ^{13}C NMR spectrum the presence of compounds, other than fatty acid residues, can be detected. In the 62-68 ppm region the carbons belonging to the glycerol backbone is found for acylglycerols. Carbons belonging to TAGs, DAGs and MAGs can separately be identified here, while in the 174 ppm region carbons belonging to FFAs can be detected.

2.2.4 Saturated triacylglycerols: ^{1}H and ^{13}C NMR spectra

The saturated standard TAGs contain long chains of carbons with no double bonds. For our studies these TAGs included trimyristin, tripalmitin, and tristearin. Figures 2.9 and 2.10 illustrate typical ^{1}H and ^{13}C NMR spectra for these standards using tripalmitin at 400 MHz and 100 MHz respectively in $CDCl_3$ at room temperature as example.

Trimyristin

Trimyristin is the TAG of the saturated, 14-carbon myristic acid, and is shown in figure 2.7. Assignments of the peaks for both the ^{1}H and ^{13}C spectra are shown

in tables 2.1 and 2.2. No references were found for the ^{1}H NMR and ^{13}C NMR spectra of trimyristin in the literature.

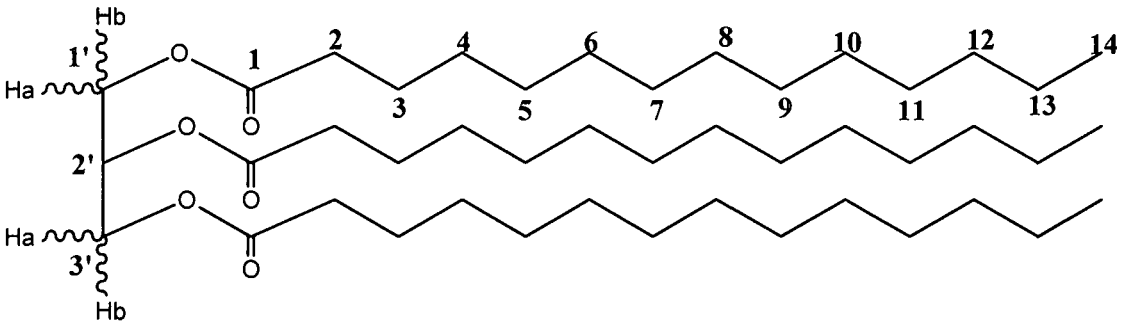


Figure 2.8: Numbered structure of trimyristin.

Table 2.1: ^{1}H NMR data obtained for trimyristin at 400 MHz in $CDCl_3$ at room temperature.

Assignment	Chemical shift (ppm)	Multiplicities	No. of H's	*J* (Hz)	
H1a'/H3a'	4.317	dd	2	^{2}J = 4.3	^{3}J = 11.9
H1b'/H3b'	4.167	dd	2	^{2}J = 6.0	^{3}J = 11.9
H2'	5.292	multiplet	1	^{2}J = 4.3	^{3}J = 5.9
H2	2.332	dt	6	^{2}J = 2.3	^{3}J = 7.4
H3	1.625	multiplet	6		
H4-H13	1.318	multiplet	60		
H14	0.896	t	9	^{3}J = 6.7	

Table 2.2: ^{13}C NMR data obtained for trimyristin at 100 MHz in $CDCl_3$ at room temperature.

Assignment	Position on glycerol backbone	Chemical shift (ppm)
C1	sn 1', 3'	173.261
	sn 2'	172.851
C2	sn 1', 3'	34.075
	sn 2'	34.242
C3	sn 1', 3'	24.896
	sn 2'	24.936
C4	sn 1', 3'	29.149
	sn 2'	29.113
C5	sn 1', 3'	29.511
	sn 2'	29.535
C6	sn 1', 3'	29.305
	sn 2'	29.329
C7	sn 1', 3'	29.655
	sn 2'	29.668
C8	sn 1', 3'	29.720
	sn 2'	29.729
C9	sn 1', 3'	29.693
	sn 2'	29.687
C10	sn 1', 3'	29.706
C11	sn 1', 3'	29.394
C12		31.957
C13		22.719
C14		14.129
CHO		68.900
CH_2O		62.115

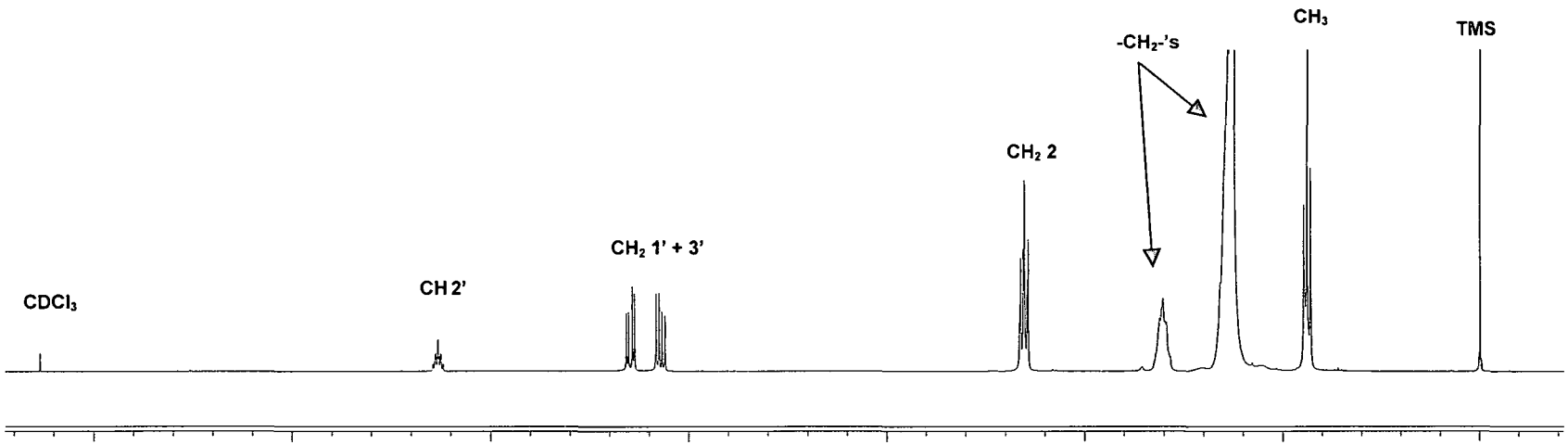


Figure 2.9: ^{1}H NMR spectrum of tripalmitin at 400 MHz in $CDCl_3$ at room temperature.

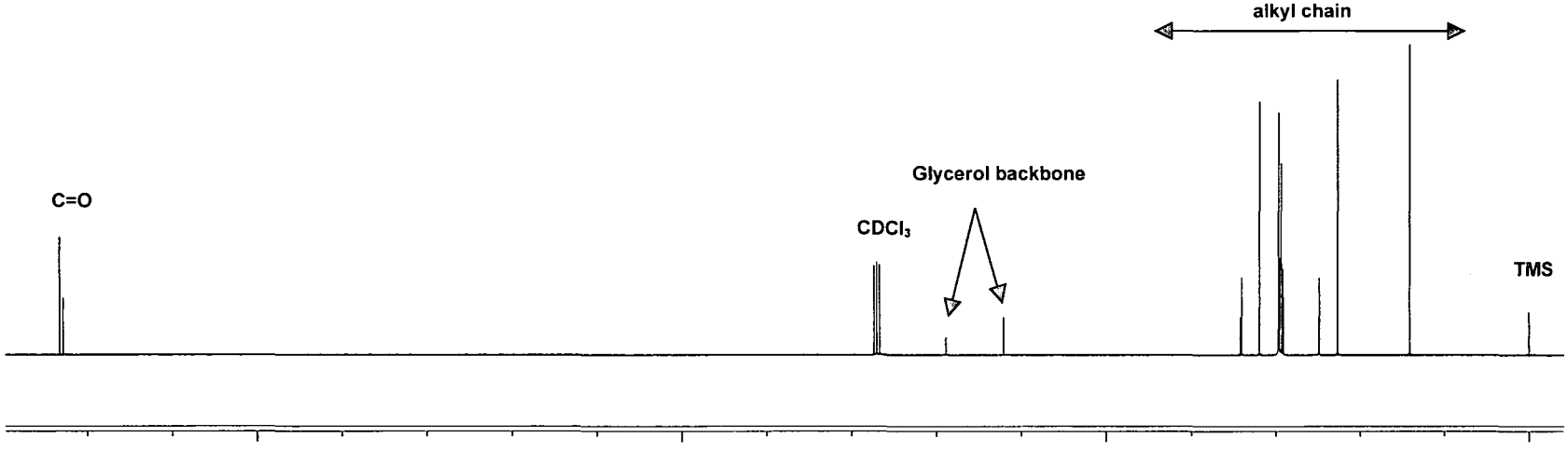


Figure 2.10: ^{13}C NMR spectrum of tripalmitin at 100 MHz in $CDCl_3$ at room temperature.

Tripalmitin

Tripalmitin is a TAG formed from the saturated, 16-carbon palmitic acid, and is a white crystalline solid at room temperature. Based on the density of tripalmitin (0.8752 g/cm^3) it was determined that a mass of 0.08752 g was equivalent to 100 µl of compound.The actual mass weighed was 0.0876 g.

The structure of tripalmitin is shown in figure 2.11. Assignments of the peaks for both ^{1}H and ^{13}C spectra are shown in tables 2.3 and 2.4. No references were found for the ^{1}H NMR spectrum of tripalmitin, however Mannina *et al.*[1] have assigned the ^{13}C NMR spectrum of this TAG. Our assignments compare well as indicated in table 2.4.

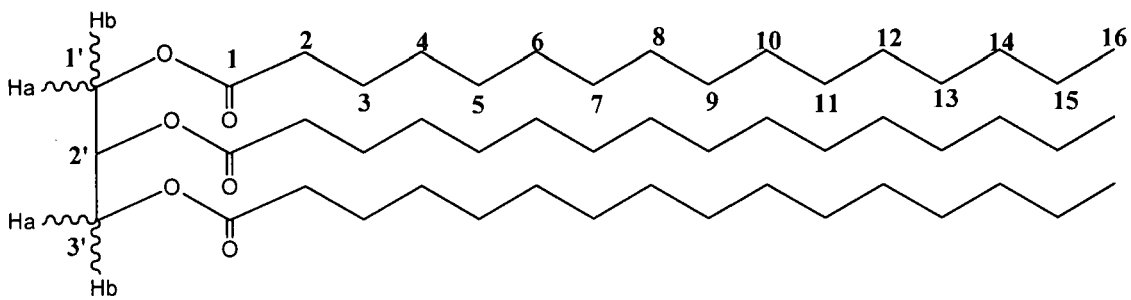


Figure 2.11: Numbered structure of tripalmitin.

Table 2.3: ^{1}H NMR data obtained for tripalmitin at 400 MHz in $CDCl_3$ at room temperature.

Assignment	Chemical shift (ppm)	Multiplicities	No. of H's	*J* (Hz)	
H1a'/H3a'	4.317	dd	2	^2J = 4.3	^3J = 11.9
H1b'/H3b'	4.167	dd	2	^2J = 6.0	^3J = 11.9
H2'	5.292	multiplet	1	^2J = 4.3	^3J = 5.9
H2	2.332	dt	6	^2J = 2.3	^3J = 7.4
H3	1.625	multiplet	6		
H4-H15	1.318	multiplet	72		
H16	0.896	t	9	^3J = 6.7	

Table 2.4: ^{13}C NMR data obtained for tripalmitin at 100 MHz in $CDCl_3$ at room temperature.

Assignment	Position on glycerol backbone	δ_1 Chemical shift (ppm) Mannina *et al.*[1]	δ_2 Chemical shift (ppm) This work	$\Delta=\delta_1-\delta_2$ (ppm)
C1	sn 1', 3'	173.285	173.256	0.029
	sn 2'	172.875	172.847	0.028
C2	sn 1', 3'	34.086	34.075	0.011
	sn 2'	34.253	34.242	0.011
C3	sn 1', 3'	24.903	24.898	0.005
	sn 2'	24.946	24.939	0.007
C4	sn 1', 3'	29.158	29.153	0.005
	sn 2'	29.121	29.116	0.005
C5	sn 1', 3'	29.517	29.517	0.000
	sn 2'	29.541	29.540	0.001
C6	sn 1', 3'	29.309	29.309	0.000
	sn 2'	29.334	29.333	0.001
C7	sn 1', 3'	29.663	29.662	0.001
	sn 2'	29.683	29.683	0.000
C8	sn 1', 3'	29.698	29.700	0.002
	sn 2'	29.698	29.700	0.002
C9	sn 1', 3'	29.701	29.715	0.014
	sn 2'	29.701	29.715	0.014
C10	sn 1', 3'	29.766	29.743	0.023
	sn 2'	29.766	29.737	0.029
C11	sn 1', 3'	29.735	29.726	0.009
	sn 2'	29.735	29.726	0.009
C12	sn 1', 3'	29.741	29.737	0.004
	sn 2'	29.741	29.737	0.004
C13	sn 1', 3'	29.399	29.403	0.004
	sn 2'	29.399	29.403	0.004
C14	sn 1', 3'	31.964	31.964	0.000
	sn 2'	31.964	31.964	0.000
C15	sn 1', 3'	22.720	22.723	0.003
	sn 2'	22.720	22.723	0.003
C16		14.125	14.131	0.006
CHO		68.921	68.902	0.019
CH_2O		62.130	62.114	0.016
average				0.008

Tristearin

Tristearin is a TAG formed from the 18-carbon stearic acid and is a white crystalline solid at room temperature. Based on the density of tristearin (0.8559 g/cm^3) it was determined that a mass of 0.08559 g was equivalent to 100 µl of compound. The actual mass weighed was 0.0852 g.

The structure of the compound is shown in figure 2.12 with corresponding tables 2.5 and 2.6 containing the ^{1}H and ^{13}C NMR data. Note that according to Mannina *et al.*[1] a single peak was found in the ^{13}C NMR spectrum to represent C8 and C9 and also a single peak to represent C11-14. However during our assignment we were able to resolve the two peaks representing C8 and C9, and also two peaks representing C11-14. It is however not clear exactly which carbons each of these peaks are due to. Satisfactory proton correlations with Guillen *et al.*[3] for H14-17 and H18 were obtained for the ^{1}H NMR spectrum.

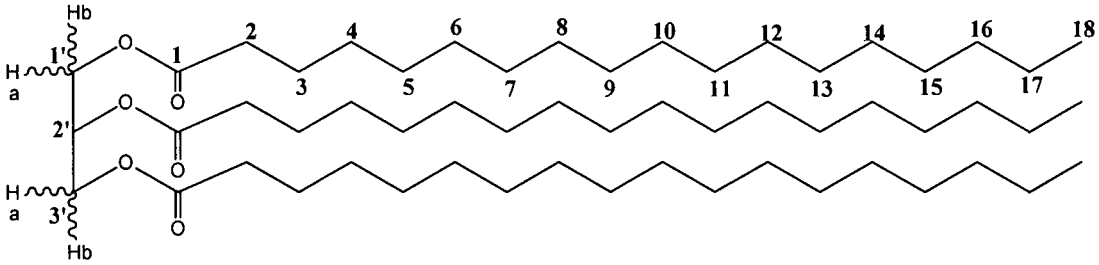


Figure 2.12: Numbered structure of tristearin.

Table 2.5: ^{1}H NMR data obtained for tristearin at 400 MHz in $CDCl_3$ at room temperature.

Assignment	Chemical shift (ppm)	Multiplicities	No. of H's	*J* (Hz)	
H1a'/H3a'	4.317	dd	2	^2J = 4.3	^3J = 11.9
H1b'/H3b'	4.167	dd	2	^2J = 6.0	^3J = 11.9
H2'	5.292	multiplet	1	^2J = 4.3	^3J = 5.9
H2	2.331	dt	6	^2J = 2.2	^3J = 7.5
H3	1.625	multiplet	6		
H4-H17	1.256	multiplet	84		
H18	0.896	t	9	^3J = 6.7	

Table 2.6: ^{13}C NMR data obtained for tristearin at 100 MHz in $CDCl_3$ at room temperature.

Assignments	Position on glycerol backbone	δ_1 Chemical shift (ppm) Mannina *et al.*[1]	δ_2 Chemical shift (ppm) This work		$\Delta=\delta_1-\delta_2$ (ppm)
C1	sn 1', 3'	173.253	173.254		0.001
	sn 2'	172.847	172.845		0.002
C2	sn 1', 3'	34.087	34.076		0.011
	sn 2'	34.253	34.243		0.010
C3	sn 1', 3'	24.913	24.9		0.013
	sn 2'	24.954	24.942		0.012
C4	sn 1', 3'	29.169	29.158		0.011
	sn 2'	29.132	29.12		0.012
C5	sn 1', 3'	29.534	29.523		0.011
	sn 2'	29.556	29.547		0.009
C6	sn 1', 3'	29.324	29.315		0.009
	sn 2'	29.348	29.339		0.009
C7	sn 1', 3'	29.678	29.669		0.009
	sn 2'	29.699	29.69		0.009
C8	sn 1', 3'	29.718			0.010
	sn 2'	29.718		29.708	0.010
C9	sn 1', 3'	29.718		29.722	0.004
	sn 2'	29.718			0.004
C10	sn 1', 3'	29.743	29.734		0.009
	sn 2'	29.743	29.734		0.009
C11	sn 1', 3'	29.763			0.014
	sn 2'	29.763			0.014
C12	sn 1', 3'	29.763			0.014
	sn 2'	29.763		29.749	0.014
C13	sn 1', 3'	29.763		29.752	0.011
	sn 2'	29.763			0.011
C14	sn 1', 3'	29.763			0.011
	sn 2'	29.763			0.011
C15	sn 1', 3'	29.418	29.409		0.009
	sn 2'	29.418	29.409		0.009
C16		31.981	31.969		0.012
C17		22.734	22.726		0.008
C18		14.131	14.132		0.001
CHO		68.937	68.906		0.031
CH_2O		62.131	62.115		0.016
average					0.010

2.2.4 Unsaturated triacylglycerols: ^{1}H and ^{13}C NMR spectra

The unsaturated standard TAGs contain long chains of carbon atoms which differ in degree of unsaturation. For our studies these included triolein, tripalmitolein and trilinolein. Figures 2.14 and 2.15 illustrate typical ^{1}H and ^{13}C NMR spectra in $CDCl_3$ for these standards, using triolein as an example.

To determine the *cis* or *trans* orientation of the double bonds in the unsaturated chains of the TAGs, the coupling constant values in the ^{1}H NMR spectra are used. Coupling constant values falling between 11-18 Hz indicate *trans* orientation of the double bond while values between 6-15 Hz indicate *cis* orientation.[5] Figure 2.13 illustrates these cases:

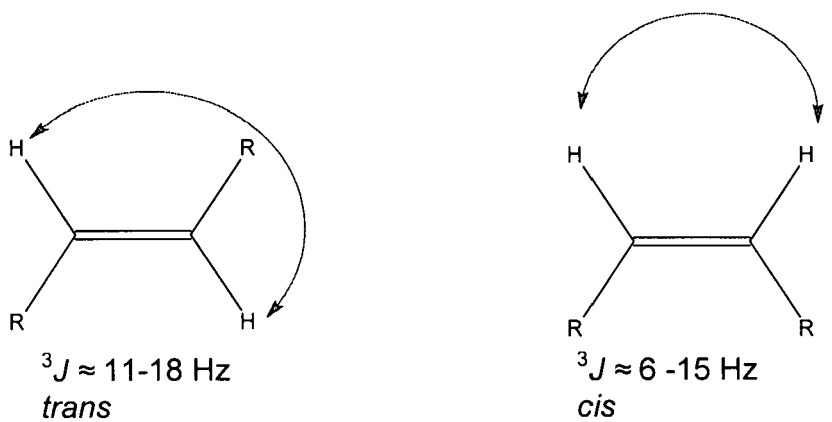


Figure 2.13: Typical ^{1}H coupling constants for *trans* and *cis* orientation of double bonds.

The resonances that represent the protons belonging to the double bond(s) of a standard TAG in the ^{1}H spectrum are used for the determination of double bond configuration. Refer to figure 2.5 where H9 and 10 are indicated for triolein as an example. The complicated splitting diagram makes it difficult to determine the coupling constants, however when calculating distances between peaks of the multiplet, coupling constant values of between 6 and 7 Hz were consistently found in each multiplet indicating a *cis* arrangement of the double bond.

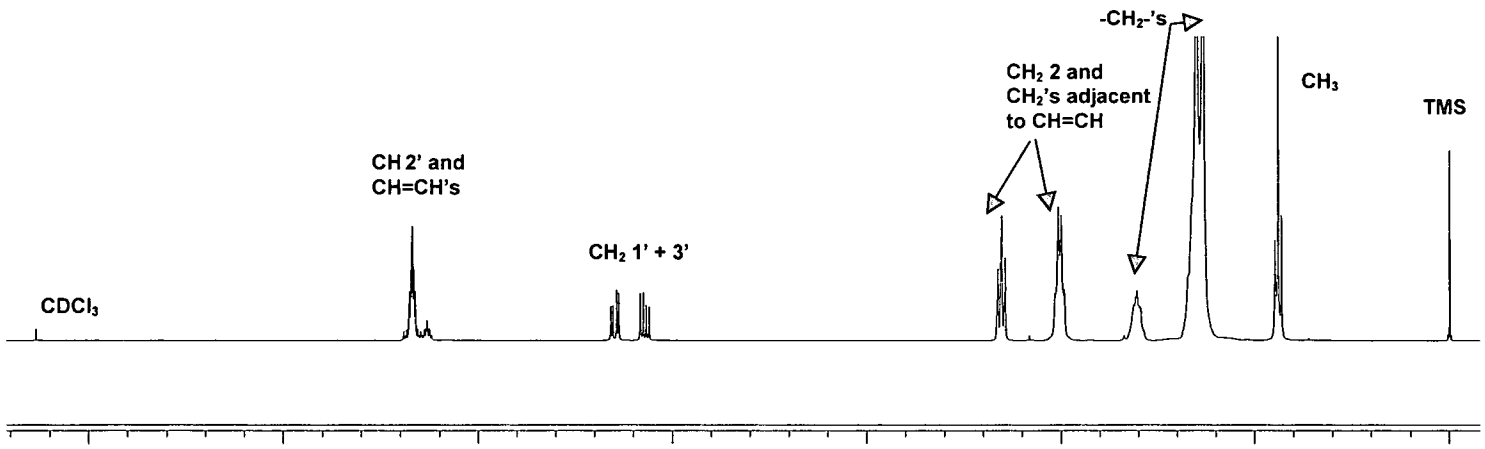


Figure 2.14: ^{1}H NMR spectrum of triolein at 400 MHz in $CDCl_3$ at room temperature.

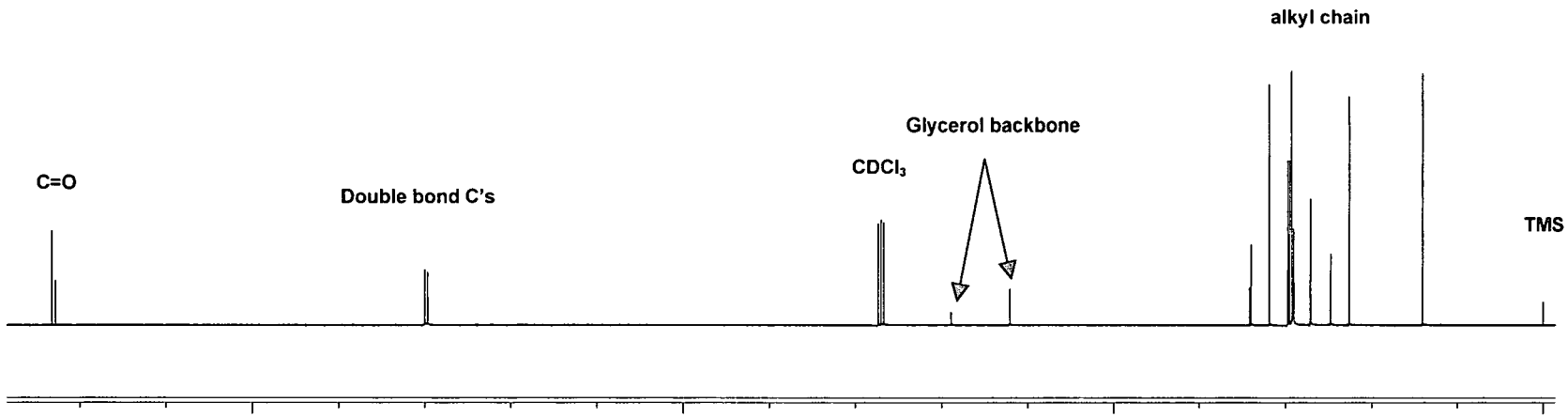


Figure 2.15: ^{13}C NMR spectrum of triolein at 100 MHz in $CDCl_3$ at room temperature.

Triolein

Triolein (figure 2.16) is a TAG formed from the 18 carbon oleic fatty acid containing one double bond which is in the *cis* orientation. This conformation was confirmed as by the coupling constant of ~6.5 Hz found from the ^{1}H spectrum. Tables 2.7 and 2.8 give the results from the ^{1}H and ^{13}C NMR spectra. H8, 11; H12-17, H4-7 and H18 chemical shifts correlated well with those of Guillen *et al.*[3] as indicated in table 2.7.

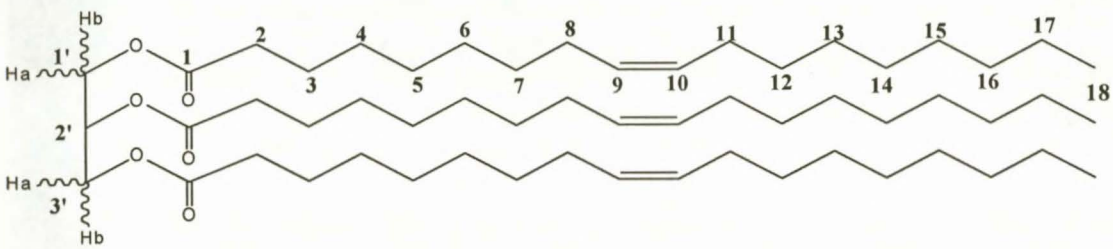


Figure 2.16: Numbered structure of triolein.

Table 2.7: ^{1}H NMR data obtained for triolein at 400 MHz in $CDCl_3$ at room temperature.

Assignment	Chemical shift (ppm)	Multiplicities	No. of H's	*J* (Hz)	
H1a'/H3a'	4.316	dd	2	$^{2}J = 4.3$	$^{3}J = 11.9$
H1b'/H3b'	4.165	dd	2	$^{2}J = 6.0$	$^{3}J = 11.9$
H2'	5.29	multiplet	1		
H2	2.332	dt	6	$^{2}J = 2.3$	$^{3}J = 7.5$
H3	1.627	multiplet	6		
H8,11	2.032	multiplet	12		
H9,10	5.383	multiplet	6		
H12-17, H4-7	1.302	multiplet	60		
H18	0.897	t	9	$^{3}J = 6.6$	

Table 2.8: ^{13}C NMR data obtained for triolein at 100 MHz in $CDCl_3$ at room temperature.

Assignments	Position on glycerol backbone	δ_1 Chemical shift (ppm) Mannina *et al.*[1]	δ_2 Chemical shift (ppm) This work	$\Delta=\delta_1-\delta_2$ (ppm)
C1	sn 1', 3'	173.262	173.206	0.056
	sn 2'	172.850	172.798	0.052
C2	sn 1', 3'	34.063	34.041	0.022
	sn 2'	34.228	34.205	0.023
C3	sn 1', 3'	24.882	24.866	0.016
	sn 2'	24.922	24.906	0.016
C4	sn 1', 3'	29.129	29.112	0.017
	sn 2'	29.090	29.073	0.017
C5	sn 1', 3'	29.214	29.201	0.013
	sn 2'	29.236	29.222	0.014
C6	sn 1', 3'	29.151	29.134	0.017
	sn 2'	29.166	29.150	0.016
C7	sn 1', 3'	29.744	29.729	0.015
	sn 2'	29.758	29.743	0.015
C8	sn 1', 3'	27.257	27.243	0.014
	sn 2'	27.261	27.247	0.014
C9	sn 1', 3'	129.736	129.702	0.034
	sn 2'	129.709	129.676	0.033
C10	sn 1', 3'	130.042	130.001	0.041
	sn 2'	130.057	130.016	0.041
C11	sn 1', 3'	27.208	27.193	0.015
	sn 2'	27.208	27.193	0.015
C12	sn 1', 3'	29.804	29.793	0.011
	sn 2'	29.804	29.793	0.011
C13	sn 1', 3'	29.362	29.354	0.008
	sn 2'	29.367	29.354	0.013
C14	sn 1', 3'	29.561	29.555	0.006
	sn 2'	29.564	29.555	0.009
C15	sn 1', 3'	29.352	29.349	0.003
	sn 2'	29.352	29.349	0.003
C16		31.942	31.935	0.007
C17		22.709	22.708	0.001
C18		14.119	14.126	0.007
CHO		68.934	68.907	0.027
CH_2O		62.136	62.106	0.030
average				0.019

Trilinolein

Trilinolein in figure 2.17 is a TAG formed from the 18 carbon linoleic fatty acid containing two double bonds which are both in a *cis* orientation due to the coupling constants of 7.0 Hz that was extracted from the ^{1}H spectrum. The ^{1}H and ^{13}C NMR data is found in tables 2.9 and 2.10. The chemical shifts obtained for H11; H18; H8, 14; H15-17, H4-7 compare well with the values by Guillen *et al.*[3]

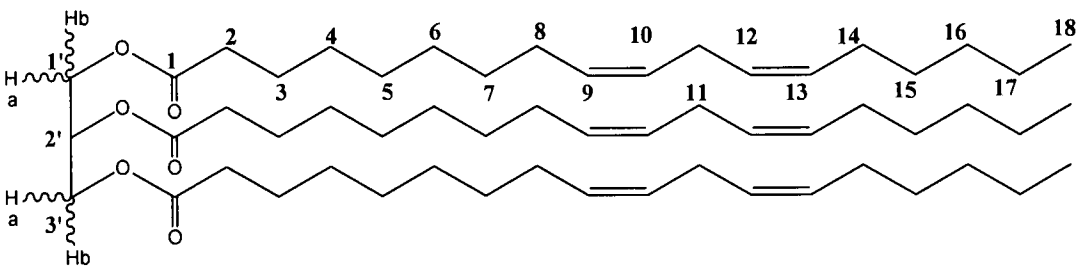


Figure 2.17: Numbered structure of trilinolein.

Table 2.9: ^{1}H NMR data obtained for trilinolein at 400 MHz in $CDCl_3$ at room temperature.

Assignment	Chemical shift (ppm)	Multiplicities	No. of H's	*J* (Hz)	
H1a'/H3a'	4.317	dd	2	$^{2}J = 4.3$	$^{3}J = 11.9$
H1b'/H3b'	4.166	dd	2	$^{2}J = 6.0$	$^{3}J = 11.9$
H2'	5.287	multiplet	1		
H2	2.334	dt	6	$^{2}J = 2.4$	$^{3}J = 7.4$
H3	1.629	multiplet	6		
H8,14	2.074	multiplet	12		
H9,10,12,13	5.417	multiplet	12		
H15-17, H4-7	1.357	multiplet	42		
H11	2.785	t	6	$^{3}J = 6.7$	
H18	0.907	t	9	$^{3}J = 6.9$	

Table 2.10: ^{13}C NMR data obtained for trilinolein at 100 MHz in $CDCl_3$ at room temperature.

Assignments	Position on glycerol backbone	δ_1 Chemical shift (ppm) Mannina *et al.*[1]	δ_2 Chemical shift (ppm) This work	$\Delta=\delta_1-\delta_2$ (ppm)
C1	sn 1', 3'	173.218	173.203	0.015
	sn 2'	172.811	172.795	0.016
C2	sn 1', 3'	34.039	34.031	0.008
	sn 2'	34.204	34.195	0.009
C3	sn 1', 3'	24.860	24.854	0.006
	sn 2'	24.898	24.892	0.006
C4	sn 1', 3'	29.105	29.100	0.005
	sn 2'	29.066	29.062	0.004
C5	sn 1', 3'	29.194	29.194	0.000
	sn 2'	29.215	29.214	0.001
C6	sn 1', 3'	29.136	29.133	0.003
	sn 2'	29.150	29.146	0.004
C7	sn 1', 3'	29.631	29.626	0.005
	sn 2'	29.645	29.640	0.005
C8	sn 1', 3'	27.225	27.219	0.006
	sn 2'	27.225	27.219	0.006
C9	sn 1', 3'	130.005	129.988	0.017
	sn 2'	129.987	129.963	0.024
C10	sn 1', 3'	128.096	128.074	0.022
	sn 2'	128.115	128.092	0.023
C11	sn 1', 3'	25.657	25.647	0.010
	sn 2'	25.657	25.647	0.010
C12	sn 1', 3'	127.929	127.907	0.022
	sn 2'	127.917	127.897	0.020
C13	sn 1', 3'	130.213	130.198	0.015
	sn 2'	130.222	130.198	0.024
C14	sn 1', 3'	27.213	27.208	0.005
	sn 2'	27.213	27.208	0.005
C15	sn 1', 3'	29.370	29.366	0.004
	sn 2'	29.370	29.366	0.004
C16		31.550	31.543	0.007
C17		22.593	22.593	0.000
C18		14.079	14.082	0.003
CHO		68.931	68.908	0.023
CH_2O		62.121	62.106	0.015
average				0.010

Tripalmitolein

Although no palmitoleic acid residues have ever been detected in olive oil, the presence of this compound was detected by GLC-MS in two of the oils that we studied, macadamia nut and avocado pear oil. Therefore we obtained the ^{1}H and ^{13}C NMR spectra of the standard tripalmitolein which has one double bond (refer to figure 2.18).

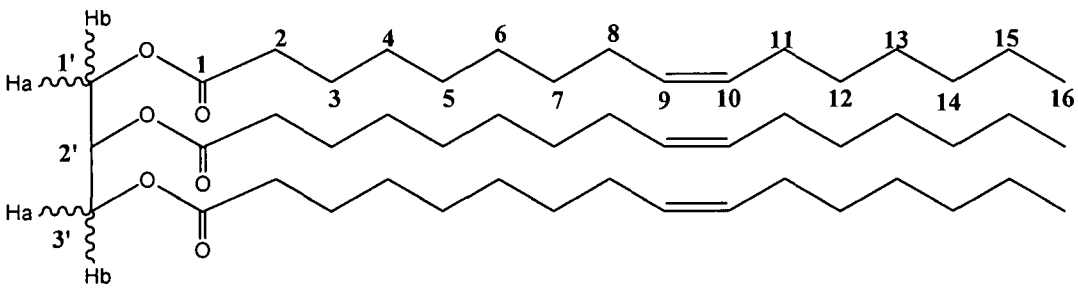


Figure 2.18: Numbered structure of tripalmitolein.

Since there is no literature assignment for the ^{13}C NMR spectra of tripalmitolein, the spectra of triolein and tripalmitin were used as references as aids in the assignment of this standard. This was decided since the oleic acid residue also contains one double bond while the palmitic acid residue contains the same amount of carbons, namely sixteen. The chemical shifts of the unsaturated carbons of tripalmitolein were assigned by comparison to those of triolein, while most of the chemical shifts of the saturated carbons were assigned by referring to tripalmitin. The 28-30 ppm region of the tripalmitolein spectrum however was a cause for concern. It was expected that ten resonances would be observed due to C4, 5, 6, 7, 12 and 13. Carbons 4, 5, 6 and 7 were expected to give two resonances each, one resonance representing the carbons in position sn 1',3' and one for carbons in position sn 2' of the glycerol backbone, while carbons 12 and 13 would only be represented by one resonance each. However thirteen resonances were observed in the 28-30 ppm region (refer to figure 2.19) and not the expected ten. Since no literature assignment for this compound could be obtained, we were unable to accurately assign this region by simple inspection.

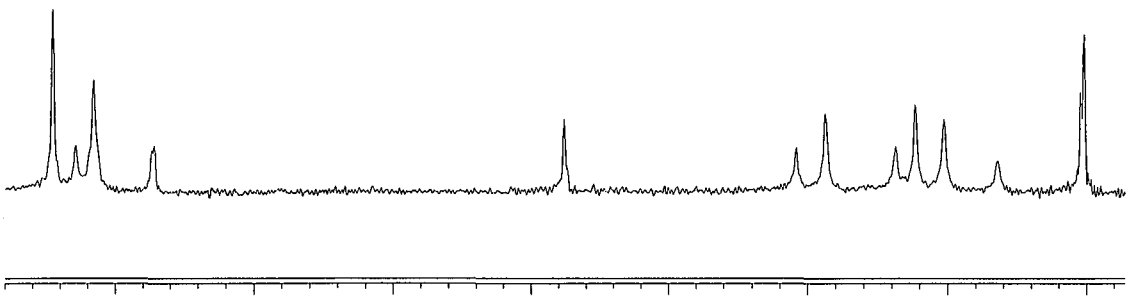


Figure 2.19: Expanded region, 28-30 ppm, of the ^{13}C NMR spectrum of tripalmitolein showing 13 peaks instead of the expected 10. At this stage it was not know which the "extra" peaks were.

The next step was to determine whether the sample of tripalmitolein obtained was possibly contaminated and therefore the purity of the standard TAG, which was labeled as ≥98% pure, was investigated. Although a 2% impurity is unlikely to show up in a ^{13}C NMR spectrum, if it was observed it would be an indication that the quantity of impurity was larger causing the presence of the two extra peaks in the ^{13}C NMR spectrum.

If the tripalmitolein sample was indeed contaminated, spiking of a vegetable oil containing palmitoleic acid residues, with tripalmitolein might lead to the identification of the peaks belonging to the standard and therefore also those peaks belonging to the contaminant. The ^{13}C NMR spectrum of a sample of 200 µl avocado pear oil in 700 µl $CDCl_3$ with TMS as reference was obtained. The same sample was then spiked with 20 µl of the standard tripalmitolein and the ^{13}C NMR spectrum obtained. The resonances belonging to the palmitoleic acid residue were expected to be enhanced in the spectrum after spiking which would be detected upon comparison between the spiked and unspiked spectra, either by inspection or by deconvolution (refer to figure 2.20).

It was however difficult to determine any increase in resonance height by inspection and therefore deconvolution was used. However the addition of 20µl was not sufficient to detect any enhancements by this technique either.

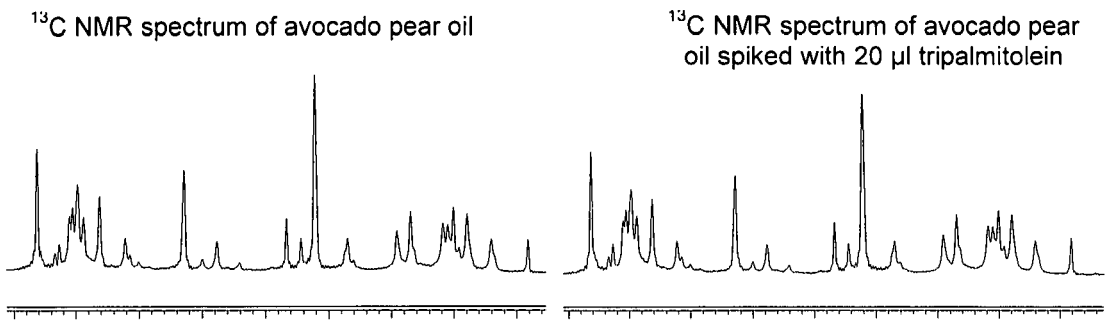


Figure 2.20: Expansion of 28-30 ppm region of avocado pear oil ^{13}C NMR spectrum on left, spiked with 20 µl tripalmitolein on right. As can be seen the spectra appear in no way different from one another and the "extra" peaks can still not be identified.

An additional 40µl was added, and once again a ^{13}C NMR spectrum was obtained under the same conditions as mentioned above (refer to figure 2.21). Unfortunately the detection of any increase in resonance heights by either inspection or deconvolution was still difficult, this being the case since we were working with such small quantities. Further spiking of the oil with tripalmitolein would however have caused the oil/solvent mixture to become too concentrated, which would hinder obtaining a well resolved ^{13}C NMR spectrum of well separated resonances. Therefore this attempt to determine the resonances belonging to the palmitoleic acid residue was unsuccessful and a different method for the solution of our problem was investigated.

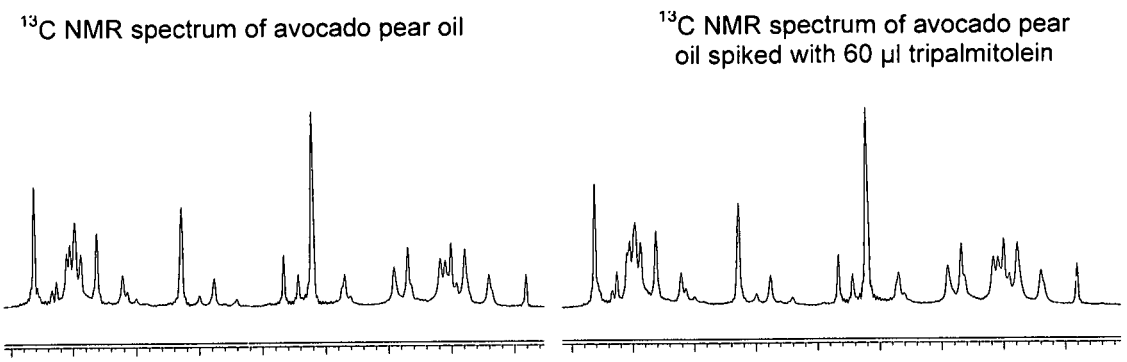
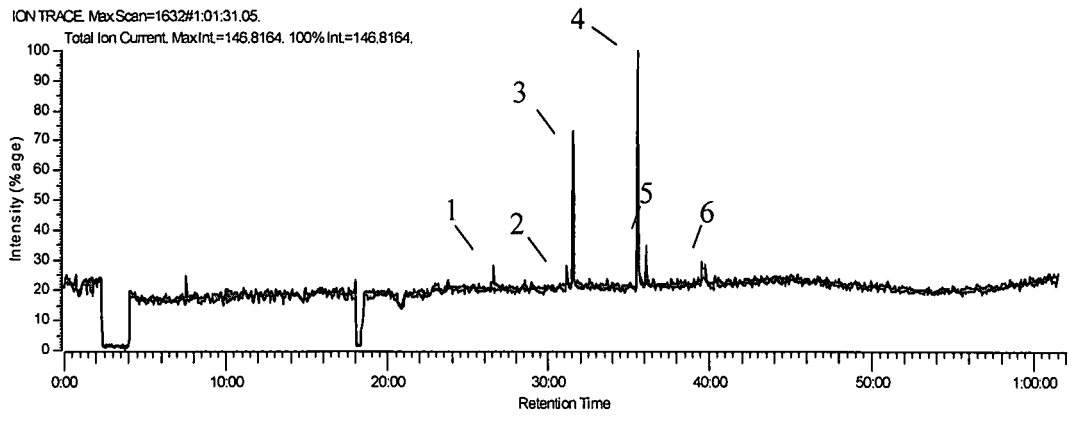


Figure 2.21: Expansion of 28-30 ppm region of avocado pear oil ^{13}C NMR spectrum on the left, with the spiking of 60 µl tripalmitolein on the right.

A sample of the TAG was sent for LC-MS and GLC-MS analysis in the hope that the impurity could be characterized. A small amount of tripalmitolein was dissolved in DMSO and further diluted in a 50 % acetonitrile/water solution for LC-MS analysis. The results however were inconclusive as no molecule other than tripalmitolein could be observed. For GLC-MS analysis, although an esterified sample of tripalmitolein in theory is required (refer to chapter 3), no esterification of the standard TAG was performed, due to the limited amount available and the hope that any impurities would be detected regardless of whether tripalimitolein was esterified or not. Therefore 1 µl of the sample was injected into a gas chromatograph inlet (no sample preparation was performed). GLC analysis indicated the presence of six compounds, refer to figure 2.22 where peak 1 represents background interference, peak 2 possibly an unsaturated aliphatic alcohol, peak 3 methyl tetradecanoate, peak 4 methyl palmitoleate, peak 5 methyl palmitate, peak 6 possibly an unsaturated hydrocarbon. However the major compounds were methyl palmitoleate (methyl ester of palmitoleic acid) and methyl tetradecanoate (methyl ester of myristic acid). These are indicative of the fatty acid residues in tripalmitolein.



Fi

gure 2.22: GLC chromatogram of an esterified tripalmitolein sample.

Since palmitoleic acid residues would be expected to be present in tripalmitolein, our focus turned to the presence of myristic acid and this prompted us to investigate the possibility of this compound as a contaminant in the tripalmitolein sample. Therefore ^{13}C NMR analysis was done on tripalmitolein by spiking the sample with trimyristin. Two experiments were run, firstly a sample of pure tripalmitolein and secondly the standard spiked with trimyristin. A ^{13}C NMR spectrum of 200 µl of tripalmitolein in 700 µl of $CDCl_3$ with TMS as reference was collected. The same sample was then taken and spiked with 50 µl of trimyristin and a second ^{13}C NMR spectrum was obtained. The main interest was specifically in the 28-30 ppm region to see if the spiking of tripalmitolein with trimyristin would cause the presence of the the extra peaks in figure 2.19. Although this experiment turned out to solve the problem of contamination (see below), it was with unexpected results. Upon obtaining the spectrum of tripalmitolein even before spiking with trimyristin, the ^{13}C NMR spectrum differed from the previous original spectrum obtained for this TAG as fewer peaks were observed in the 28-30 ppm region than before.

This led to the discovery that the solvent, deuterated chloroform, was in fact contaminated leading to the detection of two extra peaks in the ^{13}C NMR spectrum of tripalmitolein. However, no further investigation was done into the source of this contamination since ten other researchers, working on platinum

group metals, shared that same solvent and an endless number of compounds could have been involved. This process was however useful in showing that the most obvious potential source of contamination should perhaps be investigated first and not last.

For the purpose of accuracy, the experiment was repeated using a different $CDCl_3$ sample and before use, the $CDCl_3$ solvent was run through basic alumina to remove the presence of any trace HCl possibly formed. ^{1}H and ^{13}C NMR spectra were obtained for tripalmitolein and figure 2.23 indicates the absence of those two peaks at 29.672 ppm and 29.376 ppm in the ^{13}C NMR spectrum. However looking closer at the spectrum in figure 2.23 eleven ^{13}C resonances were observed instead of the expected ten. This eleventh resonance was due to the splitting of C13 at 29.98 ppm for the carbons in positions sn 2' and sn 1',3' on the glycerol backbone.

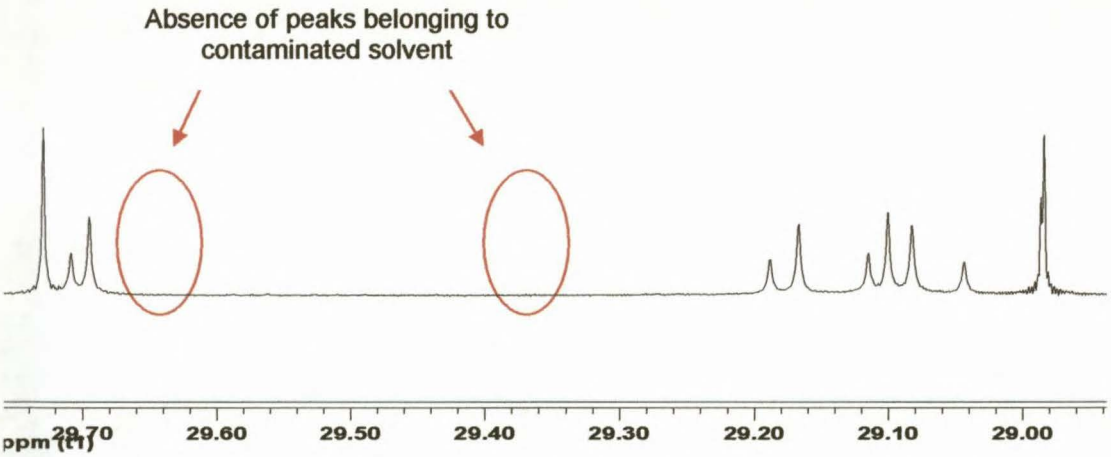


Figure 2.23: Expanded region (28-30 ppm) of the ^{13}C NMR spectrum of tripalmitolein with uncontaminated solvent at 100 MHz in $CDCl_3$ at room temperature. Refer back to figure 2.18 for the contaminated spectrum.

The ^{1}H and ^{13}C NMR spectra obtained using the uncontaminated solvent, were analyzed and the compound was found to be in the *cis* orientation. This was confirmed by the coupling constant of ~7 Hz found from the ^{1}H NMR spectrum. Tables 2.11 and 2.12 give the results obtained. Since the ^{13}C NMR spectrum of tripalmitolein had not yet been assigned, there was no literature to compare

chemical shift values with. Assignments of peaks were carried out using the assignments of triolein and tripalmitin as mentioned earlier.

Table 2.11: ^{1}H NMR data obtained for tripalmitolein at 400 MHz in $CDCl_3$ at room temperature.

Assignment	Chemical shift (ppm)	Multiplicities	No. of H's	*J* (Hz)	
H1'/H3'	4.313	dd	2	^2J = 4.3	^3J = 11.9
H1'/H3'	4.165	dd	2	^2J = 6.0	^3J = 11.9
H2'	5.263	multiplet	1		
H2	2.315	dt	6	^2J = 2.4	^3J = 7.4
H3	1.608	multiplet	6		
H8,11	2.018	multiplet	12		
H9,10	5.345	multiplet	6		
H12-15, H4-7	1.301	multiplet	48		
H16	0.881	multiplet	9		

Table 2.12: ^{13}C NMR data obtained for tripalmitolein at 100 MHz in $CDCl_3$ at room temperature.

Assignments	Position on glycerol backbone	Chemical shift (ppm)
C1	sn 1', 3'	173.205
	sn 2'	172.795
C2	sn 1', 3'	34.021
	sn 2'	34.185
C3	sn 1', 3'	24.879
C4	sn 1', 3'	29.115
	sn 2'	29.044
C5	sn 1', 3'	29.167
	sn 2'	29.188
C6	sn 1', 3'	29.082
	sn 2'	29.100
C7	sn 1', 3'	29.695
	sn 2'	29.709
C8	sn 1', 3'	27.220
	sn 2'	27.224
C9	sn 1', 3'	129.691
	sn 2'	129.665
C10	sn 1', 3'	129.984
	sn 2'	129.995
C11	sn 1', 3'	27.162
	sn 2'	27.162
C12	sn 1', 3'	29.729
	sn 2'	29.729
C13	sn 1', 3'	28.984
	sn 2'	28.986
C14	sn 1', 3'	31.783
C15	sn 1', 3'	22.655
C16	sn 1', 3'	14.094
CHO		68.877
CH_2O		62.086

2.3 ^{13}C NMR ASSIGNMENT BY STANDARD-ADDITIONS METHOD

Once the major components of the vegetable oils were known by GLC and GLC-MS analysis (refer to table 3.1 in chapter 3), we attempted to assign the ^{13}C NMR spectra of the six vegetable oils using the literature method for the assignment of ^{13}C NMR spectra of olive oil, which we refer to as the standard-additions method. In order to assign the ^{13}C NMR spectrum of a vegetable oil, standard TAGs (triolein, tristearin, etc.) are used to spike the vegetable oil sample. For instance, a ^{13}C spectrum of the oil is first obtained and then a small amount of the standard triolein is added to the vegetable oil and a second ^{13}C NMR spectrum is obtained. The two spectra, one belonging to the vegetable oil and one to the vegetable oil spiked with triolein, are then compared to determine which peaks were enhanced, which will give an indication of which peaks belong to the olein residues in the vegetable oil. This enhancement of peaks is determined by deconvolution.[1,7,8,9] This exercise is repeated for all the standard TAGs and consequently all the peaks belonging to fatty acid residues are assigned for the ^{13}C NMR spectrum of a vegetable oil.

An attempt was made to assign the ^{13}C NMR spectra of the six vegetable oils starting with marula oil by the standard additions of pure TAGs. All of the samples used contained 100 µl of marula oil, 700 µl of $CDCl_3$ and TMS as reference. Added to each sample, with the exception of the first, was 20 µl of a standard TAG. Therefore five ^{13}C NMR spectra were obtained, one of the pure marula oil, and four of marula oil with an added TAG, these being tripalmitin, tristearin, triolein or trilinolein. Parameters used were the same as described in section 2.2.1. Since tristearin and tripalmitin were in solid form, the mass representing 20 µl of solution had to be determined first. Using the densities of tripalmitin (0.8752 g/cm^3) and tristearin (0.08559 g/cm^3) the required masses were determined to be 0.0175 g and 0.0171 g respectively.

It was found that the measuring of oils by volume introduced significant inaccuracy into measurements and therefore batches of oil/$CDCl_3$ solutions were prepared for NMR samples with TMS as reference. Approximate concentrations of the batches were 0.125 g/ml.

Since the standard-additions method essentially required the measurement of the relative intensities of peaks, it was important to take certain parameters into account in order to compare the different spectra of the oil spiked with each standard. Therefore before we could attempt this standard addition method, the T_1 of the carbons for all six the oils of interest had to be determined. Relaxation processes are important since these are the methods by which the excited nuclei return to their ground states. The two relaxation processes are called spin-lattice relaxation and spin-spin relaxation. Spin-lattice relaxation processes represent those that occur in the direction of the field and the spin-lattice relaxation time, T_1, indicates the rate of this process. The second process called spin-spin relaxation occurs in the plane that is perpendicular to the direction of the field with the spin-spin relaxation time, T_2, being the associated measure of the process.[5] Determination of these T_1 values for the vegetable oils were to make sure that the total time of acquisition and relaxation delay was adequate for the complete relaxation of all carbon nuclei in the compound. Therefore the carbon nucleus that takes the longest time to relax back to its rested state (the carbon with the largest T_1 value) is used for further calculations. NMR experiments were run on the oil samples to determine their pw90 and subsequently their T_1 values. Table 2.13 indicates the largest T_1 value for each vegetable oil performed on the 400 MHz NMR spectrometer as well as the calculated time needed for that specific carbon nucleus to relax back to its rested state using a pulse of 30°. The following Ernst Equation was used for the calculations in table 2.13 and is here applied to avocado pear oil for illustration:

$$\cos\varphi = e^{-\Delta/T_1}$$

$$\cos 30 = e^{-\Delta/5.249}$$

$$\ln \cos 30 = -\Delta / 5.249$$

$$\Delta = 0.7550\,s$$

Where Δ represents the time that is needed for the carbon nucleus in its excited state to return to its ground state.

0.755 seconds is smaller than the total repetition time for the NMR experiment which was 11.5 s. This repetition time is made up out of 4.5 s for the acquisition time (at) and 7 s for the relaxation delay (d_1). Therefore the total time that was used was adequate for the relaxation of all the carbon nuclei in avocado pear oil. This same situation was found for all the vegetable oils.

Table 2.13: Determination of the time needed for full ^{13}C nuclei relaxation at a 30° pulse for vegetable oils.

	Longest T1	Calculated time needed for carbon relaxation (s)		at + d_1	Acquisition time (at in s)	Relaxation delay (d_1 in s)
Apricot kernel	4.6	0.69	≤	11.5	4.5	7
Avocado pear	5.6	0.76	≤			
Grapeseed	5.8	0.83	≤			
Macadamia nut	4.5	0.65	≤			
Mango kernel	5.0	0.72	≤			
Marula	5.3	0.76	≤			

Upon attempting this standard-additions method for the assignment of macadamia nut oil and other oils, it was found to have some disadvantages, particularly being time-consuming, since a number of separate ^{13}C NMR spectra needed to be obtained after each addition of the standard TAGs present in the vegetable oil. However the major problem experienced with this technique was

with the complete assignment of the spectra. When attempting this method for the six vegetable oils, it was found that although most resonances in the spectrum could be assigned, it could not be applied to certain areas in the spectrum, specifically for the aliphatic carbons which showed ^{13}C peaks in the 28 –30 ppm region, where significant spectral overlap occurs (figure 2.24). As mentioned, spiking of the oil with a standard leads to the increase of specific resonances, and due to the complicated spectral overlap in this region, detection of an increase in an individual resonance was impossible. This was most likely the reason why this area of the ^{13}C spectrum of olive oil has not been fully assigned in the literature. Another disadvantage of the spiking method was that the chemical shift values for the carbon atoms in the TAGs are concentration dependant i.e. the chemical shift values change with a change in concentration. Mannina *et al.*[2] investigated this phenomenon by examining the ^{13}C NMR spectra of TAGs with varying concentration in $CDCl_3$. Plotting the chemical shift values of selected individual carbons in each TAG on the y-axis against the concentration (volume) of the TAG used on the x-axis, yielded linear graphs with similar trends. Their results showed that the chemical shift of any ^{13}C in a TAG is dependant on the concentration of that TAG.

When attempting the standard-additions method of ^{13}C NMR resonance assignment for the six oils of interest, the concentration dependence of the chemical shifts mentioned above presents a significant problem for the unambiguous assignment of the ^{13}C peaks in the spectrum of vegetable oils since the addition of the standards altered the concentration of fatty acids giving ambiguous results in solution.

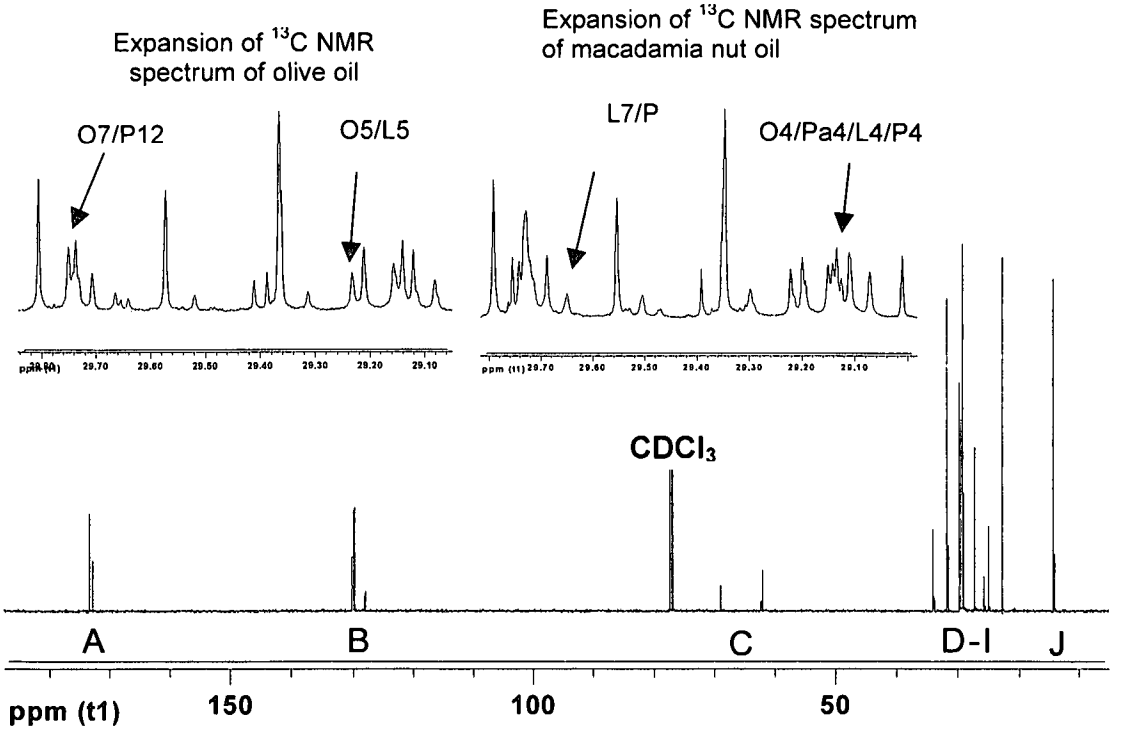


Figure 2.24: ^{13}C NMR spectrum of olive oil in $CDCl_3$ with an expansion of the crowded 28-30 ppm region of olive oil (on the left) inset and an expansion of macadamia nut oil (on right), at 100 MHz in $CDCl_3$ at room temperature.

2.4 DEVELOPEMENT OF THE "LINEAR GRAPH METHOD" FOR ^{13}C NMR ASSIGNMENTS

In view of the slight concentration dependence of ^{13}C NMR signals mentioned above, attention was focused on developing a faster, accurate method for the unambiguous assignment of the ^{13}C NMR spectra of vegetable oils, particularly of natural origin. In this context a new "'linear graph method"' was developed for the fast and facile assignment of these ^{13}C NMR spectra. This method consists of correlating the chemical shift values of the fatty acid ^{13}C resonances of the standard TAGs and those present in the vegetable oil, on the premise that whatever caused the small concentration dependence of ^{13}C NMR shifts, should be similar for a single molecule in a mixture. Thus in vegetable oils consisting of mixtures of which only one standard component, for which all ^{13}C NMR resonances are known, is present, it is reasonable to expect that the ^{13}C NMR signals in the vegetable oil should all be equally affected, by whatever causes the

concentration dependence in the first place. To test this hypothesis, the various ^{13}C resonances of a "standard" known oil component were calculated with those in a vegetable oil, for which concentration dependence is to be expected. Firstly the spectrum of the vegetable oil was divided into sections each representing different carbons of the same type present in the TAGs (figure 2.25). Section A contained the carbonyl carbon atoms, section B the olefinic carbon atoms, section C the glycerol carbon atoms and sections D-J the aliphatic carbon atoms.

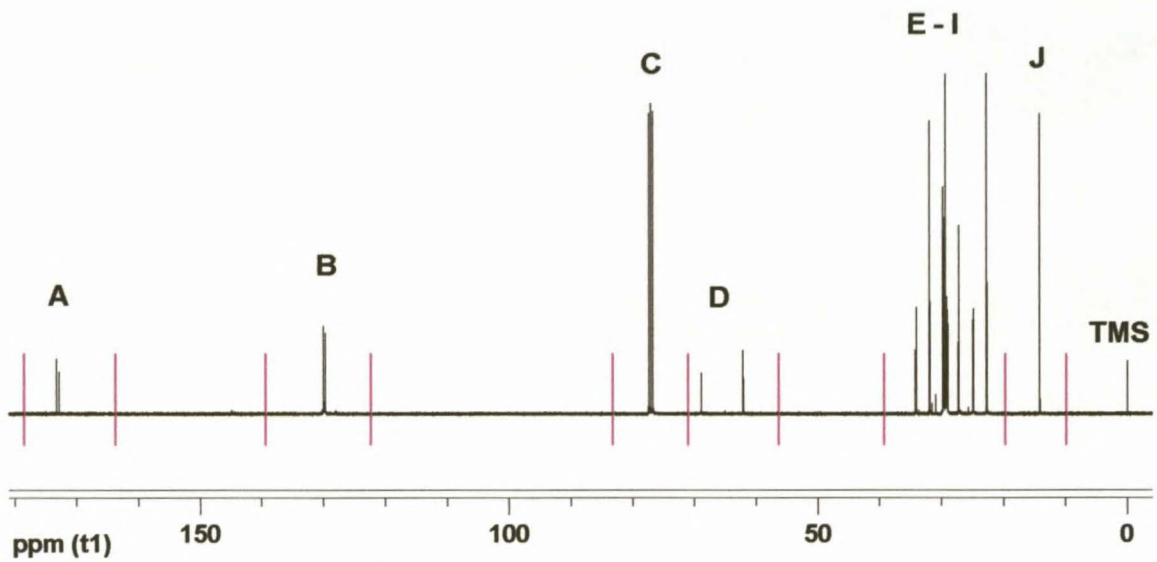


Figure 2.25: ^{13}C NMR spectrum of macadamia nut oil divided into sections A-J.

Certain of these regions were easily assigned by inspection using the relative percentages of each fatty acid residue present in the oil, determined by GC analysis (refer to chapter 3) and compared to the approximate resonance intensities in the NMR spectrum. In addition it was known from the work of Vlahov *et al.*[10] that a carbon from a saturated fatty acid chain is generally observed further downfield than the equivalent carbon in an unsaturated fatty acid chain. This assisted further in assigning additional resonances by inspection. If triolein was the main component in a certain oil, it was expected to give the most intense resonances, assuming that nOe enhancement was the same for all resonances of a particular type of carbon. These concepts were applied in order

to assign Sections A, C, E, I and J. Refer to table 2.14 for the assignment of these sections using macadamia nut oil as an example.

Table 2.14: ^{13}C NMR assignment of Sections A, C, E, I and J for macadamia nut and the corresponding standard TAGs.

Section	Carbon	Position	Macadamia nut oil ^{13}C chemical shift (ppm)	Standard triacylglycerol ^{13}C chemical shift (ppm)
A	P1	sn 1', 3'	173.256	173.256
	Pa1	sn 1', 3'	173.243	173.205
	O1			173.206
	L1	sn 1', 3'	173.226	173.203
	O1			172.798
	Pa1			172.795
	L1	sn 2'	172.818	172.795
C	CHO (L)			68.908
	CHO (O)			68.907
	CHO (P)			68.902
	CHO (Pa)		68.907	68.887
	CH_2O (Pa)			62.108
	CH_2O (O)			62.106
	CH_2O (L)		62.11	62.106
E	P14		31.955	31.964
	O16		31.933	31.935
	Pa14		31.811	31.783
	L16		31.55	31.543
I	P17		22.717	22.723
	O17		22.707	22.708
	Pa15		22.681	22.655
	L17		22.598	22.593
J	P16		14.131	14.131
	O18		14.125	14.126
	Pa16		14.117	14.094
	L18		14.085	14.082

Where O=olein; P=palmitin; L=linolein; V/E=vaccenin or eicosenoin.

A graph using all resonances shown in table 2.14 for each fatty acid residue was drawn up, such that on the x-axis the chemical shift values obtained for the known ^{13}C NMR shifts of the standard TAG were plotted, while the corresponding chemical shift values of the TAG in the vegetable oil were plotted on the y-axis. Remarkably a straight line was obtained from this data. Notice this was only done for the sections A, C, E, I and J of the vegetable oil that could easily and unambiguously be assigned by inspection. Figure 2.26 shows the case for the assignment of the linoleic fatty acid residues in macadamia nut oil.

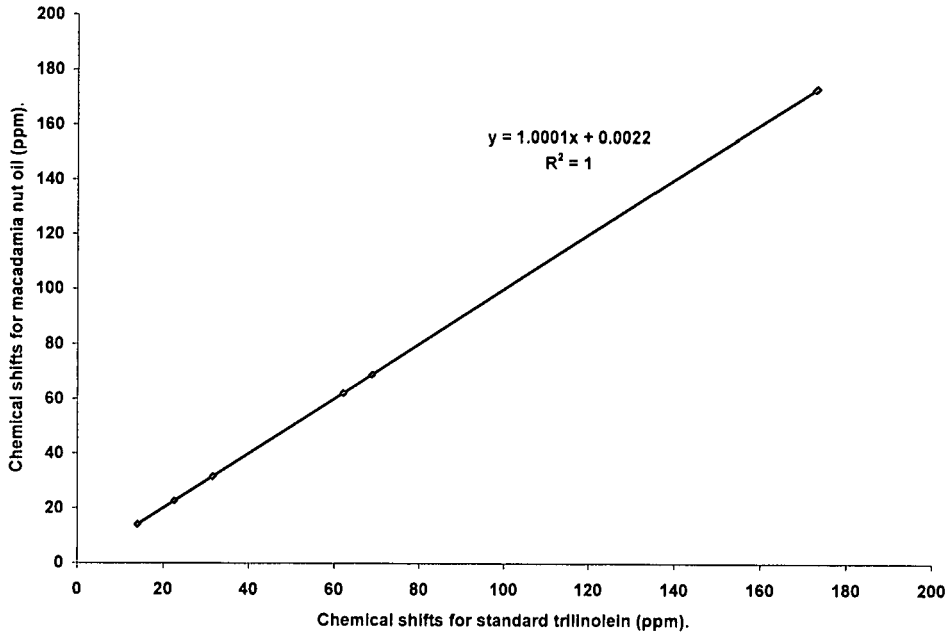


Figure 2.26: Linear graph obtained from plotting the chemical shifts of the resonances belonging to standard trilinolein against the linoleic fatty acid residues in macadamia nut oil for sections A, C, E, I and J.

The linear equation obtained from the graph was used to predict the shifts of the ^{13}C NMR peaks of the TAG in the mixture of the vegetable oil. By substituting the chemical shift values for the standard TAG ^{13}C NMR shifts into the equation (x-value), the resulting y-values calculated were the expected values for the corresponding carbon chemical shifts in the macadamia nut oil. Usually the ^{13}C chemical shifts are quoted to two decimal places, but as this was insufficient to

assign two adjacent peaks in the largely crowded areas of the ^{13}C NMR spectrum of a vegetable oil, the chemical shifts are therefore quoted to the third decimal place. As an example, C17 of the linoleic fatty acid residue for the standard TAG had a ^{13}C chemical shift value of 22.593 ppm. Substituting this value into x into the linear expression, the predicted ^{13}C peak in the oils is expected to be:

$$y = 1.0001x + 0.0022$$
$$y = 1.0001(22.593 \text{ ppm}) + 0.0022 = 22.597 \text{ ppm}$$

This value of 22.597 ppm is the expected chemical shift for the C17 resonance in the macadamia nut oil spectrum. This value closely matched an observed value of 22.598 ppm.

Use of this linear plot led to the assignment of the most of the remaining ^{13}C resonances of this component in the vegetable oil. Using the known value (obtained from the literature or from a sample of a pure TAG) for the ^{13}C chemical shift of a particular carbon atom of the standard TAG as the x-coordinate on the straight line the corresponding chemical shift value on the y-axis could be predicted even in strongly overlapped regions of the ^{13}C NMR spectrum (figure 2.27). For instance, for C4 of the oleic acid residue, which lies in the crowded region 28-30 ppm, the standard TAG value of 29.100 ppm was substituted into the linear equation, and the corresponding y-value was determined giving the expected experimental value for the chemical shift in the vegetable oil. Comparison to the actual spectrum then led to the assignment of a resonance at 29.111 ppm to C4 in the macadamia nut oil ^{13}C spectrum.

$$y = 1.0001x + 0.0022$$
$$y = 1.0001(29.100 \text{ ppm}) + 0.0022 = 29.105 \text{ ppm}$$

Although one could say that this expected y value of 29.105 ppm closely correlated to the observed value of 29.111 ppm, this discrepency is considered

too big since it gave a problem for this largely crowded region (28-30 ppm) of the spectrum, such as shown in figure 2.24. Due to the fact that the resonances are so close together an error in even the second decimal place can cause an incorrect assignment of resonances. Such discrepancies were also found for other regions. As can be seen in table 2.15 some of these values (marked in bold) we considered as too large for the accurate assignment of a resonance.

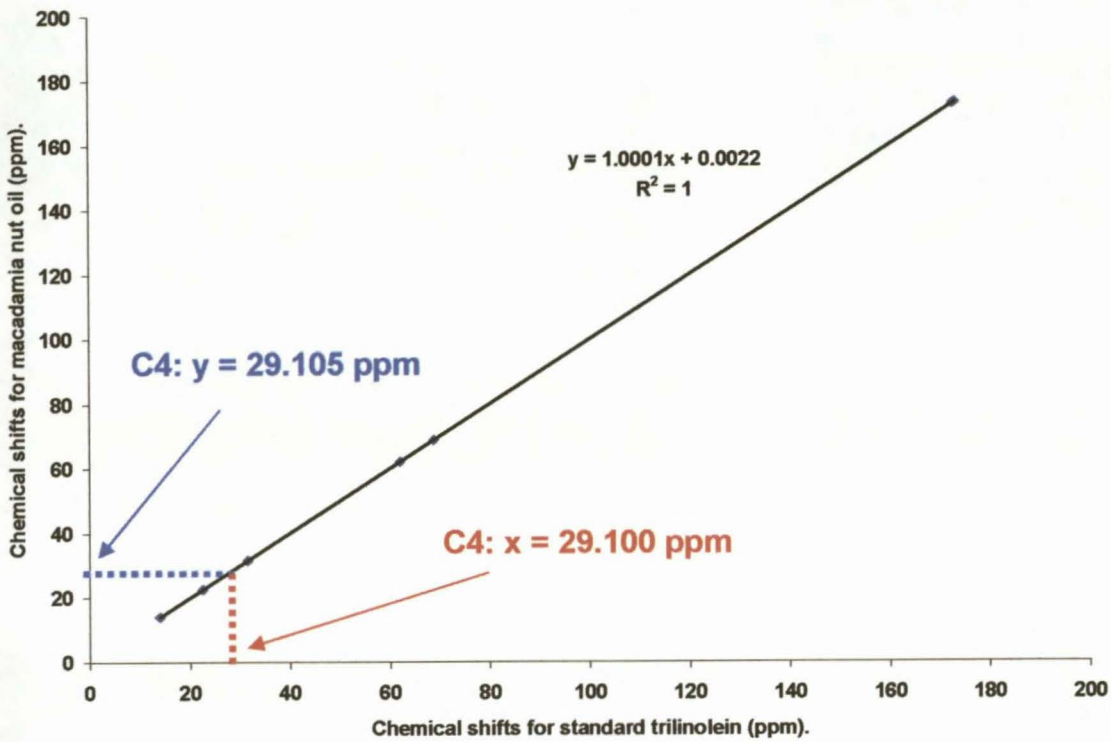


Figure 2.27: Linear graph obtained from plotting the chemical shifts of the carbon resonances belonging to standard trilinolein against those in macadamia nut oil.

Table 2.15: Difference between the calculated and observed chemical shift values of the linoleic acid carbons in macadamia nut oil.

Assign-ment	Position on glycerol backbone	Chemical shifts of standard (ppm)	δ_1 Chemical shift (ppm) expected y = 1.0001x + 0.0022	δ_2 Chemical shift (ppm) observed	$\Delta=\delta_1-\delta_2$ (ppm)
C1	sn 1', 3'	173.203	173.223	173.226	0.003
C1	sn 2'	172.795	172.814	172.818	0.004
C13		130.198	130.213	130.201	**0.012**
C9	sn 1', 3'	129.988	130.003	129.923	**0.080**
C9	sn 2'	129.963	129.978	129.824	**0.154**
C10	sn 2'	128.092	128.107	128.093	**0.014**
C10	sn 1', 3'	128.074	128.089	128.074	**0.015**
C12	sn 1', 3'	127.907	127.922	127.909	**0.013**
C12	sn 2'	127.897	127.912	127.896	**0.016**
CHO		68.908	68.917	68.907	**0.010**
CH_2O		62.106	62.114	62.110	0.004
C2	sn 2'	34.195	34.201	34.208	**0.007**
C2	sn 1', 3'	34.031	34.037	34.043	0.006
C16	sn 2'	31.543	31.548	31.550	0.002
C7	sn 2'	29.640	29.645	29.650	0.005
C7	sn 1', 3'	29.626	29.631		**0.019**
C15	sn 1', 3'	29.366	29.371	29.372	0.001
C5	sn 2'	29.214	29.219	29.220	0.001
C10	sn 1', 3'	29.200	29.205	29.200	0.005
C6	sn 2'	29.151	29.156	29.151	0.005
C6	sn 1', 3'	29.133	29.138	29.142	0.004
C4	sn 1', 3'	29.100	29.105	29.111	**0.006**
C4	sn 2'	29.062	29.067	29.072	**0.005**
C8		27.219	27.224	27.223	0.001
C13	sn 1', 3'	27.208	27.213	27.212	0.001
C11		25.647	25.652	25.651	0.001
C3	sn 2'	24.892	24.897	24.905	**0.008**
C3	sn 1', 3'	24.854	24.859	24.865	**0.006**
C17		22.593	22.597	22.598	0.001
C18		14.082	14.086	14.085	0.001

Although we assigned most of the resonances in the ^{13}C NMR spectrum of macadamia nut oil (as shown in table 2.14) by using the method above, it was clear that this method was not precise enough.

However it was still believed that the "linear graph method" would prove to be useful and therefore it was decided to use the method in a different way. It is likely that carbon type (i.e sp, sp^2 and sp^3) might play a role due to the fact that the carbonyl and olefinic carbons showed a greater difference (table 2.15) between the observed and expected chemical shift values than for the aliphatic carbons. Therefore a plot of the sp^2 hybridized carbons (the olefinic and carbonyl carbons) separately from the sp^3 hybridized carbons (aliphatic and glycerol backbone carbons) might be required, which was consequently confirmed by a great improvement in the method.
In order to test the hypothesis that the type of ^{13}C atom and thus its ^{13}C NMR chemical shift can influence the correlation between known ^{13}C and expected ^{13}C assignments of the same component in a standard compared to a vegetable oil, two separate graphs were constructed, one for sp^3 type carbon atoms (aliphatic and glycerol backbone carbon atoms) and one for sp^2 type carbon atoms (olefinic and carbonyl carbon atoms).

<u>The sp^3 hybridized carbon</u>:
Once the assignment of sections C, E, I and J were completed, the ^{13}C chemical shift values of the sp^3 carbon atoms belonging to the standard TAG resonances were graphed on the x-axis against the corresponding assigned values of the oil on the y-axis, to obtain a straight line (figure 2.28). This was done separately for each different standard TAG found to be present in the oil. Figure 2.28 illustrates the linear line obtained from plotting the chemical shifts for the sp^3 carbons atoms of the linoleic acid residue of the standard against those of macadamia nut oil (refer to table 2.14).

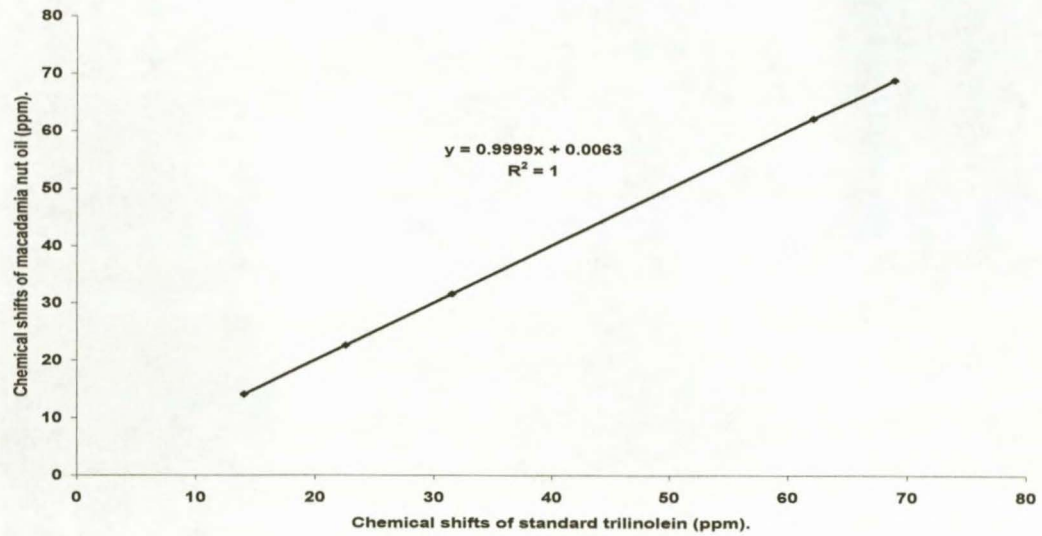


Figure 2.28: Linear graph obtained from plotting the chemical shifts of the saturated carbon resonances belonging to standard trilinolein against those assigned by inspection in macadamia nut oil.

Using this linear correlation for sp^3 carbon atoms an equation (figure 2.28) y = 0.9999x + 0.0063, was obtained. This was done section by section (sections D-J) and once a section had been assigned, the values of that section were then re-entered into the original graph to obtain a new linear correlation. The assigned chemical shifts were then confirmed by substituting the standard TAG values into the linear equation calculated from the graph. In this way sections were added until a linear graph was obtained containing all of the chemical shifts in the ^{13}C NMR spectrum. Figure 2.29 illustrates the final linear correlation obtained between ^{13}C chemical shift values for the sp^3 carbon atoms of the fully assigned ^{13}C NMR spectrum of trilinolein against macadamia nut oil (refer to table 2.16). Table 2.17 indicates the difference between the chemical shift values of the expected and observed assignments for the linoleic carbon atoms. Notice that the differences between the values are very small and therefore within achievable experimental error, so that ^{13}C NMR assignment of the signals in a spectrum can be achieved, especially in regions of significant ^{13}C NMR resonance overlap.

In this way all the relevant ^{13}C resonances of a specific TAG were assigned to the appropriate carbons present in the fatty acid residues, particularly in the crowded spectral region. This method found most application in sections D, G and H where significant spectral overlap occurred.

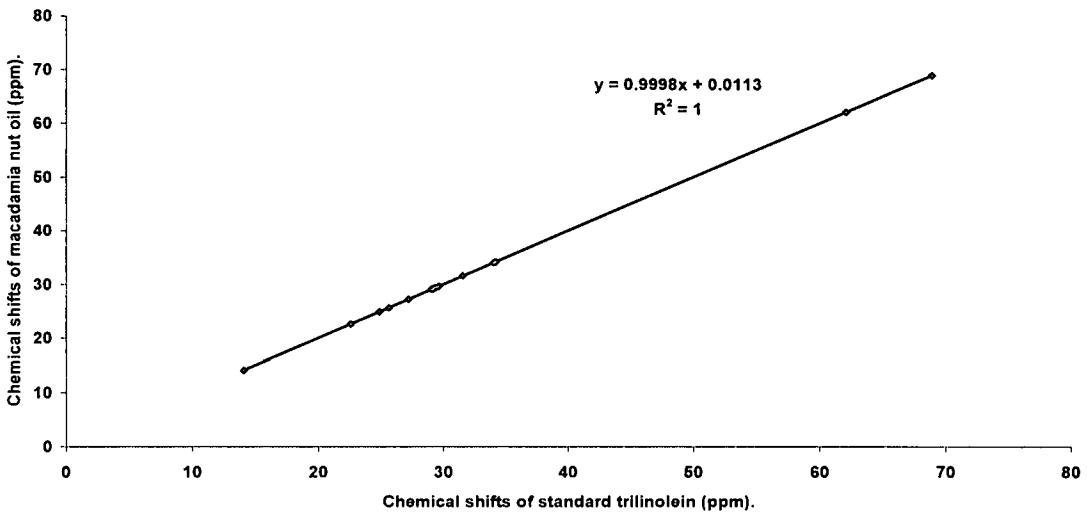


Figure 2.29: Final linear graph obtained from plotting the chemical shifts of the saturated carbon resonances belonging to standard trilinolein against those in macadamia nut oil.

Table 2.16: Assignment of ^{13}C NMR resonances of the major fatty acid residues of macadamia nut oil.

Assignment	Position on glycerol backbone	Chemical shifts (ppm)	Assignment	Position on glycerol backbone	Chemical shifts (ppm)
P1	sn 1', 3'	173.256	L7/P7		29.650
Pa1	sn 1', 3'	173.243	O14		29.554
O1/L1	sn 1', 3'	173.226	P5	sn 1', 3'	29.505
O1/Pa1/L1	sn 2'	172.818	P13		29.392
L13		130.201	L15		29.372
O10/Pa10	sn 2'	130.017	O13		29.353
O10/Pa10/L9	sn 1', 3'	130.003	O15		29.347
L9	sn 2'	129.923	P6	sn 1', 3'	29.298
V/E		129.825	O5/L5	sn 2'	29.222
V/E		129.822	Pa5	sn 2'	29.216
O9/Pa9	sn 1', 3'	129.705	O5/L5	sn 1', 3'	29.200
O9/Pa9	sn 2'	129.679	Pa5	sn 1', 3'	29.194
L10	sn 2'	128.093	O6/L6	sn 2'	29.151
L10	sn 1', 3'	128.074	Pa6/L6/P4	sn 2'/sn1,3	29.142
L12	sn 1', 3'	127.909	O6	sn 1', 3'	29.135
L12	sn 2'	127.896	Pa6	sn 1', 3'	29.126
CHO		68.907	O4/Pa4/L4/P4	sn 1', 3'	29.111
CH2O		62.110	O4/Pa4/L4	sn 2'	29.072
O2/Pa2/L2	sn 2'	34.208	Pa13		29.011
P2	sn 1', 3'	34.065	O8/Pa8	sn 2'	27.246
Pa2	sn 1', 3'	34.056	O8	sn 1', 3'	27.243
O2/L2	sn 1', 3'	34.043	L8		27.223
P14		31.955	L14		27.212
O16		31.933	O11		27.193
Pa14		31.811	Pa11		27.187
L16		31.550	L11		25.651
unassigned		30.897	O3/Pa3/L3	sn 2'	24.905
O12		29.792	P3		24.888
Pa12	sn 2'	29.763	O3/Pa3/L3/P3	sn 1', 3'	24.865
Pa12	sn 1', 3'	29.755	P17		22.717
O8/Pa7	sn 2'	29.743	O17		22.707
P10	sn1,3	29.731	Pa15		22.681
O8/P12	sn 1', 3'	29.729	L17		22.598
Pa7/P11	sn 1', 3'	29.721	P16		14.131
P9		29.714	O18		14.125
P8		29.689	Pa16		14.117
			L18		14.085

Table 2.17: Difference between the calculated and observed chemical shift values of the linoleic acid and glycerol backbone carbons in macadamia nut oil.

Assignment	Position on glycerol backbone	Chemical shifts of standard (ppm)	δ_1 Chemical shift (ppm) expected y = 0.9998x + 0.0113	δ_2 Chemical shift (ppm) observed	$\Delta=\delta_1-\delta_2$ (ppm)
CHO		68.908	68.906	68.907	0.001
CH_2O		62.106	62.105	62.110	0.005
C2	sn 2'	34.195	34.199	34.208	0.009
C2	sn 1', 3'	34.031	34.035	34.043	0.008
C16	sn 2'	31.543	31.548	31.550	0.002
C7	sn 2'	29.640	29.645	29.650	0.005
C7	sn 1', 3'	29.626	29.631		
C15	sn 1', 3'	29.366	29.371	29.372	0.001
C5	sn 2'	29.214	29.219	29.220	0.001
C10	sn 1', 3'	29.200	29.205	29.200	0.005
C6	sn 2'	29.151	29.156	29.151	0.005
C6	sn 1', 3'	29.133	29.138	29.142	0.004
C4	sn 1', 3'	29.100	29.105	29.111	0.006
C4	sn 2'	29.062	29.067	29.072	0.005
C8		27.219	27.225	27.223	0.002
C13	sn 1', 3'	27.208	27.214	27.212	0.002
C11		25.647	25.653	25.651	0.002
C3	sn 2'	24.892	24.898	24.905	0.007
C3	sn 1', 3'	24.854	24.860	24.865	0.005
C17		22.593	22.600	22.598	0.002
C18		14.082	14.090	14.085	0.005

The sp^2 hybridized carbon:

The resonances of sp^2 type ^{13}C atoms in sections A and B of the spectrum are well separated and easy to identify and the "linear graph method" was not necessary for the assignment of these chemical shifts. Nevertheless to lend support to this "linear graph method" as an assignment aid, the chemical shift values determined by inspection were still plotted and a linear graph was once again obtained. This confirmed the utility of this "linear graph method" for sp^2

hybridized carbons if simple inspection should proove to be inadequate to assign ^{13}C resonances. A similar procedure as discussed for the sp^3 carbon atoms and their resonances was applied. Figure 2.30 illustrates the final linear graph obtained for the ^{13}C chemical shift values for the sp^2 carbon atoms for triolein and that in the fully assigned ^{13}C NMR spectrum of this component in macadamia nut oil (table 2.16). Table 2.18 indicates the difference between the chemical shift values of the expected and observed assignments.

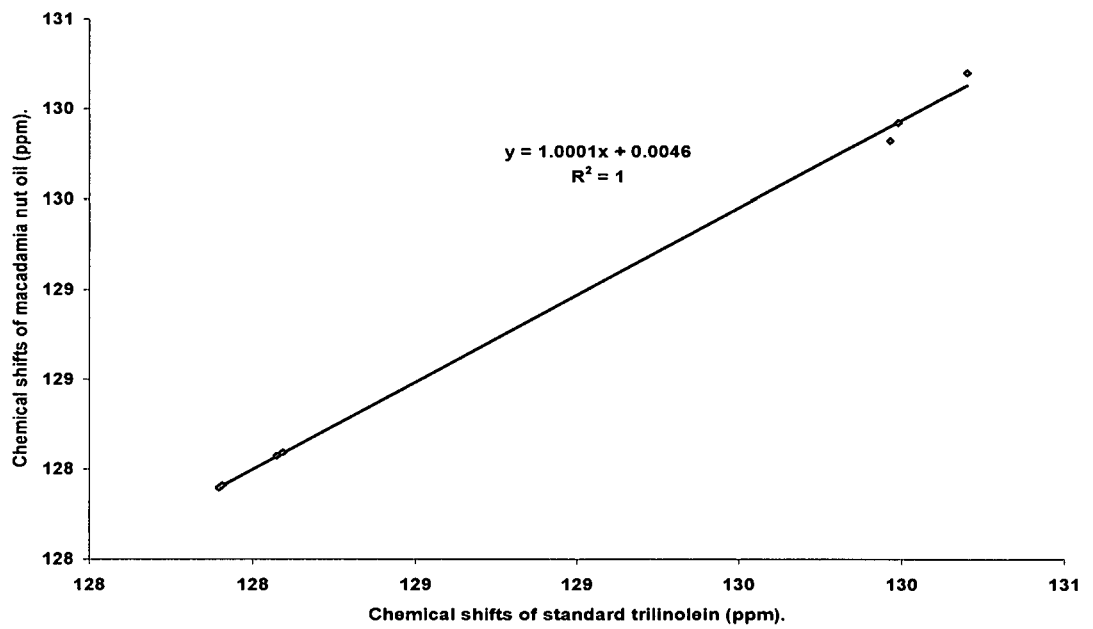


Figure 2.30: Final linear graph obtained from plotting the chemical shifts of the olefinic and carbonyl carbon resonances belonging to standard trilinolein against those in macadamia nut oil.

Table 2.18: Difference between the calculated and observed chemical shift values of the linoleic acid sp^2 carbon atoms in macadamia nut oil.

Assign-ment	Position on glycerol backbone	Chemical shifts for the standard (ppm)	δ_1 Chemical shift (ppm) expected y = 1.0001x + 0.0046	δ_2 Chemical shift (ppm) observed	$\Delta=\delta_1-\delta_2$ (ppm)
C1	sn 1', 3'	173.203	173.225	173.226	0.001
C1	sn 2'	172.795	172.817	172.818	0.001
C13		130.198	130.216	130.201	0.015
C9	sn 1', 3'	129.988	130.006	129.923	0.083
C9	sn 2'	129.963	129.981	129.824	0.157
C10	sn 2'	128.092	128.109	128.093	0.016
C10	sn 1', 3'	128.074	128.091	128.074	0.017
C12	sn 1', 3'	127.907	127.924	127.909	0.015
C12	sn 2'	127.897	127.914	127.896	0.018

There is excellent agreement between some of the observed and expected chemical shift values for the sp^2 carbon atoms. However the differences for others, e.g. C9, are large. As discussed later the sp^2 carbon ^{13}C chemical shifts do appear to be much more concentration dependent than those of the sp^3 carbon atoms. This implies that the sp^2 carbon atoms are more likely to be involved in some kind of inter-molecular interactions which we do not fully understand but in all likelihood make prediction of the chemical shifts of these carbon atoms more complicated than for the sp^3 carbon atoms. However it is still the case that in this region the difference between the observed and expected chemical shifts is not detrimental and still allows for reliable assignment of the peaks.

We concluded that by plotting the carbon atoms, according to their hybridization, on separate graphs, the "linear graph method" became more reliable. Using this method we were able to assign fully the ^{13}C NMR spectra of macadamia nut oil (refer to table 2.16).

2.5 VALIDATION OF THE "LINEAR GRAPH METHOD" WITH OLIVE OIL

Although we were able to assign all the ^{13}C resonances belonging to the TAGs in the NMR spectrum of a vegetable oil with this newly developed method, validation of the method was desirable to allow this procedure to be generally accepted. The method described was tested by applying it to the ^{13}C NMR spectrum of an olive oil, as olive oils have been best studied, and fully assigned in the literature on a number of occasions.[10,11,12,13] The assignment obtained for the ^{13}C NMR spectrum of an extra virgin olive oil using the "linear graph method" can be found in table 2.19. Once again the spectrum was divided into sections but this time comparisons of our assignments were made to those in the literature. In this way validation of the reliability of our method for the assignment of the ^{13}C NMR spectrum of a well-studied olive oil, was investigated.

The relative chemical shift values for Sections A, C, E, I and J corresponded well with those in the literature[10,11,12,13] and the order of carbons present in the spectrum for section B also corresponded well.[10,11,13] C2 of all fatty acid residues present in section D corresponded reasonably well with the literature,[11,13] with the exception of the resonances representing the carbon atoms of triolein and trilinolein in the α positions of the glycerol backbone. In our ^{13}C spectrum these were clearly separated and therefore could be separately assigned, while Shaw *et al.* observed only one resonance representing both these carbons. Although most of the assignment of section G correlated well with assignments in the literature,[10,13] there were a few differences. Two resonances were seen for C8 of the oleic fatty acid residue in our oil, indicating a separate assignment for the α and β positions where Shaw *et al.* only reported a single resonance. The same situation occurred in section H where separate resonances for the carbons of oleic and linoleic fatty acid residues were assigned. Upon first glance, section F was more difficult to assign, but by using the "linear graph method", it became fairly straight forward. Correlation with assignments given in two papers,[10,13]

confirmed that most of the carbons correlated well with the literature, but some exceptions were noted. Assignments of certain carbons were not given in the literature including carbons P4, 06, L6, O7, P10, P12, P11, and P9 but could be assigned by our method. Moreover in our ^{13}C spectra, separate resonances representing the α and β carbons were also found for O14 (29.560 ppm and 29.558 ppm), which could be due to higher resolution as we used a higher field instrument (9.4 T, 400 MHz) while a 7.05 T (300 MHz) spectrometer was used in the literature. There are however no published reports on the unambiguous assignment of these ^{13}C NMR resonance carbon atoms in a ^{13}C NMR spectrum of a vegetable oil. One of the signals at 29.653 or 29.650 ppm could however also be due to any other compound known to be present in olive oil that does not fall under the category of TAGs, namely sterols, aldehydes, polyphenols, etc. The oils were not tested for the presence of eicosenoic and vaccenic fatty acid residues, since they cannot be distinguished from each other by NMR spectroscopy in the olefinic region.[12] Some of the unassigned resonances could also be due to the vaccenic or eicosenoic (refer to figure 2.30) fatty acid residues or possibly some other saturated residues not tested which will fall mostly underneath the palmitic fatty acid resonances. However since palmitin is the major saturated component present in olive oil, the assignment of the saturated resonances was carried out using the tripalmitin chemical shifts.

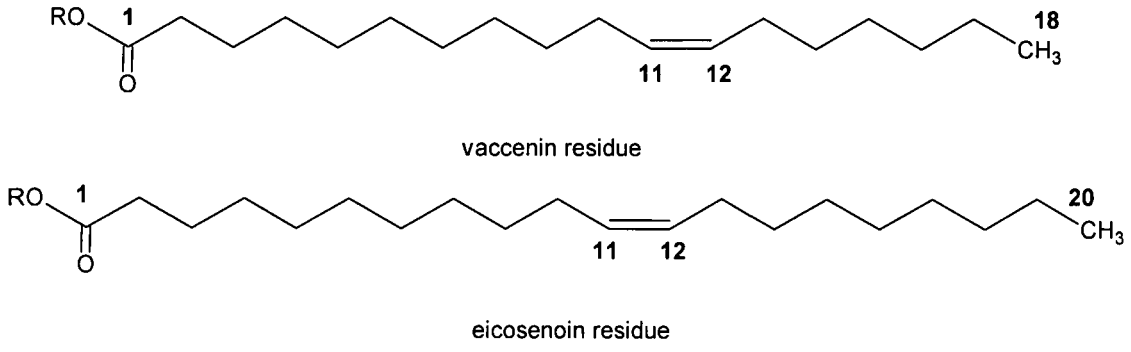


Figure 2.30: Structures of vaccenic and eicosenoic fatty acid residues with R representing where the fatty acid residues are attached to the glycerol backbone.

Table 2.19: Assignment of ^{13}C NMR resonances of the major components of fatty acids in olive oil.

Section	Assignment	Position on glycerol backbone	Chemical shift (ppm) measured	Chemical shift (ppm) Literature assignments [11,12,13]		
				Vlahov *et al.* [11]	Sacchi *et al.* [12]	Shaw *et al.* [13]
A	P1	sn 1',3'	173.236		173.27	173.113
	V/E		173.223			173.101
	O1	sn 1',3'	173.204		173.2	173.084
	L1	sn 1',3'	173.194		173.17	173.075
	O1	sn 2'	172.837		172.83	172.688
	L1	sn 2'	172.787		172.77	172.679
B	L13	sn 2'	130.198	130.15	130.22	130.105
	L13	sn 1',3'	130.191	130.15	130.22	130.097
	O10	sn 2'	130.016	129.96	130.04	129.945
	O10	sn 1',3'	130.001	129.94	130.02	129.93
	L9	sn 1',3'	129.984	129.91	129.98	129.9
	L9	sn 2'	129.958	129.89	130.01	129.874
	V/E		129.918			
	V/E		129.822			
	O9	sn 1',3'	129.702	129.65	129.69	129.64
	O9	sn 2'	129.676	129.63	129.72	129.614
	L10	sn 2'	128.095	128.07	128.12	128.053
	L10	sn 1',3'	128.077	128.05	128.11	128.035
	L12	sn 1',3'	127.911	127.89	127.94	127.874
	L12	sn 2'	127.899	127.88	127.93	127.862
C	CHO		68.911	68.90		68.8852
	CH2O		62.108	62.06		62.0478
D	O2/L2	sn 2'	34.206	34.16		34.1408
	P2	sn 1',3'	34.064			33.9972
	unassigned		34.054			
	O2/L2	sn 1',3'	34.041	33.990		33.9758
E	P14		31.959	31.940	34.26	31.9283
	O16		31.938	31.920	34.2	31.9074
	unassigned		31.818			
	L16		31.555		34.15	31.5167
F	O12		29.796	29.770		29.7586
	O7	sn 2'	29.745			
	P10		29.735	29.720		29.6637
	O7/P12	sn 1',3'	29.731			

	P11		29.718	29.68		
	P8		29.693	29.64		
	P7	sn 1',3'	29.653			29.6637
	L7	sn 2'	29.650			29.6203
	L7	sn 1',3'	29.635			
	O14	sn 2'	29.560	29.54		29.5289
	O14	sn 1',3'	29.558	29.54		29.5289
	P5		29.508	29.49		29.4748
	P13		29.397	29.38		29.3688
	L15		29.377			29.3419
	O13		29.356	29.34		29.3156
	O15		29.353	29.34		29.3156
	P6		29.302	29.28		29.2686
	O5/L5	sn 2'	29.225	29.20		29.1869
	O5/L5	sn 1',3'	29.203	29.18		29.1656
	O6/L6	sn 2'	29.153			
	P4		29.145			
	O6/L6	sn 1',3'	29.136			
	O4	sn 1',3'	29.114	29.05		29.0717
	L4	sn 1',3'	29.108	29.05		
	O4/L4	sn 2'	29.075			29.0324
G	O8	sn 2'	27.249	27.16	27.31	27.1477
	O8	sn 1',3'	27.245	27.16	27.31	27.1477
	L8		27.226		27.22	27.1657
	L14		27.214			27.1815
	O11		27.194	27.22	27.31	27.2009
H	L11		25.653		25.67	25.6084
	O3	sn 2'	24.908			24.8605
	L3	sn 2'	24.898			24.8605
	P3		24.890	24.86	24.84	24.8466
	O3	sn 1',3'	24.869	24.84	24.8	24.8239
	L3	sn 1',3'	24.861		24.7	24.8239
I	P15		22.720		22.82	22.6841
	O17		22.711		22.8	22.6743
	L17		22.602		22.61	22.5656
J	P16		14.133		14.13	14.0824
	O18		14.127		14.13	14.0769
	unassigned		14.119			
	L18		14.081		14.09	14.037

In conclusion, using the "linear graph method" to assign the ^{13}C NMR spectrum of olive oil and by comparing our results to the literature (table 2.19) we proved the precision and usefulness of this newly developed method. Not only could this "linear graph method" be used for the assignment of the ^{13}C NMR spectrum of olive oil but the method also proved to be extremely useful for distinguishing and assigning ^{13}C peaks in the largely crowded regions where the standard-additions method failed. However before we could attempt the assignment of the ^{13}C NMR spectra of the other six vegetable oils, one more question remained as to the application of this "linear graph method" and that was whether the method only worked for a certain concentration of oil or whether any reasonable concentration was possible.

2.6 EFFECT OF OIL CONCENTRATION ON THE "LINEAR GRAPH METHOD" FOR ^{13}C ASSIGNMENT

We have shown that the "linear graph method" allowed the complete assignment of two different vegetable oils, but further investigation was carried out to test whether the method is independent of the concentration of the vegetable oil that is used to collect the ^{13}C NMR spectrum. To achieve this, a series of ^{13}C NMR spectra were collected using different concentrations of olive oil of the same origin in $CDCl_3$ at room temperature. The spectra were run for a shorter period of time, roughly 30 minutes, than was the case for the full assignment of olive and macadamia nut oils, since a relative trend was the main focus in this case and not the assignment of every minor peak. Besides this, the experimental parameters described earlier in section 2.2.1 were used as well as the same brand of olive oil for these experiments, for accurate comparison purposes. The error for each point plotted was determined to be 0.001 g due to the accuracy of the balance used to measure the mass of the olive oil samples. The linoleic fatty acid residues in each of these NMR spectra were assigned using the above described "linear graph method". The gradient and intercept of each linear equation of each graph obtained were plotted against the sample concentrations

of olive oil used, yielding two separate graphs for each of the sp^2 and sp^3 carbon atom types (figures 2.31-2.34). Refer to table 2.20 for the data used to plot the graphs.

Table 2.20: Gradient and intercept values obtained using the "linear graph method" for the linolein fatty acid residue in olive oil at varying concentrations of the oil.

				sp^2 carbons of the linolein residues		**sp^3 carbons of the linolein residues**	
Sample number	Mass of oil (g)	Total mass of sample (g)	Mass of oil per 1g of sample (g/g)	Gradient	Intercept	Gradient	Intercept
1	0.1065	1.1300	0.0942	1.0005	-0.0676	0.9999	0.0027
2	0.1300	1.1519	0.1129	0.9998	0.0279	1.0000	0.0087
3	0.1614	1.1442	0.1411	0.9995	0.0651	1.0000	0.0123
4	0.1770	1.1170	0.1585	0.9992	0.0904	1.0000	0.0142
5	0.2340	1.1463	0.2041	0.9985	0.1862	1.0000	0.0249

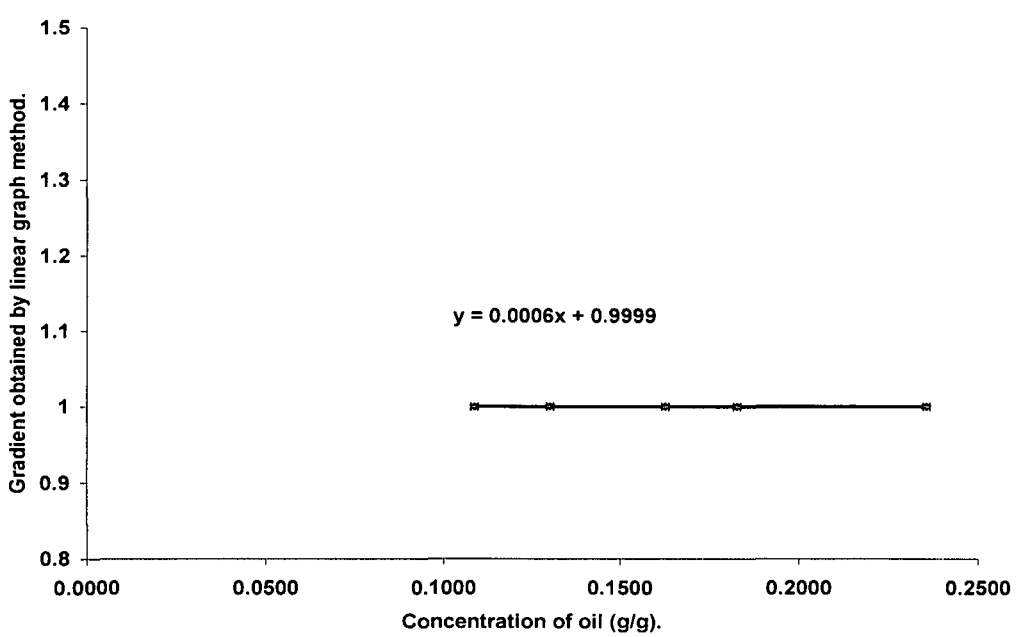


Figure 2.31: The gradient values for samples of varying concentration obtained from the "linear graph method" for the sp^3 carbons of the linoleic fatty acid residue in olive oil plotted against the concentration of the samples.

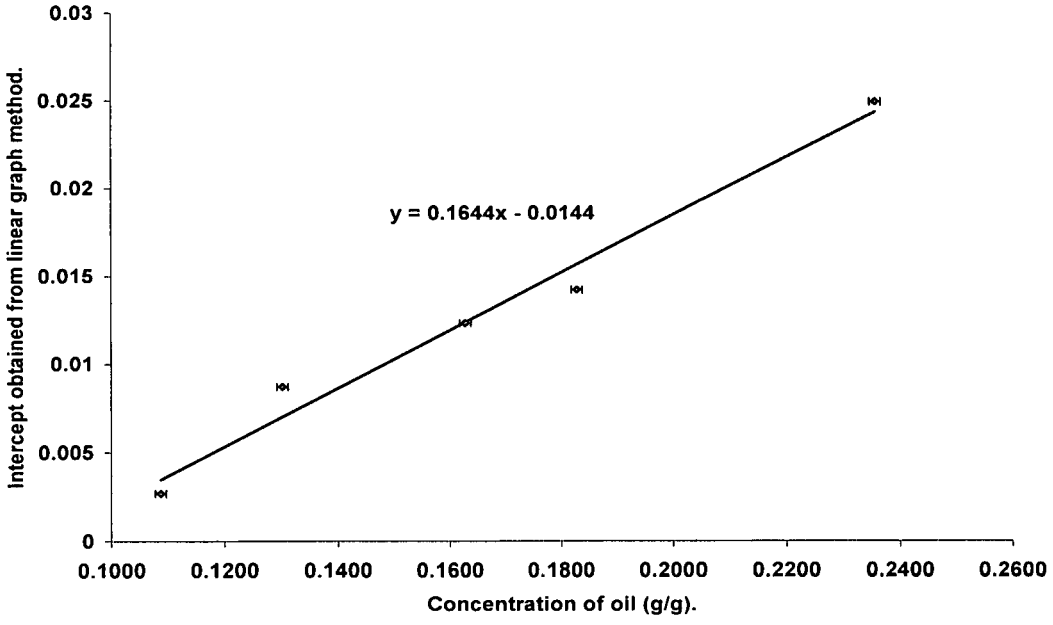


Figure 2.32: The intercept values for samples of varying concentration obtained from the "linear graph method" for the sp^3 carbons of the linoleic fatty acid residue in olive oil plotted against the concentration of the samples.

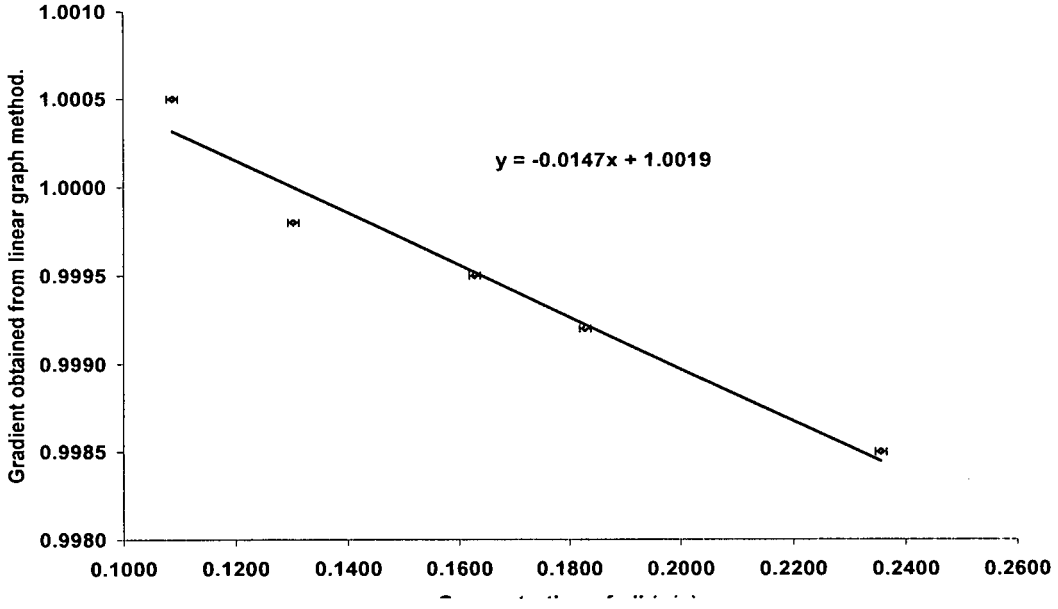


Figure 2.33: The gradient values for samples of varying concentration obtained from the "linear graph method" for the sp^2 carbons of the linoleic fatty acid residue in olive oil plotted against the concentration of the samples.

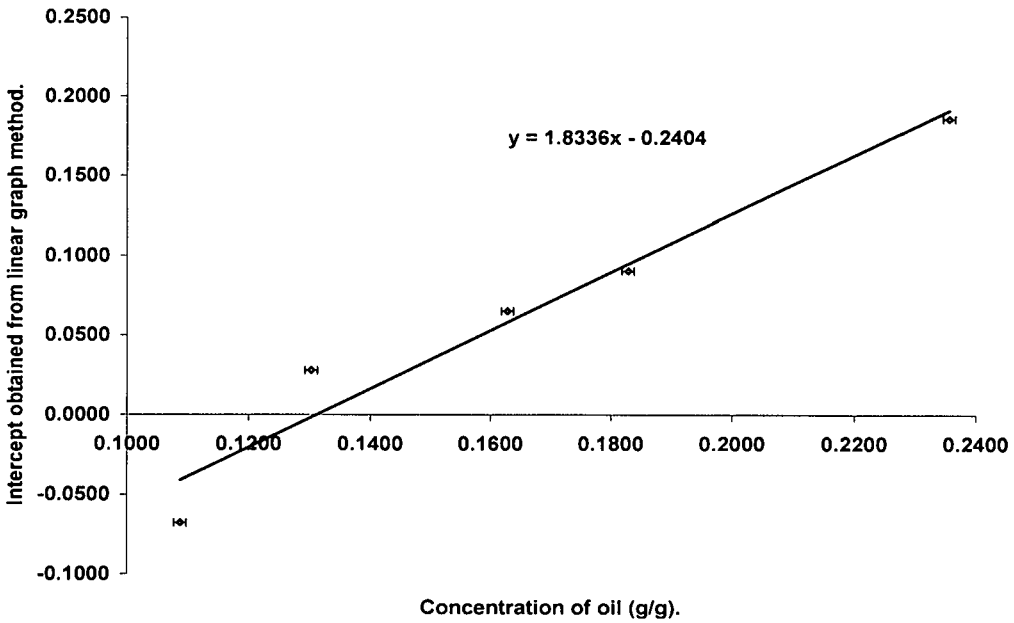


Figure 2.34: The intercept values for samples of varying concentration obtained from the "linear graph method" for the sp^2 carbons of the linoleic fatty acid residue in olive oil plotted against the concentration of the samples.

sp^3 carbon atoms:

Graph 2.31 indicates that a change in the concentration of the oil does not in any way affect the gradient of the graph obtained from the "linear graph method" for the sp^3 carbons. However graph 2.32 indicates that a change in the concentration of the oil does affect the intercept of the graph obtained from the "linear graph method" for the sp^3 carbons. Thus as the change in gradient is zero, but the change in intercept non-zero it indicates that although altering the concentration of the oil causes a change in the ^{13}C chemical shifts of the sp^3 carbon atoms, the chemical shifts of these atoms are **all affected to exactly the same degree, i.e. in a linear manner,** by a change in concentration. As the "linear graph method" compensates precisely for this effect it results in it being independent of the concentration of the oil for the assignment of the ^{13}C chemical shifts of the sp^3 carbon atoms of the fatty acid residues present in the oil.

sp^2 carbon atoms:

Graphs 2.33 and 2.34 indicate that a change in the concentration of the oil affects both the gradient and intercept of the graph obtained from the "linear graph method" for the sp^2 carbon atoms. Therefore a change in the concentration of the oil causes a change in the chemical shifts of the sp^2 carbon atoms, but unlike for the sp^3 chemical shifts, the chemical shifts of the sp^2 carbon atoms are not all affected to the same degree, causing the "linear graph method" not to be independent of the concentration of the oils used when assigning the ^{13}C chemical shifts of the sp^2 carbon. Although this seems to disprove the applicability of the "linear graph method" to the sp^2 carbon atoms, a closer look at the graphs solves this problem. The change in gradient and intercept may be non-zero but these values are in fact so close to zero that the method is concentration independent over the range of concentrations used. If either a much higher or lower concentrations of oil is used, this may no longer be the case. However for NMR spectroscopy one is limited in the range of concentrations that can be used: Too high a concentration will result in peak broadening and insufficient resolution for assignment of the ^{13}C chemical shifts of the fatty acids present in the oils and concentrations which are too low will cause the ^{13}C to be too time consuming to collect or else impossible to obtain as NMR spectroscopy is fundamentally not a very sensitive technique. Thus although the "linear graph method" is not strictly speaking concentration independent for the assignment of the ^{13}C chemical shifts for the sp^2 carbon atoms, over the concentration range that can be used for NMR spectroscopy the method is in fact independent of the concentration of the oil used.

Figure 29.35 illustrates further the degree of concentration dependence of each different type of carbon atom. The change in chemical shift (as observed in five ^{13}C NMR spectra at different concentrations) of each type of carbon atom is plotted on the y-axis against the mass of the oil used on the x-axis, which represents the different concentrations of each sample. Table 2.21 shows these results for sp^2 olefinic and carbonyl carbon atoms and a sp^3 aliphatic carbon

atom. The change in chemical shift values were determined relative to the most upfield ^{13}C chemical shift.

Table 2.21: Chemical shift values determined for the different types of carbon atoms at different concentrations.

Mass of oil per 1 g of sample	sp^2 Olefinic carbon L9 sn 1',3'	Change in chemical shift (ppm)	sp^2 Carbonyl carbon L1 sn 1',3'	Change in chemical shift (ppm)	sp^3 Aliphatic carbon L5 sn 2'	Change in chemical shift (ppm)
0.0942	129.983	0.027	173.243	0.116	29.217	-0.030
0.1129	129.985	0.029	173.194	0.067	29.223	-0.024
0.1411	129.972	0.016	173.174	0.047	29.230	-0.017
0.1585	129.967	0.011	173.162	0.035	29.233	-0.014
0.2041	129.956	0	173.127	0	29.247	0

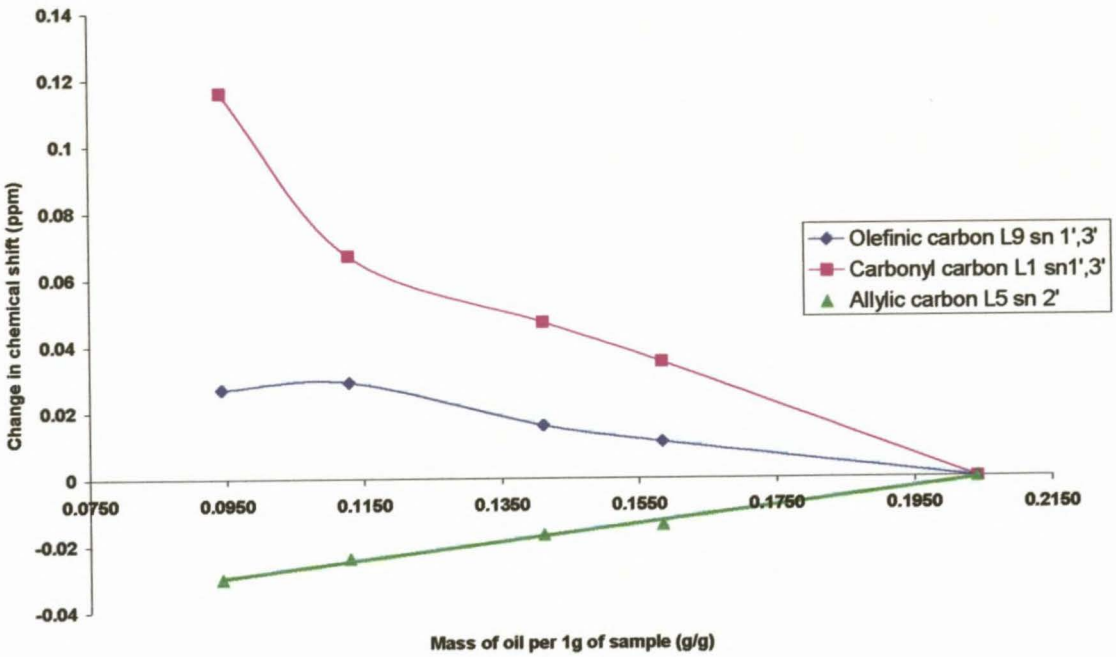


Figure 29.35: Change in chemical shift for different types of carbon atoms plotted again the mass of oil per 1g of sample.

Figure 29.35 illustrates clearly that there is a linear change in the chemical shift of the aliphatic sp^3 carbon atom showing that the sp^3 carbon atoms are affected in a linear manner by a change of concentration. So not only are all sp^3 hybridized carbon atoms affected to an equal degree by a change in

concentration as shown by figures 2.31 and 2.32, but this change is also linear for each individual carbon atom. Both types of sp^2 carbon atoms shown (carbonyl and olefinic) are not affected in a linear manner. However the "linear graph method" would be independent of concentration for these oils if the carbon atoms were still affected to the same degree, even if not in a linear way. However as the carbonyl carbon atoms are clearly more affected by a change in concentration than the olefinic carbon atoms it becomes clear that the "linear graph" method cannot be concentration independent for these atoms. Therefore these two types of carbon atoms should strictly speaking be treated separately and plotted on different graphs when using the "linear graph method". However due to the fact that there are only two chemical shift values for each carbonyl carbon atom (one for the sn 1',3' position and one for the sn 2' position of C1) of each fatty acid residue, it is therefore not possible to draw up a graph for these two points alone. However as mentioned previously, due to the fact that the sp^2 carbon atoms can be assigned by inspection, this does not pose a problem. For a more complicated mixture of fatty acids it may however be an issue. A further observation that can be made regarding figure 29.35 is that with an increase in concentration the sp^2 carbons shift upfield while the sp^3 carbons shift downfield. This confirms that the sp^2 and sp^3 carbon atoms can in no way be plotted on the same graph when applying the "linear graph method".

To conclude, the above graphs indicate that the "linear graph method" can be used for the assignment of the ^{13}C chemical shifts of the sp^3 carbon atoms of fatty acid residues in vegetable oils. Though strictly speaking the method is not concentration independent for the sp^2 carbon atoms when all treated together, over the range of concentrations suitable for NMR spectroscopy it is still applicable. Plotting of the gradients and intercepts against concentration further emphasized that the sp^3 and sp^2 hybridized carbon atoms had to be treated independently since the chemical shifts of the saturated sp^3 and unsaturated sp^2 carbon atoms are clearly affected in different ways and to different degrees by a change in concentration.

The above proof that our "linear graph method" for the concentrations used in NMR spectroscopic analysis is concentration independent does not only apply to the linoleic fatty acid residue that has been used as an example, but also to the saturated and unsaturated carbon atoms of oleic and palmitic, and in all likelihood to others as well.

2.7 APPLICATION OF THE "LINEAR GRAPH METHOD"

The above validation and concentration independent studies done on our "linear graph method" proved it to be highly successful in the complete and precise assignment of the ^{13}C NMR spectra of not only macadamia nut oil but also five other locally produced vegetable oils: apricot kernel oil, avocado pear oil, grapeseed oil, mango kernel oil and marula oil.

Full assignments of the ^{13}C NMR spectra for each of the five vegetable oils could be made with the help of our newly developed "linear graph method". Tables 2.21, 2.22 and 2.23 give the assignments of these ^{13}C NMR spectra.
Peaks representing the carbons in the sn 1', sn 2' and sn 3' positions on the glycerol backbone can usually be distinguished between by inspection. Since sn 1' and sn 3' carbons are observed at the same chemical shift position in a ^{13}C NMR spectrum, the peak height is observed as twice the size of the sn 2' carbon, due to the 2:1 situation. Also notice for mango kernel oil that although two resonances were observed in the ^{13}C NMR spectrum for C10 of the linoleic fatty acid residue, it was not possible to determine by inspection which of the two resonances represented the sn 2' or sn 1', 3' position since both these resonances were roughly the same in height. The same situation was observed for C12 of the linoleic fatty acid residue. Many of the oils, especially marula oil had very complicated ^{13}C NMR spectra and the assignments of these spectra were difficult, resulting in several unassigned resonances, not necessarily just due to TAGs but possibly also other compounds. Notice that the resonance for

C9 of the palmitic fatty acid residue was not detected in the ^{13}C NMR spectrum of several of the oils; including avocado pear, apricot kernel and grapeseed oils. Further investigation of this is required.

Table 2.21: ^{13}C NMR assignments for grapeseed and apricot kernel oil.

GRAPESEED OIL			APRICOT KERNEL OIL		
Assignment	Position on glycerol backbone	Chemical shifts (ppm) measured	Assignment	Position on glycerol backbone	Chemical shifts (ppm) measured
FFA		173.813	P1		173.244
P1		173.252	unassigned		173.231
O1	sn 1',3'	173.221	O1	sn1', 3'	173.212
L1	sn 1',3'	173.21	L1	sn1', 3'	173.202
unassigned		172.812	L1	sn 2'	172.805
L1/O1	sn 2'	172.801	O1	sn 2'	172.794
unassigned		145.14	L13	sn1', 3'	130.203
L13	sn 2'	130.204	L13	sn 2'	130.195
L13	sn 1',3'	130.196	O10	sn 2'	130.016
O10	sn 2'	130.018	O10	sn1', 3'	130.002
O10	sn 1',3'	130.002	L9	sn 2'	129.987
L9	sn 1',3'	129.988	L9	sn1', 3'	129.962
L9	sn 2'	129.962	O9	sn1', 3'	129.703
O9	sn 1',3'	129.703	O9	sn 2'	129.677
O9	sn 2'	129.676	L10	sn1', 3'	128.092
L10	sn 2'	128.091	L10	sn 2'	128.074
L10	sn 1',3'	128.072	L12	sn 2'	127.908
L12	sn 1',3'	127.906	L12	sn1', 3'	127.897
L12	sn 2'	127.894	CHO		68.908
CHO		68.908	CH2O		62.106
CH2O		62.109	O2	sn 2'	34.204
L2/O2		34.196	L2	sn 2'	34.199
P2		34.06	P2		34.062
L2/O2	sn 1',3'	34.032	O2	sn1', 3'	34.04
P14		31.948	L2	sn1', 3'	34.034
O16		31.927	P14		31.953
L16		31.545	O16		31.932
unassigned		30.899	unassigned		31.811
O12		29.787	L16		31.549
O7	sn 2'	29.737	unassigned		30.895
O7/P10	sn 1',3'	29.725	O12		29.791
P12		29.718	unassigned		29.762
P11		29.708	O7	sn 2'	29.742
P8		29.683	O7/P10,12	sn1', 3'	29.727
L7	sn 2'	29.641	P11		29.713
L7	sn 1',3'	29.627	P8	sn 2'	29.687

O14		29.548
P5		29.499
P13		29.386
L15		29.367
O13		29.347
O15		29.341
P6		29.293
L5/O5	sn 2'	29.215
L5/O5	sn 1',3'	29.194
L6/O6	sn 2'	29.147
L6/O6/P4	sn 1',3'	29.133
L4/O4	sn 1',3'	29.1
L4/O4	sn 2'	29.062
O8	sn 2'	27.242
O8	sn 1',3'	27.238
unassigned		27.231
L8		27.219
L14		27.208
O11		27.188
L11		25.647
O3	sn 2'	24.901
L3	sn 2'	24.892
P3		24.883
O3	sn 1',3'	24.861
L3	sn 1',3'	24.854
P15		22.713
O17		22.703
L17		22.595
P16		14.131
O18		14.125
L18		14.084

L7	sn1', 3'	29.645
L7	sn 2'	29.631
O14		29.553
unassigned		29.528
P5	sn1', 3'	29.503
unassigned		29.47
P13		29.391
L15		29.371
O15,13	sn 1',3'	29.346
P6		29.298
O5/L5	sn 2'	29.22
O5/L5	sn1', 3'	29.199
O6/L6/P4	sn 2'	29.15
O6/L6	sn1', 3'	29.133
O4	sn1', 3'	29.11
L4	sn1', 3'	29.105
O4	sn 2'	29.071
L4	sn 2'	29.067
unassigned		29.01
O8		27.242
L8		27.222
L14		27.211
O11		27.191
L11		25.65
O3	sn 2'	24.905
L3	sn 1', 3'	24.896
P3		24.887
O3	sn 1', 3'	24.865
L3	sn 2'	24.858
P15		22.716
O17		22.706
unassigned		22.699
unassigned		22.682
L17		22.598
unassigned		14.131
O18		14.125
P16		14.118
L18		14.085

Table 2.22: ^{13}C NMR assignments for mango kernel and marula oil.

MANGO KERNEL OIL			MARULA OIL		
Assignment	**Position on glycerol backbone**	**Chemical shifts (ppm) measured**	**Assignment**	**Position on glycerol backbone**	**Chemical shifts (ppm) measured**
unassigned		178.907	FFA		173.873
unassigned		178.885	FFA		173.842
FFA		173.898	P1	sn 1', 3'	173.265
FFA		173.866	O1	sn 1', 3'	173.234
unassigned		173.723	L1	sn 1', 3'	173.223
P1		173.287	O1	sn 2'	172.825
O1	sn 1',3'	173.255	L1	sn 2'	172.812
L1	sn 1',3'	173.244	L13	sn 2'	130.186
O1	sn 2'	172.844	L13	sn 1', 3'	130.178
L1	sn 2'	172.834	O10	sn 2'	130.013
unassigned		145.079	O10	sn 1', 3'	129.999
L13		130.199	L9	sn 1', 3'	129.973
O10	sn 1',3'	130.017	L9	sn 2'	129.948
O10	sn 2'	130.005	unassigned		129.717
L9	sn 1',3'	129.989	unassigned		129.707
L9	sn 2'	129.962	O9	sn 1', 3'	129.701
unassigned		129.725	O9	sn 2'	129.675
O9	sn 2'	129.705	L10	sn 2'	128.103
unassigned		129.689	L10	sn 1', 3'	128.083
O9	sn 1',3'	129.679	L12	sn 1', 3'	127.920
L10		128.094	L12	sn 2'	127.903
L10		128.077	CH2O		68.939
L12		127.912	unassigned		68.332
L12		127.9	unassigned		65.041
CHO		68.921	CHO		62.127
DAG		68.349	O2/L2	sn 2'	34.212
DAG		65.051	unassigned		34.105
CH2O		62.114	P2		34.069
O2/L2	sn 2'	34.215	O2/L2	sn 1', 3'	34.047
unassigned		34.122	unassigned		34.020
unassigned		34.106	unassigned		34.005
P2		34.07	P14		31.976

O2/L2	sn 1',3'	34.047	O16		31.955
unassigned		33.974	L16		31.568
unassigned		33.958	O12		29.810
P14		31.963	O7	sn 2'	29.757
O16		31.941	unassigned		29.754
L16		31.557	unassigned		29.747
O12		29.798	O7	sn 1', 3'	29.743
P10,12/O7		29.741	P10		29.736
P11		29.726	P12		29.727
P9		29.711	P11		29.719
P8		29.699	unassigned		29.711
unassigned		29.679	P9		29.704
P7/L7	sn 1',3'	29.661	P8		29.690
L7	sn 2'	29.637	unassigned		29.670
unassigned		29.632	L7	sn 2'	29.661
O14	sn 1',3'	29.565	P7		29.647
O14	sn 2'	29.562	L7	sn 1', 3'	29.643
P5		29.515	unassigned		29.578
unassigned		29.48	unassigned		29.576
P13		29.402	unassigned		29.573
L15		29.378	O14		29.571
O13		29.361	unassigned		29.525
O15		29.357	P5		29.491
P6		29.307	unassigned		29.415
unassigned		29.292	P13		29.391
O5/L5	sn 2'	29.229	O13/L15		29.370
O5/L5	sn 1',3'	29.206	O15		29.363
unassigned		29.187	unassigned		29.318
O6/L6	sn 1',3'	29.158	P6		29.301
P4/L6	sn 1',3'	29.149	O5/L5	sn 1', 3'	29.239
O6	sn 2'	29.141	unassigned		29.226
O4	sn 1',3'	29.119	O5/L5		29.217
L4	sn 2'	29.117	unassigned		29.201
unassigned		29.103	unassigned		29.196
unassigned		29.088	O6/L6	sn 1', 3'	29.164
O4/L4	sn 2'	29.08	unassigned		29.158
O8	sn 1',3'	27.252	O6/L6	sn 1', 3'	29.148

O8	sn 2'	27.248	P4		29.139
unassigned		27.242	O4		29.126
L8		27.228	L4	sn 2'	29.119
L14		27.217	unassigned		29.110
unassigned		27.208	unassigned		29.093
O11		27.199	O4/L4	sn 1', 3'	29.088
unassigned		27.188	O8		27.260
unassigned		27.183	O8		27.256
L11		25.654	unassigned		27.249
O3/L3	sn 2'	24.91	L8		27.236
P3		24.892	L14		27.223
unassigned		24.878	O11		27.204
O3/L3	sn 1',3'	24.87	unassigned		27.197
unassigned		24.753	unassigned		27.188
unassigned		24.737	L11	sn 2'	25.662
P15		22.723	O3		24.917
O17		22.713	L3	sn 1', 3'	24.906
L17		22.604	P3		24.900
P16		14.133	O3/L3		24.878
O18		14.127	unassigned		24.742
L18		14.087	unassigned		24.727
			P15		22.734
			O17		22.724
			L17		22.615
			P16		14.137
			O18		14.132
			L18		14.092

Table 2.23: Assignment of ^{13}C NMR resonances of the major components of fatty acids in avocado pear oil.

Assignment	Position on glycerol backbone	Chemical shifts (ppm) measured	Assignment	Position on glycerol backbone	Chemical shifts (ppm) measured
P1	sn 1',3'	173.266	L7	sn 2'	29.650
Pa1	sn 1',3'	173.253	L7	sn 1',3'	29.633
O1	sn 1',3'	173.235	O14		29.555
L1	sn 1',3'	173.226	P5		29.506
O1/P1/Pa1	sn 2'	172.825	P13		29.393
L1	sn 2'	172.815	L15		29.372
L13	sn 1',3'	130.202	O13/P6		29.353
L13	sn 2'	130.195	O15		29.349
O10/Pa10	sn 2'	130.017	P6	sn 1',3'	29.299
O10/Pa10	sn 1',3'	130.003	O5/L5	sn 2'	29.222
L9	sn 2'	129.989	Pa5	sn 2'	29.217
L9	sn 1',3'	129.962	O5/L5	sn 1',3'	29.201
V/E		129.920	Pa5	sn 1',3'	29.194
V/E		129.824	O6/L6	sn 2'	29.152
Pa9		129.709	L6/P4/Pa6	sn 1',3'	29.145
O9	sn 1',3'	129.704	O6	sn 1',3'	29.135
O9/Pa9	sn 2'	129.678	Pa6	sn 2'	29.126
L10	sn 1',3'	128.092	O4/L4/Pa4	sn 1,3	29.111
L10	sn 2'	128.075	O4/L4/Pa4	sn 2'	29.073
L12	sn 2'	127.910	Pa13		29.011
L12	sn 1',3'	127.898	O8/Pa8	sn 2'	27.247
CHO		68.913	O8	sn 1',3'	27.243
CH2O		62.114	L8		27.224
O,L,Pa2	sn 2'	34.208	L14		27.212
P2	sn 1',3'	34.065	O11		27.193
Pa2	sn 1',3'	34.056	Pa11		27.188
O2/L2	sn 1',3'	34.042	L11		25.651
P14		31.955	O3/Pa3	sn 2'	24.906
O16		31.934	L3	sn 2'	24.896
Pa14		31.812	P3	sn 1',3'	24.888
L3		31.551	O3/L3/Pa3	sn 1',3'	24.866
O12		29.793	P15		22.717
Pa12	sn 2'	29.763	O17		22.707
Pa12	sn 1',3'	29.756	Pa15		22.682
O7	sn 2'	29.743	L17		22.599
P10/Pa7	sn 2'	29.732	P16		14.131
O7/P12/Pa7	sn 1',3'	29.728	O18		14.125
P11		29.715	Pa16		14.116
P8		29.689	L18		14.084

2.8 CONCLUSIONS

During the development of the method to assign the ^{13}C NMR spectra of six vegetable oils several conclusions were made:

1) The literature method, referred to in this thesis as the standard-additions method, proved not only to be time-consuming and costly, but failed for the assignment of ^{13}C NMR resonances of the vegetable oils in largely crowded areas, specifically the 28-30 ppm region. The ^{13}C NMR spectra of the vegetable oils of interest could not be fully assigned using the standard-additions method.

2) The "linear graph method" which was developed proved to be precise and facile for the full assignment of the ^{13}C NMR spectra of vegetable oils provided that sp^3 and sp^2 carbon atoms were treated separately. Where the standard-additions method failed to assign certain peaks in largely crowded regions of the ^{13}C NMR spectra, the "linear graph method" solved this problem.

3) The "linear graph method" was validated by applying it to the ^{13}C NMR spectrum of olive oil, one of the best studied vegetable oils. Comparison of the ^{13}C NMR assignments obtained from the "linear graph method" with the literature assignments proved the reliability of the newly developed method. The 28-30 ppm region of olive oil, previously not fully assigned in the literature, could be precisely assigned by the "linear graph method".

4) Concentration studies using olive oil indicated that the "linear graph method" was concentration independent for the assignment of the ^{13}C chemical shifts of sp^3 carbon atoms but not for sp^2 carbon atoms. However for the concentrations used in NMR spectroscopic analysis, this concentration dependence does not play a role. Consequently, the "linear graph method" is precise for any concentration of vegetable oil, with the exception of very low (insufficient signal-to-noise of the spectra) or high (peak broadening due to high viscosity) values.

5) The ^{13}C NMR spectra of six locally produced vegetable oils; apricot kernel, avocado pear, grapeseed, macadamia nut, mango kernel and marula oils, which have been previously unassigned in the literature, were successfully assigned using the "linear graph method.

In summary, although the "linear graph method" was only applied to seven vegetable oils (the six locally produced vegetable oils and olive oil), the method should prove a useful tool in the assignment of the ^{13}C NMR spectra of a range of vegetable oils.

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CHAPTER 3

A FIRST ATTEMPT AT QUANTITATIVE ANALYSIS BY ^{13}C NMR OF THE FATTY ACID CONTENT OF VEGETABLE OILS

The fatty acid content of vegetable oils is routinely analysed by chromatographic techniques, mainly GLC and GLC-MS. Due to the TAGs lack of volatility, vegetable oils first need to be esterified before these oils can be analysed by GLC. The method of esterification has been investigated using two methods. The oil bath method was proven by GLC and ^{13}C NMR analysis to be the more suitable method for esterification of the TAGs in the vegetable oils and consequently this method was applied for further qualitative and quantitative studies. Quantification of the TAGs present in vegetable oils was investigated by two methods, the generally used GLC method and compared to ^{13}C quantitative NMR spectroscopy. Comparison of these methods indicated that ^{13}C NMR spectroscopy, although not yet an accepted method, was indeed a sufficiently accurate and precise method for the quantification of vegetable oils, giving comparable results to the traditionally used GLC method.

3.1 INTRODUCTION

As mentioned in chapter 1, vegetable oil classification and identification can be carried out by various analytical techniques such as; GLC, GLC-MS, HPLC, TLC, IR, UV, etc. Chromatographic techniques such as GLC and GLC-MS analysis are routinely used to determine the fatty acid profile of vegetable oils as well as the percentages in which these fatty acids are present.[1] Another use of these chromatographic techniques is in the quantitative analysis of vegetable oil fatty acid content. The amounts of each fatty acid present in the vegetable oil can be determined by the integration of chromatographic peak areas.[1]

Chemical modification of a vegetable oil however is required before the oil can be subjected to GLC chromatographic analysis due to the non-volatile nature of the TAGs. Generally the TAGs in a vegetable oil needs to be esterified into their individual volatile methyl fatty acid derivatives. In the following sections this esterification process is discussed especially with regard to two different methods used and consequently the fatty acid contents determined for each of the six vegetable oils. The primary objective of GLC quantification of the TAGs was to validate the results obtained by quantitative ^{13}C NMR analysis for olive oils by the method developed by Koch[2] and others[3-6] for vegetable oils. A comparison between the GLC results and those obtained by quantitative analysis of the ^{13}C NMR spectra was also made.

3.2 FORMATION OF METHYL ESTERS

3.2.1 Experimental

The method used is based on that found in the Official Journal of the European Communities which outlines procedures for the analysis of olive oil.[7] Although this procedure is mentioned only for olive oil it is however applicable to all vegetable oils.

0.5 g sodium metal was dissolved in 100 ml anhydrous methanol to form a 1.5 % sodium methoxide solution. 2 g of oil (filtered) was placed in a 5 ml glass vial. 0.3 g of the sodium methoxide solution was added to the oil and the vial

heat sealed. The vials were placed in a glass beaker containing water and heated in an oil bath at 85-90 °C for 2 hours with occasional shaking.

3.2.2 Discussion of the esterification method

The experimental method shown in section 3.2.1 was applied to the six vegetable oils using sealed vials. Upon completion of the esterification reaction two layers were observed in the vials, an upper layer containing a sediment (of unreacted sodium methoxide) and a second clear bottom layer containing the desired methyl esters and glycerol. A sample was taken of the clear layer from one of the vegetable oils, avocado pear oil, for NMR analysis to test if the reaction had been completed. The ^{13}C NMR spectrum showed that the reaction had indeed taken place due to the disappearance of certain resonances in the spectrum. As explained in chapter 2, two resonances are expected for each carbon in the TAG due to the fact that the carbon chain can be in the sn 2' or sn 1', 3' positions of the glycerol backbone. Once the TAG had been esterified only one resonance representing each carbon of a fatty acid chain was expected to be observed, since each of the fatty acid residue chains had now been removed from the glycerol backbone. This was indeed the case and only single resonances representing each carbon in the methyl esters were observed as shown in the ^{13}C NMR spectrum (figure 3.1) below, which showed two peaks around the 50 ppm region. One of these peaks was the signal expected for the methoxy carbon of the newly formed methyl esters. Upon spiking the sample with methanol it was found that the second larger peak, situated further upfield, was due to methanol. This indicated that some of the methanol was also part of the clear bottom layer.

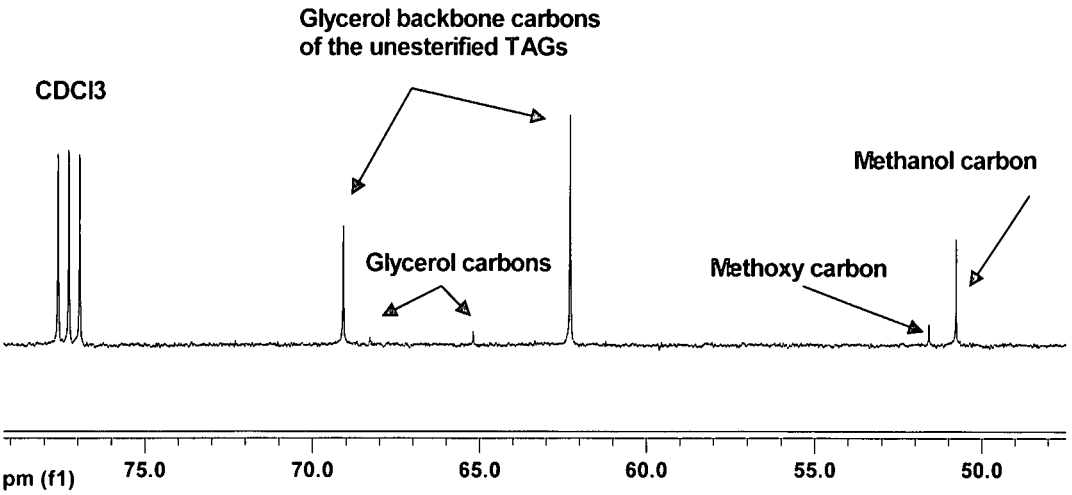


Figure 3.1: ^{13}C NMR spectrum of avocado pear oil after having been subjected to an esterification reaction where an oil bath was used for heating.

The two peaks around the 65-68 ppm region of the ^{13}C NMR spectrum in figure 3.1 could possibly represent the carbons belonging to the glycerol backbone indicating that the reaction did take place. Although this method of esterification seemed to have worked, GLC-MS analysis and ^{13}C NMR spectroscopy, indicated that only a small amount of the methyl esters had formed (discussed further in section 3.3). A second method was tried in order to see if the reaction could not be improved to give a higher conversion to methyl ester product.

An identical procedure was followed as previously used, but with the exception that an oven was used instead of an oil bath for heating the reaction. This eliminated the problem of inadequate temperature control during the use of the oil bath. However, a ^{13}C NMR spectrum of the oven sample (figure 3.2) showed only a single peak in the 50 ppm region of interest which upon spiking with methanol increased, proving that the single peak unfortunately did not belong to the desired methoxy carbon. Since there was no second peak representing the methoxy carbon, it could initially be concluded that the reaction did not take place.

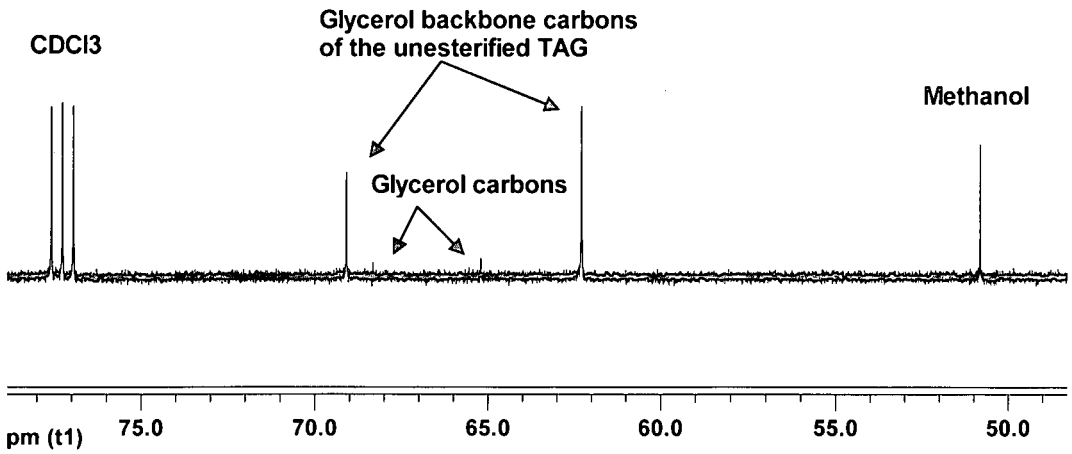
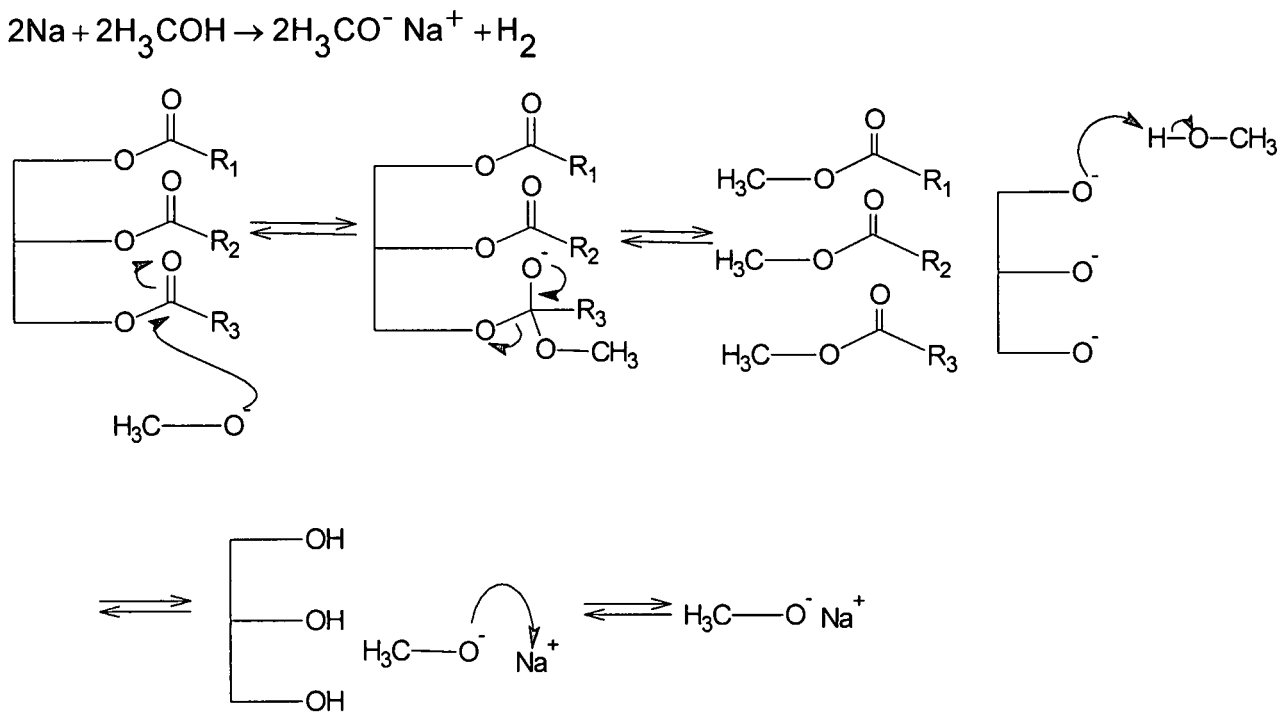


Figure 3.2: ^{13}C NMR spectrum of avocado pear oil after having been subjected to an esterification reaction where an oven was used for heating.

The ^{13}C NMR spectra of the two reaction products indicated that the method using the oil bath for the esterification of the vegetable oil TAGs was probably more effective than that which used an oven. The same situation was observed for macadamia nut oil. To confirm our assumption that the oil bath method was better than the oven based method, samples of macadamia nut and avocado pear oil were sent for GLC-MS analysis.

3.2.3 Esterification mechanism

The basic method for the esterification process is shown in Scheme 3.1. Sodium and methanol react to form sodium methoxide, with the formation of hydrogen gas as a by-product. The negatively charged methoxide ion acts as a nucleophile and attacks the carbonyl carbon attached to the glycerol backbone of the TAG yielding a tetrahedral alkoxide intermediate. Elimination of the alkoxide ion leads to the formation of two products, namely the carboxylate ion of the glycerol and the newly formed ester. Protonation of the carboxylate ion by the methanol in solution leads to the formation of glycerol.[8]



Scheme 3.1: Methyl esterification of a triacylglycerol using a sodium methoxide solution.

3.3 GLC-MS ANALYSIS OF VEGETABLE OILS

Samples of macadamia nut oil and avocado pear oil, esterified by the two separate methods, were sent for GLC-MS analysis to the GLC-MS laboratory of the Central Analytical Facility of the University of Stellenbosch in order determine which of the two above mentioned methods worked best for the esterifications of the TAGs. 50 µl of all esterified samples were diluted with 450 µl of dichloromethane and 1 µl of the solution was injected in the gas chromatograph inlet. Analysis was performed on a Carlo Erba GLC 6000 Vega Series gas chromatograph coupled to an AMD 604 high resolution mass spectrometer. The column employed was a fused silica capillary (30 m x 0.25 mm i.d., 0.2 mm film-thickness) coated with a 100 percent cyanopropylpolysiloxane non-bonded phase. The GLC oven temperature was programmed to increase from 40 to 240 °C at a rate of 4 °C/min.

According to the GLC-MS results (refer to figures 3.3 and 3.4), all the oil samples were successfully esterified, even though NMR spectroscopy indicated otherwise. Comparing the two chromatograms closely it can be seen

by inspection that the oven method indeed yielded a smaller quantity of the fatty acid methyl esters (FAMEs). This explains why it appeared from NMR spectroscopy that the esterification had not taken place as it is intrinsically a less sensitive technique than GLC and the FAMEs were in all likelihood below the detection limit.

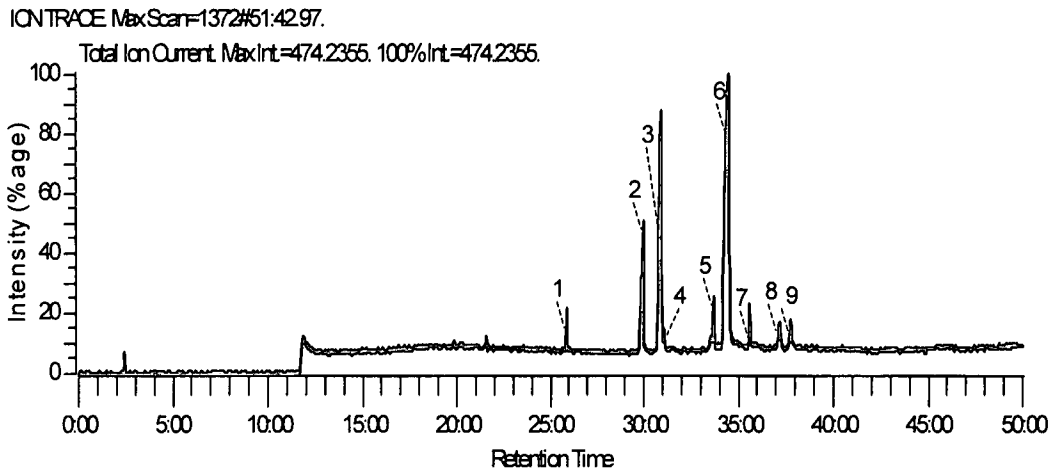


Figure 3.3: Gas chromatogram of esterified macadamia nut oil where oven heating was used for esterification where: peak 1 represents a methyl tetradecanoate, peak 2 a methyl hexadecanoate, peak 3 a methyl hexadecenoate (possibly methyl-(*Z*)-9-hexadecenoate), peak 4 a methyl hexadecenoate, peak 5 a methyl octadecanoate, peak 6 a methyl octadecenoate (possibly methyl-(*Z*)-9-octadecenoate), peak 7 a methyl octadecadienoate (possibly methyl-(*Z,Z*)-9,12-octadecadienoate, peak 8 a methyl eicosanoate and peak 9 a methyl eicosenoate (possibly methyl-(*Z*)-11-eicosenoate).

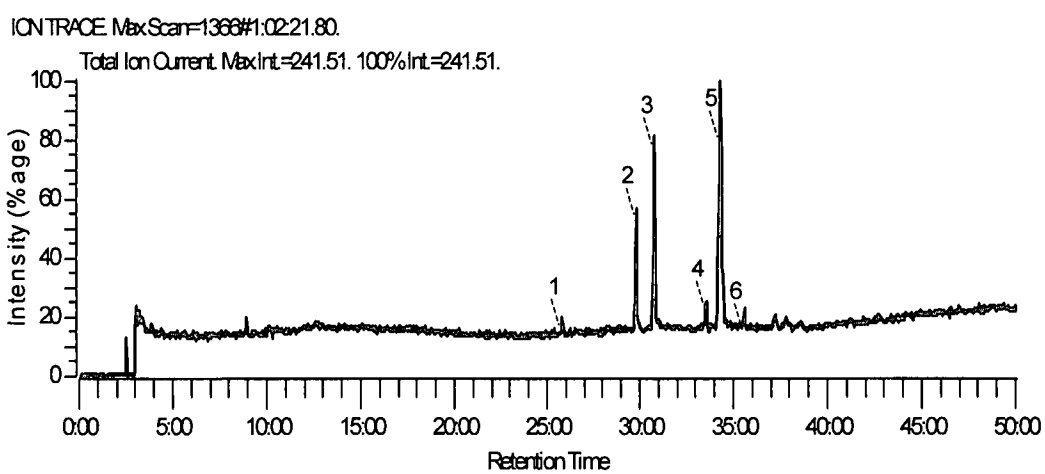


Figure 3.4: Gas chromatogram of esterified macadamia nut oil where an oil bath was used for heating where: peak 1 represents an aliphatic ester, peak 2 a methyl hexadecanoate, peak 3 a methyl hexadecenoate (possibly methyl-(Z)-9-hexadecenoate),

peak 4 a methyl octadecanoate, peak 5 a methyl octadecenoate (possibly methyl-(Z)-9-octadecenoate) and peak 6 another aliphatic ester.

In conclusion, although it originally appeared that from NMR data the oven based method for esterification of the TAGs in vegetable oils was easier but did not give the desired results and that the method given by the Official Journal of the European communities was the only one that yielded the expected results, GLC-MS analysis proved that both methods worked successfully in the esterification of the fatty acids present as TAGs in the vegetable oils. The results obtained by GLC-MS analysis and NMR analysis however agreed in that the oil bath method is the preferred method as it yields a greater quantity of product. Using this information we could continue with the determination of the fatty acid content of the six vegetable oils.

3.4 QUALITATIVE AND QUANTITATIVE DETERMINATION OF FATTY ACID CONTENT OF SIX VEGETABLE OILS

Qualitative and quantitative studies of vegetable oils can be done by GLC analysis. Therefore both these studies were done on the determination of fatty acid content of the six vegetable oils.

Qualitatively, GLC-MS indicated the presence of linoleic acid and palmitic acids for all the oils (refer to table 3.1). Oleic acid was found to be present in the largest amount for all the oils, as in olive oil, with the exception of grapeseed oil. Grapeseed oil contained linoleic acid as the main compound. One fatty acid that was found to be present in macadamia nut oil and avocado pear oil but not present in the other oils was palmitoleic acid. Other saturated compounds were also found to be present in the oils in large quantities, but palmitic acid was overall the highest.

Although the quantity of each fatty acid residue present in vegetable oils can be determined by the classic GLC method, this method however has a few disadvantages. As mentioned before, the TAGs present in vegetable oils cannot be directly detected by GLC analysis since these compounds are non-

volatile. Therefore the TAGs first need to be esterified into their FAME equivalents for detection by GLC analysis. The amount of methyl esters are not necessarily an accurate representation of the true amount of fatty acid residues in the vegetable oil, due to the possibility of preferentional esterification and incomplete esterification, which based on our experience with the esterification method is not impossible. A better method would therefore not require the chemical modification of the TAGs but would allow the oil to be analysed directly. ^{13}C NMR spectroscopy is such a technique. Several quantification studies using ^{13}C NMR spectroscopy have been done on olive oil,[2-6,9-14] but as yet this technique is not a generally accepted method for the determination of fatty acid residue content in vegetable oils. This is due to the fact that though in some respects the NMR technique has distinct advantages, the cost of an NMR instrument versus a gas chromatograph is probably the major deciding factor in GLC remaining the technique of choice.
In the next few sections the quantification of the six vegetable oils of interest by GLC and ^{13}C NMR spectroscopy are compared and discussed.

3.4.1 Quantification by GLC analysis

The six vegetable oils of interest were esterified and submitted for quantification analysis by GLC. Table 3.1 gives the quantification values obtained from the GLC chromatograms. 20 µl of all FAME samples were diluted with 1 ml of dichloromethane and 1 µl of the solution was injected in the gas chromatograph inlet. Analysis was performed on a Carlo Erba GLC 6000 Vega Series gas chromatograph. The column employed was a fused silica capillary (30 m x 0.25 mm i.d., 0.2 mm film-thickness) coated with a 100 percent cyanopropylpolysiloxane non-bonded phase. The GLC oven temperature was programmed to increase from 40 to 240 °C at a rate of 4 °C/min.

Table 3.1: Percentages (from area integration of GLC peaks) of fatty acids present in vegetable oils determined by GLC analysis.

Fatty acid	Apricot kernel oil %	Avocado pear oil %	Grapeseed oil %	Macadamia nut %	Mango kernel oil %	Marula oil %
unknown						0.26
laurate	0.01	5.21	0.15	0.70	2.20	3.20
unknown		0.22				0.40
tridecanoate	<0.01	1.14		0.40	0.11	2.83
unknown	0.01	1.52			0.17	1.04
myristic	0.02	3.92	0.53	0.17	0.27	3.47
unknown	0.01	1.11	0.09	1.03		
palmitic	4.51	19.37	7.30	9.04	9.24	13.46
hexadecenoate			0.27			
palimitoleic	0.74	7.36	0.12	22.47	0.17	0.28
stearic	0.57	0.69	3.05	2.32	28.59	3.96
oleic	62.71	49.83	12.37	59.97	49.70	66.86
vaccenic		3.49	0.41	0.58	0.89	0.66
linoleic	31.27	6.16	72.49	1.26	7.28	2.78
arachidic	0.06		2.94	0.99	0.95	0.27
eicosenoic	0.08		0.28	1.07	0.43	0.53
Total saturated	5.25	30.32	14.24	14.69	41.79	27.72

The values in table 3.2 were used to determine the percentages of each fatty acid present in each oil. These values are the integration values for each peak observed in the GLC spectrum. Each of these integration values was then expressed as a percentage of the total number of integrations, yielding the percentage of each fatty acid present in each oil (table 3.1). However it is important to realize that for GLC, peak integration values are only approximately proportional to the total concentration of the substance, unless the system has been calibrated and response factors are known. Since the system was not calibrated for this study, the results in tables 3.1 and 3.2 are approximations.

Our interest was to also use ^{13}C NMR spectroscopy to quantify the fatty acids present as TAGs in the oil and compare these values with those obtained by GLC analysis.

Table 3.2: Peak integration values obtained from GLC analysis for vegetable oils.

Peak number (increasing retention time)	Peak assignment	Apricot kernel oil	Avocado pear oil	Grapeseed oil	Macadamia nut oil	Mango kernel oil	Marula oil
1	unknown						108
2	laurate	82	1877	206	280	1304	1332
3	unknown		79				166
4	tridecanoate	36	409		160	65	1175
5	unknown	79	546			98	432
6	myristic	152	1414	706	66	163	1443
7	unknown	104	399	125	412		
8	palmitic	33613	6980	9786	3609	5486	5593
9	hexadecenoate			367			
10	palimitoleic	5517	2651	162	8967	102	116
11	stearic	4284	247	4089	925	16982	1645
12	oleic	467569	17955	16587	23933	29518	27793
13	vaccenic		1259	549	231	530	273
14	linoleic	233118	2218	97173	501	4326	1156
15	arachidic	439		3935	397	564	112
16	eicosenoic	562		374	426	253	222
	total	745555	36034	134059	39907	59391	41566

3.4.2 Quantification by ^{13}C NMR spectroscopy

Quantification by NMR spectroscopy requires the integration of ^{13}C resonances. Typically this integration is applied to ^{1}H NMR spectra. The integration of proton resonances gives values for the relative number of protons in a molecule and can give the relative concentrations for a mixture of molecules. One problem encountered with ^{1}H NMR quantification of mixtures is that the spectral range in a ^{1}H NMR spectrum is narrow, typically 0-15 ppm and together with inter-nuclear ^{1}H -^{1}H scalar *J* coupling, significant overlap of the proton resonances is observed hindering quantitative analysis of complex spectra. This situation is troublesome when a mixture containing various compounds (such as a vegetable oil) is the object of the analysis.

Therefore ^{13}C NMR is more useful for mixtures such as vegetable oils. Even though the ^{13}C nuclei have a natural abundance of only 1.11% compared to ^{1}H nuclei's 99.98%, the ^{13}C NMR spectral range is larger, typically around 0-180 ppm for a vegetable oil. This results in a spectrum where all the chemically unique carbon atoms of the major compounds (major fatty acid residues) can be detected as separate peaks in certain areas of the spectra. There are two important factors that need to be taken into account before quantification of ^{13}C NMR spectra can be undertaken.[3]

a) One of these is that if continuous ^{1}H decoupling is applied the peak areas are not necessarily an accurate indication of the amount of carbons represented by that resonance as is the case in a ^{1}H spectrum, due to the effect of nuclear Overhauser effect which results in ^{13}C resonances to be enhanced by various amounts if ^{1}H are decoupled from the ^{13}C nucleus.

b) The other important factor is that sufficient time should be given for the carbon nuclei to relax back to their ground states after excitation. Refer to section 2.3 on the relaxation of carbon nuclei.

If these two factors are taken into account, by (a) using decoupling only during the acquisition time to avoid nuclear Overhauser enchancement and (b) to allow for full relaxation of the nuclei between pulses, the distortion of ^{13}C resonances will be minimized and successful quantification can be obtained.[3]

Samples for ^{13}C NMR were prepared on a mass per unit mass basis where roughly about 0.4 g of oil was dissolved in 0.6 g of $CDCl_3$ in order to get a high

concentration of fatty acids present. TMS was once again added as reference. Due to the fact that mango kernel oil was in solid form, the sample originally prepared was difficult to analyze with NMR spectroscopy since 0.4 g oil did not dissolve in the solvent. Therefore 0.2 g mango kernel oil was dissolved in 0.8 g $CDCl_3$. The resulting solution was filtered to rid the excess undissolved oil.

Although the pw90 and T1 values were previously determined for the six vegetable oils (refer to 2.3), these values had to be re-determined for each sample due to the fact that the ^{13}C NMR experiments were to be run using a stronger magnet. A 600 MHz NMR spectrometer was used with the following parameters: nt=100; d1=10; dm='nny'; bs=4; at=5.44; spectral width of 200,-5 ppm. Refer to table 3.3 for the longest T1 values obtained representing the unsaturated and carbonyl carbons that were of interest for quantification. This largest T1 value of each vegetable oil was then further used, as explained in section 2.3, to determine if the acquisition and relaxation times that were used were adequate for complete carbon nuclei relaxation.

Table 3.3: Determination of the time needed for carbonyl and olefinic carbon nuclei relaxation with a 30° pulse for vegetable oils.

	Longest T1	**Assignment**	**Calculated time needed for carbon relaxation (s)**		**at + d_1**	**Acquisition time (at in s)**	**Relaxation delay (d_1 in s)**
Apricot kernel	3.2	L12 sn 2'	0.46	≤	15.44	5.44	10
Avocado pear	3.5	L12 sn 1', 3'	0.50	≤			
Grapeseed	2.8	L13	0.40	≤			
Macadamia nut	2.4	P1	0.35	≤			
Mango kernel	5.0*	P1	0.72	≤			
Marula	4.2	P1	0.60	≤			

*T1 value determined on 400 MHz NMR spectrometer.

Deconvolutions of the ^{13}C NMR spectra were done using the NMR software available on the spectrometer. For the formatting of the spectrum a Gaussian

function (gf) of around 0.2 times the acquisition time and line broadening of 0.0092 was used. Table 3.4 indicates the quantification values obtained. The integration values indicated are only those required to determine the percentage of fatty acid residues present in vegetable oils. These are mainly the resonances in the olefinic region (126 ppm – 130 ppm) except for the saturated fatty acids (in this case palmitic acid). The palmitic acid residue that is used for further calculations is the resonance in the carbonyl carbon region (172 – 173 ppm) due to the fact that the resonances were well separated in this region. A process of deconvolution was carried out using the NMR instrument's spectroscopic software. Deconvolution is used to reverse effects of convolution, which means to uncomplicated a spectrum and separate overlapping resonances and consequently used as an integration technique for the ^{13}C atoms. The peak areas are calculated by width and height at peak half height. Table 3.4 indicates the peak area by deconvolution analysis obtained and the average of each fatty acid residue that was determined. This was done per carbon and not for the position on the glycerol backbone, therefore L10a and L10b is considered as one carbon, where "a" represents the carbon in the alpha position and "b" represents the carbon in the beta position of the glycerol backbone.

The mole percentage of each fatty acid residue was determined from the relative integral areas. The percentage values are given in table 3.5 and were determined from the deconvolution values in table 3.4. For instance for the oleic acid residue present in marula oil:

$$\text{Oleic mol \%} = \frac{\text{oleic deconvolution value}}{\text{total acid deconvolution value}} \times 100$$

Where total acid value = oleic value + palmitoleic value + linoleic value + palmitic value

$$\text{Oleic mol \%} = \frac{148.63}{148.63 + 0 + 14.43 + 49.73} \times 100$$

$$\text{Oleic mol \%} = 69.85$$

Table 3.4: Peak integral values determined by deconvolution obtained for the fatty acid residues present in vegetable oils by ^{13}C NMR spectroscopy from the 600 MHz instrument.

Assignment	Apricot kernel oil	Avocado oil	Grapeseed oil	Macadamia nut oil	Marula oil	Mango kernel oil
Sat C=O	10.4	62.2	25.2	43.7	64.6	243.8
L13b	18.5	14.7	53.6		23.9	
L13a	24.8	27.6	124.9		76.2	69.6
O10b	21.1	70.6	13.7	58.8	12.5	181.9
O10a	59.8	119.0	18.0	97.8	200.7	241.2
L9a	24.8	21.0	106.5		32.3	
L9b	16.8	11.0	62.1		12.2	22.0
V/E		15.4		15.8		
V/E		17.0		14.8		
O9a	59.3		15.5	64.4	137.3	207.2
Pa		115.5		23.8		
O9b	20.1		14.7	52.3	97.6	165.8
Pb		65.5		9.9		
L10b	16.9	7.9	57.7		9.5	37.5
L10a	22.9	23.4	107.0		14.2	29.3
L12a	23.4	25.4	116.2		13.6	75.9
L12b	18.1	10.9	64.9		8.2	
Oleic ave	80.2		30.9		224.1	398.0
Palmitoleic ave		185.3		153.5		
Linoleic ave	41.6	35.5	173.2		22.8	68.2
Saturated ave	10.4	62.2	25.2	43.7	64.6	243.8
Vaccenic/Eicosenoic		16.2		15.3		

Table 3.5: Percentages of fatty acid residues present in vegetable oils by quantification of the ^{13}C NMR spectrum obtained from 600 MHz instrument.

	Apricot kernel %	Avocado pear %	Grapeseed %	Macadamia nut %	Mango kernel %	Marula %
Oleic	61	62	14	56	56	72
Palmitoleic				16		
Linoleic	31	12	76		10	7
Vaccenic/Eicosenoic		5		7		
Saturated	8	21	12	21	34	21

Notice that for macadamia nut oil and avocado pear oil the resonances representing the oleic and palmitoleic acid residues in the olefinic region (126 – 130 ppm) of the spectrum lay close to each other and although they could be distinguished by inspection, for deconvolution purposes this overlapping was sometimes a problem. For macadamia nut oil the resonances could be separately integrated but this was not the case for avocado pear oil. Therefore the values in tables 3.4 and 3.5 were separately indicated for the two fatty acid residues in macadamia nut oil while they were not for avocado pear oil.

In table 3.5 there is no percentage determined for linoleic acid in macadamia nut oil although we have assigned this compound in the spectrum in chapter 2. However the resonances representing these carbons in the olefinic region were too small for reliable quantification.

3.4.3 Comparison between the two methods.

Comparing the results obtained from NMR and GLC analysis, both techniques gave slightly different values of fatty acid percentages (table 3.6).

Table 3.6: Comparison of fatty acid percentages determined by GLC and NMR analysis.

		Oleic %	Palmitoleic %	Linoleic %	Vaccenic/ Eicosenoic %	Saturated %
Apricot kernel	**GLC**	63	1	31	trace[+]	5
	NMR	61		31		8
Avocado pear	**GLC**	50	7	6	trace[+]	30
	NMR		62	12	5	21
Grapeseed	**GLC**	12	trace[+]	72	1	14
	NMR	14		75		12
Macadamia nut	**GLC**	60	22	1	trace[+]	15
	NMR	56	16		7	21
Mango kernel	**GLC**	50	trace[+]	7		42
	NMR	56		10		34
Marula	**GLC**	67	trace[+]	3	1	28
	NMR	72		7		21

[+]Where trace amounts represent a percentage below 1%.

For some of the oils there was a large difference in saturated percentage results between the two methods. This was due to the fact that NMR spectroscopy can only determine the major fatty acid residues that are

present. Therefore the saturated percentage obtained by NMR spectroscopy represents palmitic acid residues mostly while the GLC results represents a range of saturated compounds which are also present in small amounts in the oil (refer to table 3.2) as well. Since the percentages were determined as fractions, the minor percentages were calculated as part of the total saturated percentage in the NMR results and would affect the results of the other fatty acid percentages such as oleic acid, and explain the difference between the results for oleic acid, palmitoleic acid and linoleic acid obtained by NMR and GLC analysis. The small values obtained for vaccenic/eicosenoic acid in some of the oils by GLC were too small to detect by NMR analysis, yielding these as minor components. According to NMR analysis palmitoleic acid was only detected in two vegetable oils, namely macadamia nut and avocado pear oil, but GLC quantification indicated small quantities of this compound in the rest of the oils as well. This indicates the possibility that other vegetable oils do contain palmitoleic acid residues on their glycerol backbones but in minor quantities that can not be detected by high resolution NMR spectroscopy.

For apricot kernel and grapeseed oils the NMR and GLC results compared well with about a 2% difference in oleic and saturated fatty acid percentages. NMR was however unable to detect palmitoleic and vaccenic/eicosenoic residues in these oils. Marula oil results compared fairly well although the difference in results was a bit larger with about a 4% difference for oleic and linoleic. The saturated fatty acid difference of about 7% could be an indication of several saturated residues present in the oil that were not detected by NMR analysis. Due to the fact that the NMR spectrum obtained for marula oil was very complicated, it could be assumed that there were many other unassigned fatty acids (saturated and/or unsaturated in smaller quantities) and other compounds in the oil that would affect the quantification values. For macadamia nut oil and avocado pear oils, most of the percentage values compared fairly well with again the exception of a large difference in saturated values, most likely due to the minor components that were detected by GLC and not NMR analysis. The vaccenic/eicosenoic value difference is however a concern. Since vaccenic and eicosenoic residues are found in the same area of the NMR spectrum and cannot be distinguished by NMR analysis (as

mentioned earlier) and it is as yet unknown whether other unsaturated fatty acid residues also may be present in this area of the spectrum influencing quantification values, these results cannot truly be used for comparison purposes. Mango kernel oil, although originally a problem to analyze, also gave good comparative results. The difference of 7% between the results for oleic and saturated acids between GLC and NMR analysis may seem at first glance to be rather large, but once again the saturated fatty acids play an even more important role. Mango kernel oil was found not to be in a liquid form, as most vegetable oils are, but a waxy solid. This was probably due to its high percentage of saturated fatty acid content (42% from GLC results). Due to the solid state of this oil, it could not be filtered and is therefore unknown if it was truly pure. The complex ^{13}C NMR spectrum suggests that other unknown compounds are also present (refer to the assignment of this spectrum in table 2.22). Differences could however also be due to "biased" esterification for GLC as discussed earlier. As mentioned earlier since the GLC was not calibrated the response factors for the various components were not known. It is thus assumed that all the components in the oil have the response factors but this may in fact not be the causing the GLC and NMR values to differ (no such response factors need to be determined for NMR spectroscopy). However both the NMR and GLC results are relative values and as NMR spectroscopy is less sensitive the minor components have not been included in calculating the relative fatty acid mole percentages for the NMR data thus introducing further error between the two sets of results.

3.6 CONCLUSIONS

In conclusion, we proved that quantification of the major fatty acid residues in six vegetable oils can be reasonably successfully determined by ^{13}C NMR spectroscopy. Results for oleic and linoleic fatty acids correlated well with those values obtained by GLC analysis. As the number of percentages of saturated fatty acids in an oil increase the NMR and GLC results deviation increases. So NMR spectroscopy is best for low fatty acid oils, such as olive oil and apricot kernel oil. The big advantage of NMR analysis over GLC analysis is that is does not require any chemical modification of the TAGs in

the vegetable oils. As mentioned in chapter two the other advantage of NMR is that you can calculate α/β ratios for oleic and linoleic fatty acids, although it wasn't done in this particular study due to insufficient resolution.

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CHAPTER 4

TITRIMETRIC ANALYSIS FOR THE PRESENCE OF FREE FATTY ACIDS IN VEGETABLE OILS

As resonances were detected in the ^{13}C NMR spectra of the six vegetable oils indicating the presence of FFAs, a titration method was used to determine the percentages of these FFAs present in the oils. Experiments were however first carried out to compare the use of two burettes and two indicators to determine which would yield the more accurate results. It was found that for standardization a 50 ml burette was more accurate than a 5 ml burette. Titration of olive oil indicated that alkaline blue was the preferred indicator for this oil. However once the titration of FFAs in the six vegetable oils was performed, phenolphthalein proved to yield better results.

4.1 INTRODUCTION

The quality of a vegetable oil is determined by several factors. It is important to assess these factors as the higher the quality of an oil, the higher its commercial value. For instance "extra virgin olive oil", which is the name given to olive oil of the highest quality, is significantly more valuable than "virgin olive oil" or "olive oil". Some of the factors determining olive oil quality (the most regulated vegetable oil) are:[1,2,3]

- Iodine number
- Sensory qualities
- Percentage FFA
- Peroxide value
- Melting behaviour/point
- Viscosity
- Smoke point
- Moisture and volatile matter
- Acidity
- Saponification value
- Thiocyanogen value
- Soluble mineral matter
- Softening point
- Turbidity point
- Slipping point
- Titer test

As can be seen the quantity of FFAs present in olive oils is one of the factors used in determining the quality of these oils. For instance, high quality extra virgin olive oil is defined as having a FFA percentage of below 1%. Literature research indicated a few methods for %FFA determination which include titration,[1] GC,[4,5] and FTIR.[6,7]

The ^{13}C NMR spectra that we obtained for the six vegetable oils analysed in this project indicated the presence of FFAs. Signals representing the carbons of FFAs in the ^{13}C NMR spectrum are usually found at around 174 ppm which is further downfield, compared to the carbonyl carbon atoms belonging to the fatty acid residues which are bound to the acylglycerol backbone (TAG). For marula and mango kernel oil these peaks were clearly evident but in the other oils they were not significant. However the presence of ^{13}C resonances at about 174 ppm (figure 4.1) in two of the vegetable oils were thought to be due to FFAs, which

then led to the determination of the %FFA by titration of all six the oils. Figure 4.1 illustrates the presence of FFAs for mango kernel oil.

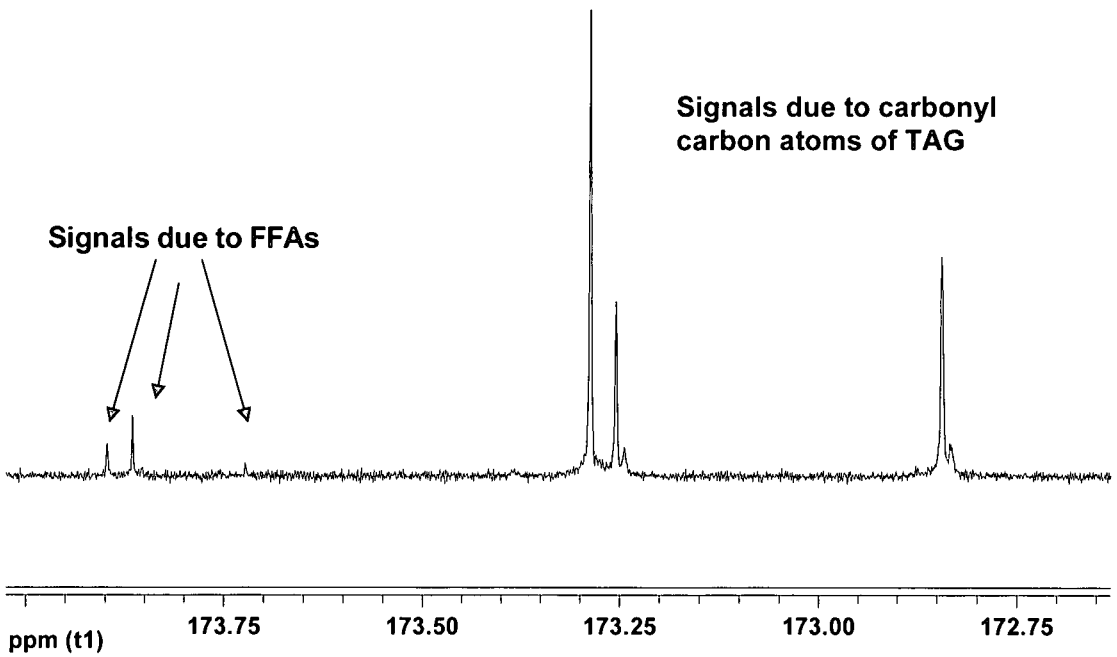

Figure 4.1: Expansion of the ^{13}C NMR spectrum of mango kernel oil with peaks belonging to FFAs indicated.

It was thus decided to determine the %FFAs of all the oils via a titration method. For a limited number of oils the titration method was preferred over instrumental techniques. One might expect that the determination of FFAs in vegetable oils using a standard titration method should be facile, but it was found that this was not in fact the case and that obtaining accurate and repeatable results for the range of vegetable oils of interest in this thesis was not straightforward and a significant amount of time on method development was spent. In the following sections the various experiments that were carried out to obtain the best methods for standardization and determination of the FFAs present are discussed, as well as the application of these methods to the six vegetable oils of interest.

4.2 EXPERIMENTAL

4.2.1 Method for the determination of %FFAs

The determination of the %FFAs of all the vegetable oils was carried out using the method described in the Official Journal of the European Communities:[1]

0.1 M KOH dissolved in distilled water was standardized with a 0.05 M oxalic acid solution. A 50/50 ethanol/toluene mixture was prepared as solvent for the vegetable oils. Depending on the color of the oil and therefore the amount of ethanol/toluene solvent that would be used, 0.3 ml phenolphthalein was added per 100 ml ethanol/toluene (50/50) solution and neutralized by the KOH solution. The required amount of vegetable oil was dissolved in the neutralized ethanol/ toluene solution (50-100 ml as required) and titrated with 0.1 M KOH in triplicate. The required amount of vegetable oil, as referred to in the method above, was determined according to table 4.1 below. The table indicates the amount of sample needed for titration purposes according to the expected %FFA in the oil. During all our experiments, 2.5 g (4-15% FFA) was used as starting point. The values obtained were used to determine the %FFAs and if this value did not fall into the expected %FFA range, the experiment was repeated using either a higher or lower amount of sample as required.

Table 4.1: Table of the mass of oil required for FFA titration.

Expected acid value	Mass of sample (g)	Weighing accuracy required (g)
< 1	20	0.05
1 to 4	10	0.02
4 to 15	2.5	0.01
15 to 75	0.5	0.001
> 75	0.1	0.0002

Each experiment was done in triplicate and for each the %FFAs was determined whereupon the average of the three percentages was taken. The following equation was used:

$$\%FFA = \frac{VCM}{10(m)}$$

Where V represent the average volume of the titrated KOH solution in milliliters; C represents the concentration of the KOH solution (as determined by standardization) in moles per liter; M represents the molar weight of oleic acid in grams per mole and m represents the weight of the oil sample in grams. The acidity of vegetable oils is always expressed as a percentage of one of the fatty acids, usually oleic acid. All %FFA results in this thesis are expressed as a percentage of oleic acid.

The confidence limits of the mean was calculated as follows with a 95% competency (p=0.05):

$$\mu = \bar{\chi} \pm t(s/\sqrt{n})$$

where μ represents the 95 % confidence interval (CI), $\bar{\chi}$ the mean of the %FFA values, t the t-distribution value, s the standard deviation and n the number of repeated experiments (titrations).[8,9]

For a triplicate experiment (n=3) the degrees of freedom (n-1) are 2 and therefore the corresponding t value was found to be 4.30. For a duplicate experiment (n=2) the degrees of freedom are 1 and therefore the corresponding t value is 12.71. [8]

4.2.2 Preparation of distilled ethanol

In order to determine the %FFAs of the vegetable oils as described in section 4.2.1 using an ethanolic KOH solution, the ethanol had to be distilled before use to remove any excess water which could lead to phase separation. The following method was used:

16 g KOH and 1 g aluminum shavings were added to 2 L ethanol and refluxed for 1.5 hours at 110 °C. The solution was then distilled immediately (78-92 °C) and stored in a dark cupboard over activated 4 molecular sieves. An ethanolic KOH

solution was also prepared from this distilled ethanol solution and left for several days in a dark cupboard after which the clear supernatant liquid was decanted from the precipitate of potassium carbonate, if any was present.

4.3 METHOD DEVELOPMENT

4.3.1 Standardization of KOH solution and evaluation of burettes

In order to determine the best method for determination of the %FFAs, experiments were carried out where two variables were taken into account, the indicators used as well as the volume of the burettes used. Two indicators, phenolphthalein and alkaline blue, were compared, since choice of indicator is especially important when working with darker oils. In the case of the burettes, a 50 ml burette marked off in 0.1 ml graduations was compared to a 5 ml burette marked off in 0.01 ml graduations. This comparison was not only applied to the determination of %FFAs in olive oil but also used during the standardization of the base, KOH. The volume of the burette used significantly affects the repeatability of results, affecting the achievable precision.

Fresh standardization of the ethanolic KOH solution is important as KOH has a tendency to absorb water. Therefore the mass of the KOH pellets weighed can not be used when calculating the concentration of the subsequent solution, since upon contact with air water is absorbed by the pellets. Titration with a known concentration of oxalic acid in ethanol is used to determine the accurate concentration of the KOH solution that is made up, before using this solution for the %FFA titrations.

A series of experiments was carried out for the KOH standardization using phenolphthalein as the indicator, but with both 50 ml and 5 ml burettes. Solutions of oxalic acid and KOH first had to be prepared. Titration of these two solutions would yield the following:

$$HOOCCOOH + 2KOH \rightarrow K^+ \ ^-OOCCOO^- \ K^+ + 2H_2O$$

Therefore a one mol ratio of oxalic acid reacts with two moles of KOH during the standardization reaction.

It was found that in most cases a volume to volume (oxalic acid/potassium hydroxide) ratio (refer to table 4.2) with 0.01 difference was obtained with the 50 ml burettes. Although it was possible to get a 0.1 difference with the 5 ml burettes (refer to table 4.3), reproducibility was very difficult, time-consuming and eventually frustrating. In most cases not even end-points of nearly the same color were observed, and the results obtained could not be used for standardization interpretation. The reason problems were encountered with the 5 ml burettes is due to the sizes of the drops for each type of burette. One drop (determined for distilled water) from the 50 ml burette weighed 37.5 mg while a drop from the 5 ml burette weighed 50.5 mg. Therefore the drops are much larger coming out of the tip of the 5 ml burette resulting in the endpoint being easily overshot and hence it is difficult to observe where precisely the end-point is. Due to the above reasons, it was concluded that although the 5 ml burettes would obviously give a more accurate indication of the concentration of KOH, it was however in a commercial sense, better to use the 50 ml burettes.

Table 4.2: Three standardizations of KOH with oxalic acid using a 50 ml burette.

Titration 1				Titration 2				Titration 3			
Oxalic acid (ml)	**KOH (ml)**	**Oxalic acid/KOH (ml/ml)**	**KOH concen-tration (g/dm^3)**	**Oxalic acid (ml)**	**KOH (ml)**	**Oxalic acid/KOH (ml/ml)**	**KOH concen-tration (g/dm^3)**	**Oxalic acid (ml)**	**KOH (ml)**	**Oxalic acid/KOH (ml/ml)**	**KOH concen-tration (g/dm^3)**
12.1	12.8	0.95	0.095	14.1	15.3	0.92	0.0936	18.6	20.6	0.90	0.087
18.15	19.2	0.95	0.095	20.0	21.7	0.92	0.0936	20.6	22.8	0.90	0.087
20.4	21.6	0.94	0.094	28.1	30.5	0.92	0.0936	22.6	25.0	0.90	0.088
22.1	23.4	0.94	0.094	30.1	32.7	0.92	0.0936	29.1	32.2	0.90	0.087
26.1	27.65	0.94	0.094	34.1	37.0	0.92	0.0935	31.0	34.3	0.90	0.087
		± 0.01				± 0.01				± 0.01	

Table 4.3: Two standardizations of KOH with oxalic acid using 5 ml burette.

Titration 1				Titration 2			
Oxalic acid (ml)	**KOH (ml)**	**Oxalic acid/KOH (ml/ml)**	**KOH concen-tration (g/dm^3)**	**Oxalic acid (ml)**	**KOH (ml)**	**Oxalic acid/KOH (ml/ml)**	**KOH concen-tration (g/dm^3)**
2.38	2.22	1.072	0.104	2.96	1.98	1.495	0.145
2.91	2.90	1.003	0.097	3.48	2.46	1.415	0.137
3.37	3.43	0.983	0.095	4.04	2.92	1.384	0.134
3.90	4.80	0.813	0.079	4.51	3.44	1.311	0.127
4.38	4.68	0.936	0.091				
		± 0.1				± 0.1	

4.3.2 %FFA determination of olive oils and evaluation of indicators

A second comparison was made between two different indicators. According to the Official Journal of the European Communities,[1] the official method for the determination of the FFA content in olive oils, a 10 ml burette marked off in 0.05 ml graduations is recommended. Due to availability, we however used a 5 ml burette marked off in 0.01 ml graduations. The Official Journal of the European Communities also stated that in the case of oils that are of a darker color, alkaline blue rather than phenolphthalein as the indicator should be used. Most vegetable oils are yellowish-red or amber in color. This color is due to carotenoids and/or chlorophyll present in the oils.[8] Chlorophyll gives oils a green color and is most likely present in some of the oils we worked with based on their appearance. The color of vegetable oils is significant, since it can be an indication of field damage, improper storage or faulty handling during crushing, extraction or rendering processes.[8] We decided to test three different olive oil samples, two of a light yellow color and one of a darker green color.

The indicator phenolphthalein changes from colorless to pink when the endpoint is reached in an acid-base titration. The values in table 4.4, especially those for two of the oils, are not very precise since the color change was difficult to observe. When the oil was added to the ethanol/toluene mixture, the presence of the FFAs should give a colorless liquid due to the indicator and upon the

endpoint being reached after addition of the base, a pink color should be observed. However since these oils are colored yellow or green, the addition of a base did not render a pink color at the end-point, but more of an orange color. This color change was very difficult to observe and the endpoint was easily overshot.

A mass of 0.58 g of KOH was used to prepare the KOH solution, upon which standardization led to the concentration of KOH to be 0.0936 M.

Looking closely at table 4.4, it can be seen that it was only possible to succesfully observe a color change for one of the three oils. The amount of KOH added each time may seem to be close enough to each other, but compared to table 4.5 where alkaline blue was used, it was clear that the %FFAs obtained differed according to the indicator used. In this case however better precision is obtained. The titration for the other two darker oils could not be completed, since it was very difficult to see a color change at the end point.

Table 4.4: Titration of three olive oils with KOH using phenolphthalein as the indicator.

Olive oil	Color of oil	Mass of oil (g)	Ethanol/Toluene solution used (ml)	KOH used (ml)	Individual %FFAs	% FFA with 95% CI
1	yellow	20.11	100	1.09	14.31	14.69 ± 0.41
		20.13	100	1.17	15.34	
		20.13	100	1.10	14.42	
2	dark green	20.18	150	1.06	13.86	12.11 ± 7.89
		20.14	150	0.79	10.35	
3	yellow	20.05	150	1.34	17.64	16.25 ± 6.27
		21.86	150	1.23	14.85	

By contrast to phenolphtalein, the indicator alkaline blue was easy to work with. a 0.5 g portion of alkaline blue was dissolved in 25 ml ethanol to correspond to the 20 g/l value given by the method. Alkaline blue causes a solution to turn from a distinctive blue to a peach color when the end point is reached. Even with the addition of darker olive oils, a blue color is observed in the presence of FFAs. When the base is added dropwise, the blue solution becomes lighter in color turning to green and finally to the peach color at the end point, which is easy to

observe. The results obtained from titrating the three olive oils using alkaline blue as the indicator are given in table 4.5.

Table 4.5: Titration of three olive oils with KOH using alkaline blue as the indicator.

Olive oil	Color of oil	Mass of oil (g)	Ethanol/Toluene solution used (ml)	KOH used (ml)	Individual %FFAs	% FFA with 95% CI
1	yellow	20.00	100	1.02	13.46	13.11 ± 0.02
		19.96	100	0.97	12.83	
		20.05	100	0.99	13.03	
2	dark green	20.01	150	0.41	5.41	5.36 ± 0.01
		19.97	150	0.41	5.42	
		20.05	150	0.40	5.27	
3	yellow	19.99	150	0.82	10.83	11.05 ± 0.01
		19.97	150	0.84	11.10	
		19.98	150	0.85	11.23	

Looking at table 4.5, it seems not only are the results carried out in triplicate close to each other, but the reproducibility (calculations shown in section 4.2.1) obtained in table 4.5 is much better than in table 4.4. These values therefore definitely gave a more acceptable account of the amount of base needed to neutralize the FFAs in the oils.

Comparing tables 4.4 and 4.5 and taking into consideration the easy observable color change of alkaline blue over phenolphthalein for the darker oils and the error values obtained, it is reasonable to conclude that the former indicator is more effective to work with for this procedure, not only in the case of the darker oils, but also for the lighter colored olive oils. When standardization of the KOH was however done, the choice of indicator did not matter and phenolphthalein served its purpose effectively.

4.4 DETERMINATION OF %FFAs IN VEGETABLE OILS OF INTEREST

After determination of the best burette for standardization and best indicator for the titrations, the quantity of FFAs in the six vegetable oils could be determined

based on the method described by the Official Journal of the European Communities (refer to section 4.2.1).[1]

The Official Journal of the European Communities recommended the use of an ethanolic KOH solution but does state that an aqueous KOH solution may be used as long as phase separation does not occur. Since the %FFAs of olive oil was previously determined (refer to section 4.2) using a KOH solution prepared with water, an aqueous KOH solution was also initially used for titration against the six other vegetable oils. Application of this method to all six of the vegetable oils of interest however proved to be problematic. For all six of the oils, upon addition of the KOH solution, phase separation occurred and the aqueous KOH solution did not mix with the ethanol/toluene solution that the oil was dissolved in. The phase separation probably occurs due to compounds that are less soluble in the aqueous KOH solution than the ethanolic solution. The six oils thus most likely contain compounds that are more soluble in ethanol than in water. But these vegetable oils have the same fatty acid residues as olive oil, therefore it is most likely not the compounds that cause phase separation but the amount of the compounds. The amount of saturated fatty acids in olive oils are usually very low, typically these values are 0.1-0.2% myristic, 7-16% palmitic, 1-3% stearic and 0.1-0.3% arachidic, yielding a total amount below 20%.[10] Several of the six vegetable oils, such as marula and mango kernel oils, were found to contain a very high percentage of saturated fatty acids compared to that of olive oil (refer to table 3.7). Saturated fatty acids are less polar than unsaturated fatty acids due to their lack of double bonds yielding longer non polar carbon chains. The polarity of these saturated fatty acids possibly plays a role in the polarity of the solvent used. The dielectric constant of a solvent is an indication of its polarity, the higher the dielectric constant the better a solvent is at insulating (solvating) charges and therefore its polarity is higher. Water has a dielectric constant of 79 which makes it more polar than ethanol with its dielectric constant of 25. The vegetable oils which contain a higher percentage of saturated fatty acid residues in the TAGs cause the oil to be less polar than olive oil and consequently these vegetable oils dissolve better in ethanol than in water. The use of water would therefore cause

phase separation for oils containing a high percentage of saturated fatty acid residues.[11] Some of the oils such as apricot kernel on the other hand had a low percentage of saturated fatty acids but also indicated to be less soluble in water than in ethanol. It is therefore unknown if other compounds also play a role in the solubility of the vegetable oils.

Due to this problem an ethanolic KOH solution instead of an aqueous solution was prepared to determine the %FFAs in the six vegetable oils. This preparation has been detailed in the experimental section 4.2.2.

4.4.1 Determination of %FFAs using alkaline blue as the indicator

Since some of the vegetable oils studied in this work were darker in color and since experiments were carried out on olive oil (section 4.3.2) to determine which indicator was best for the titration of the darker oils it was decided to use alkaline blue as the indicator for the %FFA determination of all the oils. However, not only did this indicator prove to be troublesome with the darker oils but also with the lighter ones. Due to the color of the oils (especially the very dark oils such as avocado pear oil) it was difficult to determine the endpoint consistently. Dark green avocado pear oil, upon addition of the indicator became even darker. The point at which the solution changed color was difficult to determine since several color changes occurred only to lighter greens and never truly became a peach color as seen for olive oils. This resulted in the endpoint being difficult to observe consistently; with the consequence that %FFAs for these oils would have been unprecise and inaccurate. The use of alkaline blue as the indicator, although it worked well for olive oil, was not succesfull for the determination of the %FFAs for the other six vegetable oils. Some of the oils such as avocado pear oil were much darker than the darkest olive oil worked with, and the darker the vegetable oil, the more difficult it was to observe a color change. Figure 4.2 shows a picture of the six vegetable oils indicating some of the darker problematic colored oils. Although it is understandable that the darker color of oils such as avocado pear oil was a problem when using alkaline blue as the indicator, it is unclear why this

indicator was also problematic for some of the lighter colored oils such as apricot kernel oil. It is possible that this oil contained substances which might readily change color during the titration with KOH, so making the %FFA titration with an acid/base indicator not applicable.

4.4.2 Determination of %FFAs using phenolphthalein as the indicator

The %FFAs of the six vegetable oils was re-determined using phenolphthalein as the indicator and this time consistent results were obtained. The problems with endpoint color found while using alkaline blue as the indicator were eliminated with the use of phenolphthalein. Only a single color change occurred. Alkaline blue does not have a distinctive endpoint where it changes from blue to peach but instead has a degree of different colors, such as blue going to lighter blue, then to varying colors of green until it eventually reaches a peach color. Phenolphthalein does not have this problem since it goes directly from pink to colorless. Although still sometimes difficult to observe, there was no degree of lighter colors of oil, which could have made the determination of the endpoint difficult. The %FFAs were successfully determined (refer to table 4.6) by applying the equation as explained in section 4.2.1. It is however important to realize that although alkaline blue as the indicator did not work, phenolphthalein also gave problems (as had been found with olive oil). The endpoint was sometimes difficult to observe due to the darker color of some of the oils, this being the reason why alkaline blue was suggested by the Official Journal of the European Communities. However we still found phenolphthalein to be easier to use than alkaline blue, since a color change representing the end point could be more distinctly observed.

The 5 ml burette worked well for oils where the volume of KOH that was needed to neutralize the FFAs in the oil was less than 5 ml. As can be seen in table 4.6 some oils required more than 5 ml of KOH for neutralization and therefore the 50 ml burette was used. Although the accuracy of volumes decreased, due to the fact that the 50 ml burette has 0.1 ml graduations and not 0.01 ml as the 5 ml

burette did, the larger volumes of KOH used indicated a higher percentage of FFAs present in the oils.

Table 4.6: % FFAs for the six vegetable oils by a titration method.

Oil	Color of oil	Color change observed	Mass of oil (g)	Ethanol/ toluene solution used (ml)	KOH used (ml)	Individual %FFA	%FFAs
Apricot kernel	Light yellow	Pink to yellow	10.03 10.09 10.07	100 100 100	0.75 0.78 0.76	0.178 0.184 0.179	0.18 ± 0.01
Avocado pear	Dark green	Dark green to dark orange/green	9.98 9.99 9.99	~ 150 150 150	6.30 5.55 5.30	1.479 1.302 1.243	1.34 ± 0.35
Grapeseed	Light green	Light pink to green/yellow	19.98 19.99 19.98	150 150 150	0.70 0.80 0.85	0.083 0.095 0.101	0.09 ± 0.03
Macadamia nut	Light yellow	Light yellow to pink	9.98 9.98 9.99	100 100 100	8.05 8.00 7.90	1.890 1.878 1.853	1.87 ± 0.05
Mango kernel	Brown/ yellow	Murky yellow to orange	2.49 2.53 2.50	100 100 100	10.30 10.60 9.70	9.694 9.818 9.213	9.57 ± 0.92
Marula	Yellow	Clear light yellow to pink	2.50 2.50 2.55	100 100 100	14.00 13.90 14.00	13.297 13.202 13.036	13.18 ± 0.38

4.4.3 Results and discussion

In the ^{13}C NMR spectra each of the six vegetable, shown in figure 4.2, the FFA resonances as well as the carbonyl carbon resonances of the fatty acid residues (refer to figure 4.1) are illustrated. By inspection of the peak intensities, comparing the FFA resonance height (circled in red) relative to the carbonyl carbon resonance height for the same oil, the ^{13}C NMR spectra of the six vegetable oils seem to correlate well with the %FFAs determined in table 4.6. The resonances in the ^{13}C spectra indicating the presence of FFAs in the vegetable oils for apricot kernel, grapeseed, macadamia nut and avocado pear oils were very small in comparison to the resonances representing the fatty acid residues. Upon enhancement of the spectrum they could however be observed. This is verified by the very small values in table 4.6 obtained for the %FFAs of these two oils. Marula and mango kernel oils however had larger peaks indicating the presence of FFAs. These peaks were observed as medium in height compared to the major fatty acid residue, olein. In fact the resonances representing the FFAs were larger than the resonances representing the linolein residue for both oils. The percentage linolein determined in table 3.7, roughly around 7%, for these two oils were indeed smaller than the %FFAs determined in table 4.6 of 13.18 and 9.57% for marula and mango kernel oil respectively. It is considered that the lower the %FFAs for a vegetable oil, the higher is the quality of the oil.

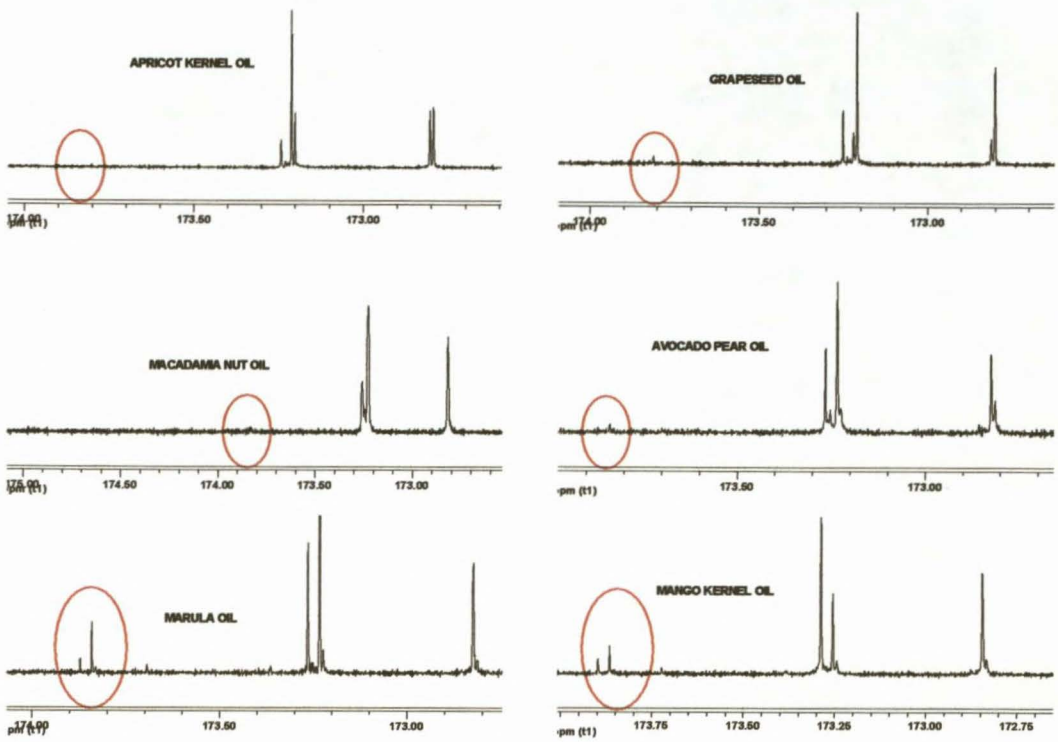

Figure 4.2: Expansion of the ^{13}C NMR spectra of the six vegetable oils illustrating the different amounts of FFA present compared to the carbonyl carbon atoms of the fatty acid residues.

4.5 CONCLUSIONS

Several experiments led to the conclusion that the 50 ml burettes were more accurate for standardization purposes of the prepared KOH solution. Unexpectedly although alkaline blue was proven to be the best indicator for the determination of %FFAs in olive oil, phenolphthalein proved to be more accurate for titration against the other six vegetable oils. We discovered that although an aqueous KOH solution was sufficient for FFA titration for olive oil, phase separation occurred for all six the other vegetable oils. This could quite possibly be due to their higher saturated content. The determination of the %FFAs is however largely dependant on the chemist's abilities and sense of color change.

We found this experiment to be very challenging, whether alkaline blue or phenolphthalein was used, and can easily question the accuracy of the results obtained when represented with data for %FFA values, not only for our own work. In future it will be worth while investigating the precision and accuracy of the other techniques available for FFA determination, i.e. GC and FTIR. However troublesome the titration method was, it proved that the FFAs that were detected by inspection in the ^{13}C NMR spectra were indeed quite substantial in percentage. Although most of the vegetable oils contained very low amount of FFAs (apricot kernel, macadamia nut, avocado pear and grapeseed oil), mango kernel and marula oils indicated rather high values of FFAs, compared to that of a good olive oil. Even FFA values below 2 %, although small, could still be observed in the ^{13}C NMR spectra of the vegetable oils.

4.6 REFERENCES

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CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE WORK

The aim of this project was to assign the ^{13}C NMR spectra of six locally produced vegetable oils upon which no NMR spectroscopic studies have previously been carried out. The oils of interest were apricot kernel, avocado pear, grapeseed, macadamia nut, marula and mango kernel oils.

As a start the fatty acid content of the oils had to be qualitatively determined and it was chosen to do this by GLC-MS analysis for which esterification of the TAGs was first required. Two methods for the esterification were compared and it was shown that esterification using an oil bath is more effective than using an oven for heating. After the esterification was achieved the GLC-MS analysis could be carried out and the fatty acids contained in the oils were identified.

In order to assign the ^{13}C NMR spectra of the major fatty acids in the oils as identified by GLC-MS analysis, a literature method was used which entailed addition of standard TAGs to each oil. This method was referred to as the standard-additions method and it was found to have several disadvantages. Not only did it require expensive standard TAGs but the method was time-consuming requiring ^{13}C NMR spectra to be run overnight. In addition a major concern about this method was that for largely crowded regions of the ^{13}C NMR spectra, in particular the 28-30 ppm region, this standard-additions method failed completely in facilitating the assignment of individual ^{13}C resonances.

As a result of the failure of the standard additions method, an alternative method was developed during this study for the precise assignment of the ^{13}C NMR spectra of these six vegetable oils. This "linear graph method" proved to be highly successful for the assignment of all the TAG fatty acids in the ^{13}C NMR spectra of the vegetable oils of interest, especially in the crowded 28-30 ppm region, particularly when the different type of carbon atoms (i.e. sp^2 and sp^3) were treated separately. Not only was it possible to assign the ^{13}C resonances in this region separately but upon validation of the method using olive oil, the "linear graph method" proved to be

precise and reliable for the assignment of ^{13}C NMR spectra of vegetable oils. As it is known that the ^{13}C chemicals shifts of the fatty acids present as TAGs in vegetable oils are concentration dependent, studies of the effect of concentration changes on the "linear graph method" itself were carried out. These studies indicated that for assignment of the ^{13}C chemical shifts of sp^3 carbon atoms the method is concentration independent while for sp^2 carbon atoms the method is not concentration independent. However over concentration ranges suitable for NMR spectroscopy the method can still be applied to the assignment of the ^{13}C chemical shifts of sp^2 carbons.

Using the newly developed "linear graph method" it was possible to assign the ^{13}C NMR spectra of all six vegetable oils of interest, with some new findings made along the way that require further investigation. During the assignment of the ^{13}C NMR spectra of the six vegetable oils we were unable to assign several of the resonances, especially for marula oil and mango kernel oil which indicates additional unexpected components in the oil. Although to the best of our knowledge these resonances have not yet been to date assigned in the literature, this is a question for further investigation. These resonances could represent possibly other uninvestigated fatty acids (although unlikely due to the fact that they would have been identified during the GLC-MS studies), but most likely are due to other components which are not fatty acids present in these oils.

Another observation made while assigning the ^{13}C NMR spectra of the vegetable oils was that α and β resonances belonging to certain unsaturated carbon atoms appear to be reversed, when compared to their corresponding carbons in the standard TAGs. Further investigation into possible concentration effects or maybe even the influence of other compounds present in the oils could explain this occurrence. For some of the oils (including avocado pear, apricot kernel and grapeseed oils) the resonance representing C9 of the palmitic fatty acid residue could not be detected in the ^{13}C NMR spectra. It would be interesting to investigate why only in some of the vegetable oils this peak is absent and not in all six of the oils.

During the investigation of the fatty acid content of the six vegetable oils ^{13}C NMR analysis detected resonances, which according to literature, could be due to vaccenic

and eicosenoic acid. There is thus some interest in determining which of these two compounds if not both are actually present in the oils. Since these compounds cannot be distinguished by ^{13}C NMR spectroscopy, investigation will continue using GC and GC-MS analysis.

Quantitative studies on these fatty acid components also showed tentatively that ^{13}C NMR spectroscopy could be an effective tool for determining the relative percentages of the major fatty acid residues present in the different oils. This preliminary attempt, although already compared to GLC-MS analysis, showed further validation is required by a more carefully executed GLC-MS study. Another potential application of ^{13}C NMR spectroscopy is for the quantification of α/β ratios of individual fatty acids for oil quality monitoring and authentication. Since this was not carried out for the six vegetable oils in this study, this will be the subject of investigation at a later time.

The FFA content of each of the oils was also determined using a titration method. Several studies were carried out and it was concluded that although olive oil could be titrated using an aqueous KOH solution, the other six vegetable oils could not be as phase separation occurred when the aqueous solution was used. Therefore an ethanolic KOH solution was used for the titrations and this proved successful. Inspection of the relative peaks heights of the FFA ^{13}C signals of each oil compared well to the relative percentages of FFA that were determined as being present in each oil using the titration method.

When considering additional studies that can be carried out apart from those mentioned above, there is scope for the use of ^{1}H NMR spectra for the analysis of these six vegetable oils. Similar studies have already been carried out on olive oil and these showed the power of using ^{1}H NMR spectroscopy for the identification of minor components in the oil which are responsible for attributes of the oil such as its color and taste. In addition it has been found for olive oil that these minor components may have significant health benefits. Thus such similar studies on the vegetable oils that have been researched during this work could yield interesting results.

There are still a wide range of further studies that need to be embarked on in the field of the chemistry of vegetable oils. Our hope it that once we understand the components making up these oils and how they behave in varying conditions, the information obtained can be used to control the quality of these oils for the benefit of the health of our community.