CHARACTERISATION AND OXIDATIVE STABILITY OF SPECIALITY PLANT SEED OILS

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Summary

The past decade has seen an influx of speciality plant seed oils arriving into the market place. The need to characterise these oils has become an important aspect of the oil industry. The characterisation of the oils allows for the physical and chemical properties of the oil to be determined. Speciality oils were characterised based on their lipid and fatty acid profiles and categorised as monounsaturated rich (oleic acid as the major acyl components e.g. Moringa and Marula oil), linoleic acid rich (Grape seed and Evening Primrose oil) or linolenic acid rich (Flaxseed and Kiwi oil). The quality of the oils was evaluated by determining the free fatty acid content, the peroxide value (that measures initial oxidation) and *p*-anisidine values (that determines secondary oxidation products containing the carbonyl function). A reference database was constructed for the oils in order to compare batches of oils for their overall quality including oxidative stability.

For some of the speciality oils, the stereochemistry of the triacylglycerols was determined. Calophyllum, Coffee, Poppy and Sea Buckthorn oils stereochemistry was determined. The oils were enriched with saturated and/or a monounsaturated fatty acids at position *sn*-1 and *sn*-3. The *sn*-2 position of the four oils was esterified with a polyunsaturated and/or a monounsaturated fatty acid indicating that they follow a typical acylation pathway and no novel acylation activity was evident from these studies (e.g enrichment of saturates at the *sn*-2 position).

The oxidative stability of the oils was evaluated at 18^{o}C and 60^{o}C and the effect of adding α-tocopherol at commercially used level i.e 750ppm was assessed. The addition of 750ppm of α-tocopherol at 18^{o}C increased the oxidative stability of Brown flax, Moringa,

Wheat germ and Yangu oils. At 60^{o}C Brown Flax, Manketti and Pomegranate oil polymerised after 48 hours. The addition of 750ppm α-tocopherol delayed the onset of polymerisation by up to 48 hours in Brown Flax seed oil.

Pomegranate oil showed a high resistance to oxidation, and was blended into other speciality oils at 1%. Pomegranate oil increased the oxidative stability of Yangu oil at 18^{o}C. The addition of Pomegranate oil to Wheat germ oil at 60^{o}C, decreased the peroxide content by 10%. In Manketti and Brown Flaxseed oil at elevated temperatures, Pomegranate oil delayed the onset of polymerisation. Preliminary studies of Pomegranate oil blending to Moringa and Borage oil showed it to be more effective than α-tocopherol for certain oils. The antioxidant effects observed following the addition of Pomegranate oil may be due to its conjugated linolenic acid fatty acid, punicic acid.

Key Words: Stereochemistry, Pomegranate, α-Tocopherol, Punicic acid

Dedication

The thesis is dedicated to my late mother and father, Meena and Jagdish Chander. Years have passed since we were all together, you both are deeply missed. I hope I am making you proud.

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List of Contents

List of Figures

List of Tables

List of Graphs

Abbreviations

ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

ACP – Acyl carrier protein

AOCS – American oil chemist's society

AT1 – Glycerol phosphate acyltransferase

AT2 – Lysophosphatidic acid transferase

ATP – Adenosine-5'-triphosphate

BHA – Butylated hydroxyanisole

BHT – Butyl hydroxy toluene

CLA – Conjugated linoleic acid

CLnA – Conjugated linolenic acid

CoA – Coenzyme A

CPT – Cholinephosphotransferase

Cys – Cysteine

DAG- Diacylglycerol

DG- Diacylglycerol

DHA – Docosahexaenoic acid

EI – Electron ionization

EPA – Eicosapentaenoic acid

EPO – Evening primrose oil

ER – Endoplasmic reticulum

FAD – Fatty acid desaturase

FAS – Fatty acid synthase

FFA – Free fatty acid value

FOX – Ferrous oxidation in xylenol orange

GC-FID – Gas chromatography-flame ionisation detection

GC-MS – Gas chromatography-mass spectrometer

GP3 – Glycerol-3-phosphate

HPLC – High pressure liquid chromatography

LDL – Low density lipoprotein

LPA – Lysophosphatidic acid

LPC – Lysophosphatidylcholine

LPCAT – Lysophosphatidylcholine

M/P – Monounsaturated fatty acid and polyunsaturated fatty acid ratio

MA – Malonaldehyde

MAG – Monoacylglycerol

MG - Monoacylglycerol

MUFA – Monounsaturated fatty acid

NADPH – Nicotinamide adenine dinucleotide phosphate

NMR – Nuclear magnetic resonance

NVAs – Non volatile aldehydes

OSI – Oil stability index

PA – Phosphatidic acid

p-A.V. – *p*-anisidine value

PAP – Phosphatidic acid phosphohydrolase

PC – Phosphatidylcholine

PDAT – Phospholipids:DAG acyltransferase

PE – Phosphatidylethanolime

PG – Propyl gallate

PL - Phospholipid

PO – Pomegranate Oil

ppm – Parts per million

PUFA - Polyunsaturated fatty acid

PV - Peroxide value

SE – Sterol Ester

SH – Sulphydryl residue

TAG- Triacylglycerol

TBA – 2-Thiobarbituric Acid

TLC – Thin layer chromatography

TOC- Tocopherol molecule

UFA- Unesterified fatty acid

VLCFAs – Very long chain fatty acids

α-TOC- α-tocopherol

Chapter 1

Introduction

1.1 The Chemistry of Fats and Oils

1.1.1 Lipids, fatty acids and triacylglycerols

Fats and oils are energy rich molecules and in plants are stored in seeds as triacylglycerols (TAGs). Triacylglycerols are the main constituent of seed oils, and are composed of three fatty acids esterified to a glycerol molecule. Fats and oils are categorised as lipids, which underpins their solubility properties [1]. Lipids often encompass a wide range of non polar organic molecular species extracted in chloroform or hexane.

Fatty acids contain a mono-carboxylic group at the end of an acyl chain which can vary in chain length from 4 to 36 carbons, Table 1. Although in plants 16 to 18 carbons are by far the most common fatty acids [2]. Fatty acids can be saturated with a straight chain or unsaturated which have a slight kink, due to the double bond(s), Figure 1.

Fatty acids can be categorised chemically through two different nomenclature systems, the delta (Δ) and omega (ω) system. The delta (Δ) nomenclature shows the position of the double bond from the carboxylic end. For example oleic acid contains 18 carbons with one double bond in the C9 position; this would be given as 18:1(Δ^9). The omega (ω) nomenclature counts from the methyl end, and numbers the double bond positions accordingly. For example α-linolenic acid which contains 18 carbons and three double bonds, at 3, 6 and 9 would be shown as 18:3 ($\omega^{3,6,9}$).

The carboxylic end of the fatty acid is polar, whilst the non polar hydrocarbon chain gives the fatty acid poor solubility in water. The solubility decreases with increasing chain length and degree of saturation. Double bonds in the majority of plants are of *cis* configuration, although *trans* fatty acids have been reported in a number of species for example *Butyrospermum parkii* and *Dolichos lablab* [3]. Non conjugated fatty acids are by far the most common fatty acids found in plant seed oils, although conjugated polyunsaturated fatty acids exist in a small proportion of plants. Punicic acid and α-eleostearic acid are conjugated fatty acids found in Pomegranate and Manketti respectively [2].

Triacylglycerols (TAGs) are composed of three fatty acids that are ester linked to a glycerol; the majority of plant TAGs contain a variety of fatty acids at each position of the glycerol backbone Figure 2. A specific labelling system indicating the attachment of the fatty acids to the glycerol backbone has been employed. Each carbon atom of the glycerol backbone is given a stereospecific number, indicated as *sn*-1, *sn*-2 and *sn*-3 respectively [4].

Figure 1. Saturated and unsaturated fatty acids

A straight chain saturated fatty acid, a) palmitic acid, and a mono unsaturated fatty acid b) oleic acid, which has a slight kink, due to the double bond [2].

Figure 2. Triacylglycerol

The structure of triacylglycerol triolein, with the stereospecific labelling system. [4].

Table 1. Fatty acids found in plant seed oils

Systematic Name	**Common Name**	**Structure**
Dodecanoic acid	Lauric acid	12:0
Tetradecanoic acid	Myristic acid	14:0
Hexadecanoic acid	Palmitic acid	16:0
Hexadec-*cis*-9-monoenoic acid	Palmitoleic acid	$16:1^{\Delta 9}$
Octadecanoic acid	Stearic acid	18:0
Octadeca-9-*cis*-monoenoic acid	Oleic acid	$C18:1^{\Delta 9}$
Octadeca-*cis*-9,12-dienoic acid	Linoleic acid	$18:1^{\Delta 9,12}$
Octadeca-*cis*-9,12,15-trienoic acid	α-Linolenic acid	$18:3^{\Delta 9,12,15}$
Octadeca-*cis*-6,9,12-trienoic acid	γ-Linolenic acid	$18:3^{\Delta 6,9,12}$
Octadeca-*cis*-6,9,12,15-tetraenoic acid	Stearidonic acid	$18:4^{\Delta 6,9,12,15}$
Eicosanoic acid	Arachidic acid	20:0
Eicosa-*cis*-9-monoenoic acid	Gadoleic acid	$20:1^{\Delta 9}$
Docosanoic acid	Behnic acid	22:0
Docsa-*cis*-9-monoenoic acid	Erucic acid	$22:1^{\Delta 9}$

Common saturated and unsaturated fatty acid found in plant seed oils [4].

1.1.2 Fatty acid biosynthesis

The first step for the biosynthesis of fatty acids is the formation of malonyl-CoA from acetyl-CoA [5]. Acetyl-CoA is formed in the plastid of plants through two different pathways. Firstly acetyl-CoA can be formed from the oxidation of pyruvate, which is catalysed by pyruvate dehydrogenase [6]. Secondly acetyl-CoA can be formed from the reaction of acetate with acetyl-CoA synthase, with the consumption ATP, Figure 3 [6]. The formation of malonyl-CoA is an irreversible process, formed from acetyl-CoA and catalysed by acetyl-CoA carboxylase [7]. All three sub units of the enzyme acetyl-CoA carboxylase are employed in the formation of malonyl-CoA, Figure 4 [2].

The four step sequence of fatty acid biosynthesis requires the coordination of separate enzymes collectively termed as fatty acid synthase (FAS). The newly formed malonyl-CoA and acetyl-CoA must first be bound to the two thiols of the FAS. The acetyl of acetyl-CoA is transferred to the Cys sulphydryl (-SH) residue of the β-ketoacyl-ACP synthase, catalysed by acetyl-CoA-ACP transacetylase. The malonyl group from malonyl-CoA to the –SH of ACP, the reaction is catalyzed by malonyl-CoA-ACP transferase [2, 8].

Figure 3. Formation of acetyl-CoA.

Formation of Acetyl-CoA, through the oxidation of pyruvate catalysed by pyruvate dehydrogenase, or the reaction of acetate with acetyl-CoA synthase, with the consumption ATP [6].

Figure 4. Formation of malonyl-CoA

The formation of malonyl-CoA, an irreversible process, formed from acetyl-CoA and catalysed by acetyl-CoA carboxylase [2].

The four step sequence begins with sequential steps of condensation, reduction of carbonyl group, dehydration and reduction of the double bond, Figure 5 and Table 2 [2, 8, 9].

Condensation of the activated acetyl and malonyl groups forms acetoacetyl-ACP, with the loss of CO_2, the reaction is catalyzed by *β*-ketoacyl-ACP synthase III [2, 10, 11]. The reduction of the carbonyl group of acetoacetyl-ACP, yields D-*β*-hydoxybutyryl-ACP. *β*-ketoacyl-ACP reductase catalyses the reaction, with the electron donated from NADPH. Dehydration of D-*β*-hydoxybutyryl-ACP leads to the loss of water and the formation of a double bond. The corresponding *trans*-Δ^2-butenoyl-ACP, is catalysed by *β*-hydroxyacyl-ACP dehydratase [2]. Reduction of the double bond of *trans*-Δ^2-butenoyl-ACP is the final step in the four step sequence. The reaction is catalysed by enoyl-ACP reductase, and the electron is donated by NADPH [2]. The resulting butyryl-ACP is again catalysed by the *β*-ketoacyl-ACP Synthase I. The four step sequence continues, until the 16 carbon acyl chain has been created. The 16 carbon acyl chain can be further extended by two carbons with *β*-ketoacyl-ACP Synthase II to yield 18:0-ACP [8, 9]. The 18:0-ACP can be reduced to 18:1-ACP with stearoyl-ACP reductase [8, 9]. The 16 carbon acyl chain can be desaturated by palmitoyl-ACP Δ^4 desaturase, yielding 16:1-ACP, which has been observed in coriander [9].

The fatty acids can be hydrolysed from the ACP with specific thioesterases. In plants thioesterases have been observed to release fatty acids from ACP at chain lengths 8 carbons and greater [9]. Plant acyl-ACP thioesterases fall into to two distinct categories, FatA and FatB [4]. FatA has shows selectivity towards palmitoyl-, stearoyl-, and oleoyl-ACP [12]. FatB is involved in specialised roles, with a strong selectivity towards

palmitoyl-ACP, specific saturated fatty acids, and a broader specificity for long chain saturated and unsaturated fatty acids [13].

The carnitine shuttle mechanism is responsible for transporting the free fatty acids from the plastid envelope into the eukaryotic lipid pathway. The carnitine shuttle mechanism involves carnitine acyltransferase which allows the reversible exchange of CoA and carnitine onto the fatty acids [14, 15]. The fatty acids enter the endoplasmic reticulum where they are esterified to glycerolipids and further modified by membrane bound enzymes of the endoplasmic reticulum [8, 9].

Desaturation and Elongation

The plastid is the site for fatty acid biosynthesis, such as palmitic (C16:0), stearic (18:0), and oleic (18:1). These fatty acids are transferred to the endoplasmic reticulum for triacylglycerol assembly and formation of modified fatty acids. Unsaturated fatty acids are synthesised by the insertion of double bonds into a saturated precursor [4].

The first double bond introduced to C18 stearic acid is at the Δ^9 position, yielding oleic acid (C18:1Δ^9). The reaction is catalysed by Δ^9 desaturase, a soluble plastidic protein, utilizing 18:0-ACP as its substrate [4]. Studies carried out on the Δ^9 desaturase of castor bean, showed the desaturase to contain a di-iron cluster at the active site [16]. The proposed mechanism of action of the desaturase indicates the removal of two hydrogen atoms, forming a transient di-radical that spontaneously recombines to yield Δ^9 double bond [16, 17]. The desaturase enzyme of the plastid requires reduced ferredoxin as an electron donor and a soluble enzyme which acts on the saturated 18:0-ACP, Figure 6 [6, 18].

Figure 5. Biosynthesis of fatty acids

The four step sequence of fatty acid biosynthesis, increasing the carbon by two carbons through each pass [2, 10, 11].

Table 2. Enzymes of fatty acid biosynthesis

Number	Enzyme
1	Acyl-CoA Carboxylase
2	Acetyl-CoA-ACP transacetylase
3	Malonyl-CoA-ACP transferase
4	β-Ketoacyl-ACP Synthase III
5	β-Ketoacyl-ACP reductase
6	β-Hydroxyacyl-ACP dehydratase
7	Enoyl-reductase
8	β-Ketoacyl-ACP Synthase I
9	Palmitoyl-ACP Δ4 Desaturase
10	β-Ketoacyl-ACP Synthase II
11	Stearoyl-ACP Reductase
12	Acyl-ACP Thioesterase

Table 2. Enzymes required for fatty acid biosynthesis [2, 10, 11].

The oleate formed in the plastid is incorporated into a phospholipid, mainly phosphatidylcholine of the ER. In the ER it can be further desaturated, sequentially by Δ^{12} and Δ^{15} desaturases [19]. The reactions of desaturation are also involved in the synthesis of less common polyunsaturated fatty acids. The synthesis of γ-linolenic acid at position *sn*-2, found in Evening Primrose oil and Borage oil, is attributed to the activity of the Δ^6 desaturase [20]. The Δ^6 desaturase also forms stearidonic acid (C18:4 $^{\Delta 6, 9, 12, 15}$) [21].

The elongation of long chain fatty acids to yield very long chain fatty acids (VLCFAs), requires the use of a sequential four step reaction, which adds two carbons to the chain length. The reactions are similar to the sequential biosynthesis of fatty acids, although there are several differences. A notable difference is that the elongase enzymes, which are membrane bound, and use acyl-CoA and malonyl-CoA substrates directly as the source for the two carbon elongation [22].

The elongases and desaturases from marine algae are involved in the biosynthesis of VLCFAs such as EPA and DHA. The elongases and desaturases have been isolated [23-25]. Algal PUFA elongases have been recently characterised from the marine microalgae *Isochrysis galbana*, *Pavlova lutheri* and *Thalassiosira pseudonana* [26]. The pathway to metabolically engineer plants to biosynthesise VLCFAs is complexed. A recent artificial metabolic pathway has lead to the production of 26% EPA in leaf triacylglycerols [27]. The pathway used the newly identified Δ^6 desaturase from the marine microalgae *Micromonas pusilla* [27]. It was demonstrated that the enzyme appeared to function as an acyl-CoA desaturase, with a preference for ω3 substrates in both *planta* and yeast [27].

Figure 6. Desaturation of C18:0-ACP

C18:0ACP desaturase catalyses the desaturation of C18:0-ACP to C18:1-ACP, with reduced ferredoxin donating the electron [6].

1.1.3 Fatty acid derivatives

Saturated and polyunsaturated fatty acids are the most common and abundant fatty acids in plant seed oils, typically 16:0, 18:0, 18:1, 18:2 and α-18:3. However in nature over 1000 fatty acids have bee identified, some of them are described below, Table 3 [3].

Cyclic fatty acids

Cyclic fatty acids exist in bacteria and plants. The alkyl chain contains a carbon ring either along the chain or at the end of the chain [1, 3]. Cyclopropane and cyclopropene are cyclic fatty acid derivates which are in low abundance in plants and bacteria.[3]. A common cyclopropane fatty acid is lactobacilic acid, found in bacteria. A common cyclopropene is sterculic acid found in plants of the Malvales family, Figure 7 [1, 3]

The biosynthesis of cyclopropane fatty acids has been reported to involve the addition of a methylene group, derived from *S*-adenosylmethione to an existing double bond [28]. The proposed pathway involves the formation of dihydrosterculic acid from oleic acid, followed by the desaturation of the subsequent sterculic acid, Figure 8 [3, 29, 30].

Fatty acids which contain a terminal ring at the end of the alkyl chain are thought to be produced by incorporation of the cyclic acid, rather than acetate at the start of the chain, although the biosynthesis has not been clearly established [3]. Cyclic fatty acids with the terminal group at the end of the alkyl chain are present it the seed oil of *Hydnocarpus* species. Plants of the Flacourtiaceae family contain terminal cyclopentenyl acids of various chain lengths [3].

Table 3. Fatty acid derivatives

Fatty acid derivatives present in plants, bacteria, fungi and mammalian species [1, 3].

Figure 7. Cyclic fatty acids cylcopropane and cyclopropene

Proposed pathway for the biosynthesis of cylcopropene fatty acid Sterculic acid from oleic acid [3].

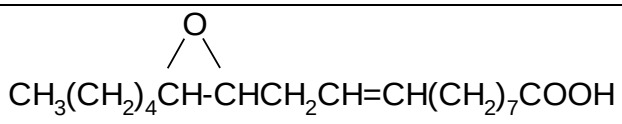
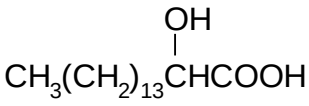
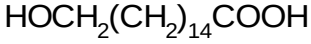
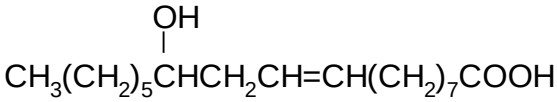
Oxy fatty acids

The occurrence of oxygenated fatty acids in plants is not uncommon. Many fatty acids and their metabolites have oxygen containing functional groups, including hydroxyl and epoxides, Table 4 [1]. The oxygen can be introduced through an enzyme mediated oxidation, with the use of lipoxygenases. The absence of enzymes autoxidation can yield oxygenated fatty acids, of the hydroxyl, keto, and epoxy variety [3].

The introduction of a hydroxy group to the mid chain of fatty acids is through the activity of enzymes. Castor oil, which is rich in ricinoleic acid (12-OH 18:1 Δ^9), contains a hydroxyl rather than a second double bond [31]. The formation of ricinoleic acid occurs in the developing endosperm of castor bean. The direct hydroxylation of oleic acid, which is esterified to the *sn*-2 position of the membrane lipid phosphatidylcholine [31]. The Δ^{12} hydroxylase is a defective desaturase, and does not allow for the insertion of a double bond, leaving a hydroxy group on the corresponding carbon [32, 33]. The hydroxy is a transient intermediate normally formed during double bond formation. *Lesquerella fendleri* seed oil contains almost 60% hydroxy fatty acids, the majority being 20 carbon hydroxy fatty acid lequerolic acid (14-OH 20:1 Δ^{11}) [34]. The developing embryos of *Lesquerella* species have shown that they hydroxylate oleic acid at C12 to form ricinoleic acid. The corresponding ricinoleic acid can be desaturated and elongated to form densipolic (12-OH 18:2 $\Delta^{9, 15}$), lesquerolic and auricolic acid (14-OH 20:2 $\Delta^{11, 17}$) [35, 36].

Epoxy fatty acids can also be found in plant oils. Vernolic acid (12-Epoxy 18:1 Δ^9) being the most common. Vernolic acid can be found in members of the Compositae, Malvaceae and Euphorbiaceae species [3].

Table 4. Oxy fatty acids

Name	Structure
Vernolic acid	
α-hydroxy palmitate	
ω-hydroxy palmitate	
Ricinoleic acid	

Oxy fatty acids structure and name [1].

Vernolic acid biosynthesis has been investigated in *Crepis palaestina* and *Vernonia gaamensis*. The introduction of the epoxide group is through the activity of a Δ^{12} oleic desaturase like enzyme [37, 38]. The cytochrome P450 enzyme is involved in the biosynthesis of vernolic acid in the seeds of *Euphorbia lagascae* [38].

Methylene interrupted fatty acids can form a variety of oxygen containing fatty acid derivatives. The formation of hydroperoxides or endoperoxides catalysed by lipoxygenase enzymes can lead to the formation of, jasmonates, divinyl ether fatty acids and furanoid fatty acids Figure 9 [3].

Jasmonates are formed from the lipoxygenase activity yielding (*9S*)-hydroperoxy and (13S)-hydroperoxy derivates of PUFAs [39]. The corresponding hydroperoxides follow the AOS (allene oxide synthase) pathway and forms unstable allene oxides. The allene oxides are metabolized to (*9S, 13S*)-12-oxophytodienoic acid (OPDA) by an allene oxide cyclase [39, 40]. The hormonal properties of jasmonates help regulate plant growth and development, and are involved in the defence of pathogens and in wound signalling [41]. Jasmonic acid is the product of β-oxidation of 12-oxophytodienoic acid (12-oxo-PDA), and is a known plant oxylipin, Figure 10 [1, 42].

Divnyl ethers are derived from the conversion of hydroperoxides generated from lipoxygenase activity. Divinyl ether synthase is present in leaves and roots, and converts hydroperoxides to divinyl ethers [3]. The 9-hydroperoxides of linoleic and linolenic acid of potatoes leads to the formation of colneleic and colnelenic acids. The presence of these fatty acids has not been detected in other higher plants, Figure 11 [3, 43].

Figure 9. Cyclic endoperoxides

Formation of hydroperoxides catalysed by lipoxygenase enzymes [44].

Figure 10. The biosynthesis of Jasmonic acid

The biosynthesis of Jasmonic acid, through lipase activity and β-oxidation [39, 40].

Hydroperoxides which are derived from the activity of lipoxygenase on linoleic and linolenic acid can also lead to the formation of furanoid fatty acids. The presence of furanoid fatty acids in plants is of low abundance. Furanoid fatty acids were initially isolated from fish and fish oils, although it is now believed that they originate from photosynthetic organisms eaten by fish [45]. Furan fatty acids, contain a furan ring attached to the acyl chain. The most abundant furan fatty acid being F_6, which is a furanoid acid with two methyl groups on the furan ring, Figure 12 [3].

Acetylene and allenic acid

Acetylenic and allenic acid are rare fatty acids, and can be found in very few animals and plants. Allenic acid, of 2,4,5-tetradecatrienoic acid, is present in insects as a sex pheromone. Fatty acids with 5,6 allene can be found in the seed of Labiatae species, laballenic acid (C18:2 $\Delta^{5,6}$), Figure 13, and some *Leucas* species, lamenallenic acid (C18:3 $\Delta^{5,6,Z16}$) [3, 46].

Fatty acids which contain the acetylenic group are tariric acid (16:1 ^{6a}) and crepenyic acid (18:2 $^{9c,\ 12a}$), of *Crepis* species, Figure 14 [3, 37]. The acetylenic bond of *C. alpina* is introduced by a Δ^{12} desaturase like enzyme [37]. The Δ^{12} desaturase is unlike the *Euphorbia lagascae* epoxygenase which uses the cytochrome P450 like enzyme. The Δ^{12} desaturase for acetylenation enzyme of Crepis species had biochemical properties similar to fatty acid desaturase. The formation of the Δ^{12} acetylenic bond forming reactions used acyl chains with a carbon double bond at the Δ^{12} position as a substrate [37].

Conjugated fatty acids

Fatty acids with two or more double bonds that are conjugated, can be found in a small number of plants and animals [1, 3]. Conjugated dienes can be detected in the fats of ruminants, and can account for up to 1% of ruminant fat [3]. The fatty acid is termed rumenic acid contains a *cis* and *trans* bond, C18:2 $\Delta^{E9c,\ Z11}$. Conjugated trienes and tetraenes can also be located in many plant species, Table 5 [3].

Conjugated trienes and tetraenes are produced from methylene interrupted polyenes by a conjugase enzyme similar to Δ^{12} desaturase [47]. The members of Δ^{12} desaturase like enzyme have a variety of functions. In addition to the synthesis of conjugated double bonds, other functions include desaturation, hydroxylation and epoxidation [47]. These collective enzymes have been termed as conjugases [47]. No specific mechanism has been identified for the formation of conjugated fatty acids, although the involvement of conjugase like enzyme has been speculated [3, 47-49]. A proposed biosynthetic pathway for conjugated linolenic acid has included an oxidase reaction [50], and the direct isomerisation of linolenic acid (5,6) [51, 52]. A recent publication has found a class of plant Δ^{12} oleic acid desaturase (FAD2) related enzymes which modifies a Δ^{9} double bond to produce the conjugated trans Δ^{8} Δ^{10} found in calendic acid (18:3 $\Delta^{Z8,\ Z10,\ E12}$) [53]. Besides introducing conjugated double bonds FAD2 class enzymes catalyze the formation of hydroxyl, epoxy and acetylenic groups respectively [32, 37, 54]. Studies of punicic acid from Pomegranate oil have shown a new type of (1,4)-acyl-lipid desaturase. The desaturase converts a *cis* double bond located at Δ^{12} position of linoleic, or γ-linolenic acids to a *cis-trans* double bond system [54]. However the desaturase does not convert α-linolenic acid to the conjugated form. The exposure of heat and light can force

the *cis* double bonds of conjugated trienes and tetranenes to isomerise to the *trans* configuration [3].

Branched fatty acids

A variety of branched chain fatty acids have been identified in bacteria and some animal sources. Branched chain fatty acids are either saturated or monoenes, with the alkyl branch being a methyl group. Branched chain fatty acids with the methyl group in the n-2 (iso) or n-3 (anteiso) carbon are common in bacteria, their presence being taxonomic indicators, Figure 15 [3].

The biosynthesis of these fatty acids follows the standard two carbon chain extension, although the starting two carbon acetate derived unit required for straight chain fatty acid is not employed. The *iso* and *anteiso* branched fatty acids require 2-methyl propionic acid (from valine) or 2 methyl butanoic acid (from leucine), respectively, as their starting unit [3, 55].

Figure 11. Fatty acid derivatives, divinyl ethers

Divinyl ethers, colneleic and colnelenic acids, present in potatoes [3].

Figure 12. Furan fatty acids

Furan fatty acid, F6, a furanoid with methyl groups attached to the furan ring [3].

Figure 13. Allenic acid

Laballenic acid an allenic fatty acid found in the seeds of labiatae species [3].

Figure 14. Acetylenic acid

Acetylenic acids, tariric acid and crepenynic acid [3].

Figure 15. Branched chain fatty acids

Branched chain fatty acids with the methyl group in the n-2 (iso) or n-3 (anteiso) carbon, found in bacteria [3].

Table 5. Conjugated fatty acids

Conjugated fatty acids, found in plants, bacteria, fungi and mammalian species [3].

1.1.4 Triacylglycerol biosynthesis and assembly

In plants the biosynthesis and assembly of TAGs takes place in the membranes of the endoplasmic reticulum (ER). The enzymes for the modification of fatty acids are present in the ER, therefore elongation and desaturation take place in the ER [4]. The Kennedy Pathway or the glycerol-3-phosphate pathway is the major pathway for the biosynthesis of TAGs, Figure 16. TAGs are assembled in a specific sequence, catalysed by a specific acyl transferase enzyme. The synthesis of TAGs also leads to the formation of diacylglycerols (DAGs), monoacyclglycerols (MAGs), and phospholipids (PLs), [4, 56, 57].

Triacylglycerol biosynthesis begins with the acylation of glycerol-3-phosphate (GP3) to form lysophosphatidic acid (LPA), catalysed by glycerol phosphate acyltransferase (AT1) [4]. Secondly LPA undergoes acylation to form phosphatidic acid (PA), catalyzed by lysophosphatidic acid transferase (AT2). AT2 exhibits strong selectivity for 18 carbon unsaturated fatty acids [58]. The PA undergoes a hydrolysis, catalysed by phosphatidic acid phosphohydrolase (PAP), yielding DAGs. DAGs are an important intermediate, from this point on several reactions occurring simultaneously [56].

Firstly the DAG can undergo acylation, catalysed by diacylglycerol acyltransferase AT3, to form TAGs [59]. Secondly the DAGs formed from the Kennedy pathway can form TAGs and MAGs, catalysed by transacylation (TA), the reaction is freely reversible [60].

The DAGs from the Kennedy pathway can also freely interconvert in a reversible reaction to form phosphatidylcholine (PC), catalysed by cholinephosphotransferase (CPT) [61]. The PC can contain oleic acid at the *sn*-2 position, which enters the acyl-CoA pool from the plastid. The reaction is reversible and is catalysed by

lysophosphatidylcholine (LPCAT)[62]. The reverse reaction of LPCAT facilitates the exchange of fatty acids from their site of desaturation and into the acyl-CoA pool for elongation [63]. The activities of LPCAT have been cloned from mammalian sources [63-65]. LPCAT demonstrate a strong selectivity of lysophosphatidylcholine (LPC) as an acyl-CoA acceptor, no data as to their reverse reaction has been forwarded [63-65].

Figure 16. Biosynthesis of triacylglycerols

Biosynthesis of triacylglycerol through the Kennedy Pathway, and the new independent pathway for the biosynthesis of triacylglycerols, this pathways sees phospholipids:DAG acyltransferase (PDAT) being employed [4, 56].

An acyl-CoA independent pathway for TAG synthesis has been discovered, phospholipids:DAG acyltransferase (PDAT), catalyses the reaction between DAG and PC to yield TAGs and lysophosphidylcholine, Figure 16 [57]. Since PC is the site for substitution for Δ^{12}, Δ^{15} and Δ^{6}, it offers the opportunity for acyl groups to be further desaturated during the exchange.

1.1.5 Oil bodies

Storage triacylglycerols are deposited in the cells of developing oil seeds. The so called oil bodies or oleosomes are spherical at early stages of seed development, and are slightly greater than 1μm diameter [66], although the size can vary depending on species [67]. The biogenesis and ontogeny of the oil body has been under research, with two accumulation pathways reported. Firstly it has been suggested that the oil body arises from the accumulation of oil between the phospholipid leaflets of the rough ER, and is then transferred to the cystol [67, 68]. Secondly it has been reported that nascent oil bodies exist in the cystol [69]. In these circumstances microsomal membranes from developing oil seeds catalysed triacylglycerol formation, and the oil accumulated in discrete droplets in the reaction mixture [70]. Isolated oil bodies are surrounded by a boundary layer of protein and phospholipids, Figure 17 [66]. The proteins surrounding the oil body are unique, and are termed oleosins [67]. Oleosins appear to stabilise the oil body surface and thus synchrony in the oil and oleosin formation would lead to deposition of discrete oil bodies found in oleaceous plant tissues [71].

Oleosins are alkaline proteins with a low molecular mass of 15 to 26 kDa [67]. The amino acid sequences of several oleosins have been obtained. The oleosins have been

obtained from maize (16 kDa), carrot (19 kDa), *Brassica* (19 kDa), and soybean (24 kDa) [72-77]. Although the oleosins vary in molecular mass, they all possess the following three structural domains. Firstly an amphipathic domain of 40-60 amino acids at the N-terminus. Secondly a totally hydrophobic domain of 68-74 amino acids located at the centre. Thirdly an amphipathic α-helical domain of 33-40 amino acids situated at or near the C-terminus. Oleosins have been proposed to serve a structural role and to act as a specific site for lipase binding during seed germination [67].

The energy source for seed germination arises from the *β*-oxidation of the free fatty acids from TAGs of the oil bodies, which have undergone lipase activity. *β*-oxidation is the reversal of fatty acid synthesis, the products of *β*-oxidation are glycerol-3 phosphate and acetyl-CoA. Although the process is loosely termed as a reversal of fatty acid biosynthesis it must be stressed that different enzymes are employed to catalyze the reactions.

1.1.6 Triacylglycerol molecular species

The composition of TAGs is dependant on the biosynthesis and the enzymes present. The triacylglycerol molecular structure is of importance in food chemistry and technology industries. The TAG molecular structure can be directly correlated to the physical and chemical properties of food. Physical properties of fats and oils, such as the crystal structure, solubility, viscosity and melting points are influenced by the molecular structure of TAGs [78]. The biosynthesis of TAGs favours a saturated fatty acid acylated in position *sn*-1.

Figure 17. Formation of Bodies, Oleosome.

Oil body formed in the ER membrane encased in a monolayer of lipids. An oil body (Oleosome), with oleosins (proteins) attached to the lipid monolayer [6].

1.2 Fats and Oils Commercialisation and Speciality Products

1.2.1 Commercialization of plant seed oils

The increased demand for fats and oils over the last decade has seen an increase in the production of vegetable oils. Commodity oils such as Palm, Soybean, Rapeseed and Sunflower have seen the majority of the growth, Table 6 [79].

Fats and oils obtained from plant sources are employed in the food and oleochemical industries, where they are used as feed stocks for a variety of products including personal care and cleaning process products. The production of biodiesel is also a contributing factor for the increased consumption of vegetable oils, other industries such as paints and inks, production of polyols, are factors which have lead to an increase in the production of commodity oils [80]. Table 7, shows the production figures for the food and non food uses of vegetable oils.

Palm oil is the highest volume vegetable oil produced and subsequently the cheapest commodity oil. The oil is produced in the developing worlds, with exports from Malaysia and Indonesia being at the forefront of production. During 2008/09 the worldwide production of Palm oil was 42.8 million tonnes, Malaysia and Indonesia combined contributed to 37.2 million tonnes of the annual production [81]. Soybean oil production was surpassed by palm oil production, making Soybean oil the second highest volume vegetable oil produced. The oil is grown in North and South America, with its main application focused on dietary consumption [82]. Rapeseed oil is predominantly grown in Western Europe, China, Canada and India, the oil is employed in the production of biodiesel. The production of biodiesel from Rapeseed oil reached 8.42 million tonnes in 2009 [83, 84]. Sunflower oil is mainly grown in

Russia, Ukraine and Argentina, some EU countries and China. The oil is rich in oleic and linoleic acid, and often used for edible applications [85].

Government biofuel targets have led to an increased demand for vegetable oils, with high inevitability of deforestation for production of rapeseed, soy and palm oils. The loss of subsistence farming has social impacts, and there have been cases of displacement of communities. The loss of agriculture land is also increasing the costs of wheat, corn and other common food amenities.

1.2.2 Speciality oils

Commodity oils are produced in large volumes, and are often know as major plant seed oils. However a wide range of oils can be obtained from lesser known plant seeds, commonly known as speciality oils. These seed oils are produced, sold and used in small quantities. Speciality oils have become of interest due to their minor components, such as the phytosterols and antioxidants [3]. The fatty acids content of speciality and commodity oils does not differ vastly, although for marketability purposes speciality oils have been exploited based on their minor components. The attractiveness to the consumer of an organic oil grown under sustainable conditions, containing natural antioxidants, having a geographical location in Asia or Africa is highly marketable. The applications of speciality oils range from health food supplements, gourmet oils, and cosmetics. Oils can be grouped in to order depending on parameters such as geographical location or physicality of the seed.

Table 6. Production of four major vegetable oils

The Production (million tonnes) of the four major vegetable oils, palm, soybean, rapeseed and sunflower oil, over 16 year period [79].

Table 7. Food and Non Food Consumption of Nine Vegetable Oils.

.Food and non-food consumption (million tonnes and %) of the 9 major vegetable oils between 1994/95 and 2007/08. (Coconut, cottonseed, olive, palm, palm Kernel, peanut, rapeseed, soybean, sunflower oils [80].

Nut Oils

Almonds (*Prunus dulcis, P. amygdalsi, Amygdalis communis*) are a major tree nut, cultivated in areas which exhibit a Mediterranean climate. The almond kernel is the edible part of the nut [86]. Almond oil is monounsaturated fatty acid (MUFA) rich, with oleic acid accounting for 65-70% of the total fatty acid content. The MUFA content of the oil is highly inversely correlated with serium cholesterol levels [3, 86, 87]. Fatty acid profile of almonds and other nut based oils may help prevent cardiovascular diseases and lower plasma cholesterol levels [88-91]. Health benefits are not only associated with the fatty acid profile of the oil but other minor components, such as phytochemicals (β-sitosterol) and antioxidants compounds (tocopherols) [90, 92-95].

The macadamia tree is a large evergreen tree indeginous to the costal rainforests of Australia. With 10 species of the oil being identified, the commercially grown macadamia are limited two species *M. tetraphylla* and *M. integrifolia*, grown in Australia, New Zealand and Hawaii [96]. The oils fatty acid content ranges from C14 to C24, with the monounsaturated content of approximately 40% of the oils content. The high MUFA content is due to the high levels of oleic acid and palmitoleic acid [96].

Hazelnut oil (*Corylus avallana*) primarily sourced from Turkey or New Zealand. The nut contains approximately 55 to 63% oil, with oleic acid being the dominant fatty acid [3]. Diets which contain hazelnuts can have health benefits which include the lowering of blood pressure, and low density lipoprotein (LDL) levels [97-102]. The β-sitosterol content of hazelnuts may help in the prevention of cancer, and reduce cholesterol [97-102].

Manketti oil (*Ricinodendron rauttanenni*) is used in the many regions of Namibia as an emollient. The oil contains conjugated linolenic acid, with eleostearic acid (C18:3 $\Delta^{E9, Z11, Z13}$) being the most abundant conjugated octadecatrienoate [3]. The conjugated linolenic acids can account for 6 to 28% of the oil content, other fatty acids include oleic and linoleic acid [3].

Berry Oils

Sea buckthorn (*Hippophae rhamnoides*) grows in the wild in several parts of Asia and Europe and the hardy bush is now cultivated in Europe, north America and Japan [3]. Two oils can be extracted from the plant, one from the seed and another from the berry (pulp/peel). The seed contains approximately 10% oil, where the peel/pulp can range from 1.4% to 13.7%, depending on morphological characteristics such as size and colour of the berries [103]. The seed oil is rich in linoleic (30-40%) and linolenic acid (20-35%), and the berry contains a significant amount of palmitoleic acid (17-47%) [104]. The seed oil contains 100-300 mg/kg of tocopherols, whilst the berry oil contains 350-500 mg/kg. The soft parts produce an oil with a yellow-orange colour, due to its high content of *β*-carotene [103, 104]. The oil from the sea buckthorn has health benefits ranging from healing wounds, burns, lowering the risk of cardiovascular and cerebrovascular diseases, regulating immunofunctions and attenuating inflammation [103, 104].

The seeds of blackcurrants (*Ribes niger*) are a by product of the juice industry, the seeds can be pressed and the oil extracted. The oil is of importance nutritionally as it contains γ-linolenic acid (C18:3 $^{\Delta 6, 9, 12}$) and stearidonic acid (C18:4 $^{\Delta 6, 9, 12, 15}$), with high levels of tocopherols (1700mg/kg) [105]. The associated health benefits of γ-linolenic acid (C18:3 $^{\Delta 6, 9, 12}$) have been reported with regards to Evening Primrose oil

and Borage oil. In humans there can be a deficiency in the conversion of linolenic acid to γ-linolenic due to the impairment of Δ^6 desaturase [106]. The inclusion of γ-linolenic in the human diet can allow for γ-linolenic to be converted to dihomo- γ-linolenic acid, yielding further metabolites such as arachidonic acids [107]. This theory can be illustrated by atopic eczema and diabetes, which may signify inherited and acquired examples of insufficient Δ^6 desaturase [106, 107].

Grape (*Vitis vinifera*) seed oil's content can range from 6 to 20%, with linoleic being the dominant fatty acid [3, 108]. As with many linoleic rich oils, grape seed shows beneficial effects once applied to the skin. Grapes have long been associated with their high abundance of phenolic compounds such as flavonoids, phenolic acids and stilbenes [109]. Health benefits associated with grape seeds antioxidant properties include prevention of cataracts [110], antihyperglycemic effects [111], and anti-inflammatory effects [112]. The use of the phenolic compounds from grape seed has recently received much interest. The phenolic compounds can be found in a wide range of health foods and dietary supplements [113].

Fruit Oils

Apricot kernel (*Prunus armeniaca*) contains approximately 27-66% oil, with an average content of 47.2%. The unsaturated fatty acid content of Apricot oil has been reported to be 91.5% to 91.8% [114], with oleic acid accounting for 58.3-73.4% and linoleic accounting for 18.8-31.7%. The neutral lipids form 95% of the oil, with glycolipids and phospholipids accounting for 1.3-1.8% and 2% respectively [3, 114]. Apricot oil is a rich source of MUFAs, tocopherols and phytosterols, which may play a beneficial role in human nutrition, by lowering cholesterols and LDL levels [114]. Oil obtained from the kernel of apricot is employed in cosmetic products, with an

emphasis on skin conditioning agents. The oil can also be used as a food source often sold as a speciality oil for cooking [3].

Avocados (*Persea americana and P. gratissima*) are grown in tropical and sub tropical regions. The lipid is often concentrated in the pulp (4 to 25%), although lipids in the seed do exist in lesser amounts (2%) [3]. Nutritionally avocados contain high levels of unsaturated fatty acids, with a significant content of vitamin E (tocopherols 130 to 200 mg/kg), ascorbic acid, vitamin B 6, β-carotene and potassium [3, 115]. Avocado oil can be found in a variety of cosmetic products with skin care applications. The unsaponifiable matter has been reported to provide protection against the sun, based upon its vitamin A and E content [3].

Pomegranate (*Punica granatum*) is a shrub which belongs to the family Punicaceae, which is found in North America, Mediterranean regions and the western part of Asia. Pomegranate oil contains conjugated linolenic acid and punicic acid (C18:3 $^{\Delta 9, 11, 13}$), which has been reported to have a positive effect on many health issues ranging from anti obesity to anti-hypertension [116]. Pomegranate seeds have a significant nutritional value and have been reported antioxidant bioactivity and anti inflammatory activities [117, 118].

Tree Oils

Moringa *(Moringa oleifera, M. stenopetala)* seed contains approximately 35% oil. The oil is rich in oleic acid, with fatty acids including palmitic, stearic, linoleic, linolenic, and eicosenoic acid [3, 119, 120]. The oil has been reported to have high oxidative stability due to its low PUFA content and the presence of a powerful antioxidant flavone myricetin [3]. Moringa belongs to the Moringaceae family, which is native to the sub-Himalayan regions of northwest India [121]. The tree is also

indigenous to many regions of Africa, Arabia, southeast Asia, the pacific, Caribbean islands and south America [122]. *Moringa oleifera* has health benefits which include medicinal treatment of ascites, rheumatism, venomous bites, and as a cardiac and circulatory stimulant [119, 123, 124].

1.3 Lipid Oxidation

1.3.1 Lipid oxidation

The oxidation of lipids, fats and oils can have a detrimental effect on their quality and marketability. The attack of oxygen on the methylene carbons between double bonds of monounsaturated and polyunsaturated fatty acid's produces hydroperoxides. The hydroperoxides can deteriorate further to potentially harmful compounds, such as aldehydes [125]. A variety parameters can cause the onset of oxidation. Free radical and photoxoxidation are to be considered for fats and oils analysed in this project.

The onset of oxidation in PUFAs such as n-3 fatty acids gives rise to products which may be detrimental to human health. The inclusion of n-3 fatty acids into human diets may prevent heart disease, and show anti inflammatory properties [126]. Therefore protecting PUFAs from oxidation is of importance, both in health and longevity of extracted oils. Inhibition of oxidation can be achieved by include the addition of antioxidants, metal inactivators and protecting the oil with nitrogen blanketing.

1.3.2 Free radical oxidation

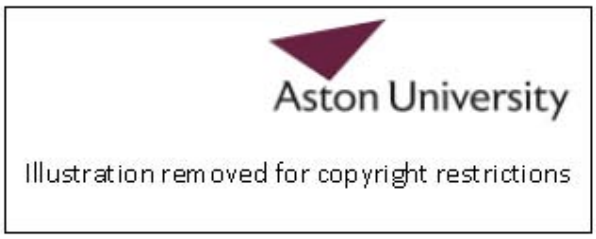
The oxidation of lipids occurs through a free radical chain mechanism, which has three phases termed initiation, propagation and termination. These phases consist of complex sequential and overlapping reactions.

Initiation reactions of an unsaturated fatty acid (LH) occur in the presence of an initiator (I), with the loss of hydrogen radical ($H^{\cdot}$), to yield a fatty acid free radical, Equation 1, [125]. Propagation of the unsaturated alkyl lipid radical ($L^{\cdot}$), occurs with the rapid reaction with molecular oxygen to form a peroxy radical, Equation 2. This

reaction occurs at a greater rate compared to the following hydrogen transfer reaction, the formation of a hydroperoxide, Equation 3, [125].

There are various termination reactions possible. The high content of peroxyl accumulation, can lead to the radicals interacting with one another to form non radical products, Equation 4, [125]. Alternatively at atmospheric pressure termination can occur firstly by the combination of peroxyl radicals, to form an unstable tetroxide intermediate, followed by rapid decomposition to yield non-radical products, Equation 5, [125]. Termination at low temperatures causes the peroxyl radicals to combine and produce dimers, peroxyl linked and molecular oxygen species, Equation 6, [125]. In an environment with low oxygen pressure and elevated temperatures, alkoxyl and alkyl radicals can combine to produce a mixture of dimers. Alkoxyl and alkyl dimers, Equation 7, and carbon carbon dimers Equation 8 [125].

Equations 1 to 8. Free radical oxidation [125]



1.3.3 Hydroperoxide formation

The oxidation of unsaturated fatty acids, initially leads to the formation of hydroperoxides. The mechanism and rate of formation is dependant on the fatty acid undergoing oxidation.

The pathway for the oxidation of oleic acid starts with the abstraction of hydrogen from the allylic carbon-8 and carbon-11, which yields two delocalised three-carbon allylic radicals. The mechanism shows oxygen attack at the end positions of these intermediates to produce an equal mixture of four allylic hydroperoxides, which contain OOH groups on carbons 8, 9, 10, and 11, Figure 18 [125, 127]. GC-MS and HPLC studies showed consistently higher amounts of 8 and 11 OOH (25-29%) compared to 22-25% of 9- and 10-OOH. The oxidation of pure methyl oleate with increasing temperature showed that the amount of *cis*-8-OOH and *cis*-11-OOH isomers decreased. The *trans*-8-OOH and *trans*-11-OOH isomers and the *cis*-9 and *cis*-10 isomers increased, whilst the *trans*-9 and *trans*-10-OOH showed no change [125, 127].

Linoleic acid contains two double bonds, and has been reported to be 40 times more reactive than oleic acid. The increase in reactivity is attributed to its active bis-allylic methylene group at C-11, situated between two double bonds. The abstraction of hydrogen from C-11, yields a hybrid pentadienyl radical, which reacts with oxygen at C-9 and C-13. This produces two conjugated diene hydroperoxides, 9 and 13, Figure 19 [125, 128]. The increased reactivity of linoleate to autoxidation is attributed to the pentadienyl radical intermediate, which can be effectively stabilized by resonance, and the dienoic hydroperoxides formed are stabilized by conjugation [125, 128].

HPLC and ^{13}C-NMR stereochemical studies showed the formation of four *cis, trans* and *trans, trans* conjugated diene hydroperoxides. At low temperatures and early

stages of oxidation, the *cis, trans*-OOH isomers are in greater abundance [125, 128, 129]. The *trans, trans*-OOH isomers increase with the increased temperature and level of oxidation. The *cis, trans* 9- and 13-OOH isomers can be inter converted into mixtures of the corresponding *trans, trans*-OOH isomers [125, 128, 129].

Methyl linolenate has two bis-allylic methylene groups therefore it reacts twice faster with oxygen than linoleate. As the reactivity is twice as fast, the assumption would be that the bis-allylic methylene groups in linolenate are reacting independently of each other. The mechanism is similar to linoleate, although two pentadienyl radicals are formed through hydrogen extraction. Hydrogen is abstracted from C-11 and C-14, with the 1,4 diene systems on C-9 and C-13 for the first radical and C-12 and C-16 on the second radical. The oxygen reacts with the end carbons of each pentadienyl radical, yielding a mixture of four peroxyl radicals, Figure 20 [125, 130, 131].

Analysis of linolenate subjected to autoxidation at various stages and temperatures showed that the 9- and 16-OOH were about four times higher than the 12- and 13-OOH [125, 130, 131].

Figure 18. Oleic acid hydroperoxide formation [125, 127]

9-hydroperoxy-*trans*-10-octadecenoate (*trans*-9-OOH)

11-hydroperoxy-*cis*-9-octadecenoate (*cis*-11-OOH)

10-hydrperoxy-*trans*-8-octadecenoate (*trans*-10-OOH)

8-hydroperoxy-*cis*-9-octadecenoate (*cis*-8-OOH)

Figure 19. Linoleic acid hydroperoxide formation [125, 128]

9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate (*cis, trans*-9-OOH)

9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate (*trans, trans*-9-OOH)

13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate (*cis, trans*-13-OOH)

13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate (*trans, trans*-13-OOH)

Figure 20. Linolenic acid hydroperoxide formation [125, 130]

9-hydroperoxy-*trans*-10, *cis*-12, *cis*-15-octadecatrienoate (*trans, cis, cis*-9-OOH)

13-hydroperoxy-*cis*-9, *trans*-11, *cis*-15-octadecatrienoate (*cis, trans, cis*-13-OOH)

12-hydroperoxy-*cis*-9, *trans*-13, *cis*-15-octadecatrienoate (cis, trans, cis-12-OOH)

16-hydroperoxy-*cis*-9, *cis*-12, *trans*-14-octadecatrienoate (*cis, cis, trans*-16-OOH)

1.3.4 Lipid oxidation detection methods

Detecting lipid oxidation can be conducted at different stages of oxidation. A variety of markers can be detected including hydroperoxides, volatiles, non-volatiles and consumption of oxygen and antioxidants, Figure 21 [132]. Detection of the hydroperoxides is the first marker measured when conducting research into the oxidation of oils. Hydroperoxide measurement coupled with several secondary oxidation compound measurements provides an in depth insight into the oxidation of oils.

Peroxide Value

The peroxide value is a widely used method, and has been used for many years to measure the extent of oxidation in oils. The iodometric procedure can be conducted via titration, colourimetric and electrometric methods. Potassium iodide is used as a reducing agent. When added to the oxidised oil, the liberated iodine can be titrated to a starch end point with sodium thiosulphate, Figure 22 [125].

A ferric thiocyanate method for peroxide value has been developed, based on the oxidation of ferrous to ferric ions. This is determined colourmetrically as ferric thiocyanate. Although it is more sensitive than the iodometric method, the values do tend to be increased by a factor of 1.5 to 2 [125]. The ferric thiocyanate method does have its advantages over the traditional titration method, as it requires a smaller amount of sample and there is minimal waste.

Figure 21. The Kinetics of oil oxidation.

Markers which can be measured throughout the oxidation of oils, including hydroperoxides, volatiles, non volatiles oxygen and antioxidant consumption [132].

Figure 22. Reaction scheme for peroxide value

The liberated iodine from the reaction of potassium iodide with hydroperoxide gives a blue colour with the addition of starch. The addition of sodium thiosulfate binds to the iodine to the sodium and turns the starch colourless [133].

The FOX (ferrous oxidation in xylenol orange) and FOX2 methods have been used to determine hydroperoxide content in edible oils, mammalian tissues and serum. The FOX method is based upon the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions by hydroperoxides , with the binding of Fe^{3+} ion to the ferrous sensitive dye xylenol orange [134]. The technique is sensitive, inexpensive and not affected by ambient oxidation concentrations. The FOX method has been exclusively used for determination of hydroperoxides in membrane systems and mammalian tissue and serum extracts [134, 135]. A modification of the FOX method was developed to determine the hydroperoxide content of edible oils, FOX2 [136].

Conjugated Diene

The conjugated diene hydroperoxides are produced from polyunsaturated lipids. The conjugated diene has a strong absorption at 234 nm, which can be observed spectrophotometerically. The method requires little sample 50-100 mg, and sensitivity makes it a reliable method for the determination of lipid hydroperoxides [137]. The method is often used in the early stages of lipid oxidation, where hydroperoxides undergo little or no decomposition. Conjugated diene hydroperoxide content can only be used with oils which have two or more double bonds (C18:2), therefore oleate rich oils are excluded.

Carbonyl Compounds

The carbonyl content of oxidized lipids can be determined through various methods, the total carbonyl content and *p*-anisidine test, are the most widely used.

The total carbonyl content of oxidized lipids is determined by a reaction of 2,4-dinitrophenylhydrazine. The coloured hydrazone (2,4-DNPH) derivatives are

measured at 430-460nm. The carbonyl value is directly related to the decomposition of the carbonyl compounds, which contributes to flavour deterioration. The assay gives an insight into relevant sensory evaluation [125].

The *p*-anisidine method measures high molecular weight saturated and unsaturated carbonyl compounds [125]. The *p*-anisidine value is defined as the absorbance of a solution resulting from the reaction of 1g fat in 100ml of isooctane solvent and *p*-anisidine reagent. Oxidised linolenic acid and other n-3 PUFAs have a tendency to produce significantly higher *p*-anisidine values than oxidized linoleic acid; due to n-3 PUFAs having a greater tendency to decompose to carbonyl compounds [125]. The reaction of *p*-anisidine with an aldehyde under acidic conditions yields yellowish products which absorb at 350nm, Figure 23 [133].

Carbonyl compounds can be measured through other methods, which involve derivitisation followed by column chromatography or by HPLC or GC [125].

2-Thiobarbituric Acid (TBA) Value

The TBA value measures the rancidity in foods and oxidation in biological systems; although the test is old it still remains popular. The oxidation of PUFAs containing three or more double bonds yields the minor component malonaldehyde (MA), an enolic form of MDA. The MA can be reacted with TBA, to give a pink complex MA-TBA, which can be measured spectrophotometerically at 530-535nm. Figure 24 [125, 133]. Precaution must be taken when using this method to determine the level of oxidation as many MA-like substances can arise in biological systems, which can lead to a false indication of the level of oxidation. Precursors of MA have been suggested including monocyclic endoperoxides and bicyclic endoperoxides, which are produced as secondary products in PUFA lipids containing three or more double bonds [125].

Consequently the TBA test is more sensitive with PUFA lipids and underestimates the oxidation products from lipid that contain oleic and linoleic acid [125]. TBA assay does have limits as it can only be used with oils which contain three or more double bonds.

Gas Chromatography

Gas chromatography has the capability of determining the volatile oxidation products that serve as markers for oxidation [125]. Determining oxidation via GC methods has many advantages, such as high sensitivity and the detection of low levels of oxidation in oils and food sources. The volatile components of oxidation include aldehydes, ketones and hydrocarbons. Volatile compounds can be determined by various GC techniques and methods, namely static headspace, dynamic headspace and direct injection methods [125].

Figure 23. Proposed reaction scheme for *p*-anisidine reaction with oxidised lipids

The possible reactions between *p*-anisidine reagents with the aldehyde malonaldehyde [133].

Figure 24. The reaction of 2-thiobarbituric acid with malonaldehyde

The reaction of TBA with MA to yield the pink complex TBA-MA, which absorbs at 530-535nm [125, 133].

1.3.5 Oxidative stability

Oxidative stability is often defined as an organic compound's resistance to oxidation, and further deterioration which causes rancidity and loss of quality. Various methods have been developed to observe the oxidative stability of lipids, and many tests have been designed to accelerate the normal oxidation process. With such procedures it is possible to understand how the lipid may deteriorate once subjected to oxidation. Understanding the kinetics of oxidative deterioration can allow for correct prevention techniques to limit deterioration.

For research purposes the oxidative stability of an oil is often tested by subjecting it to an accelerated process by elevated temperatures and bubbling with oxygen. It is possible to determine the oxidation of oils at ambient temperature and oxygen levels. Using the methods described above, a significant insight to the oil resistance to oxidation can be evaluated. To fully understand the oxidative stability of oils, measuring the hydroperoxides and secondary oxidation products is important to get an overview of its oxidative stability.

1.4 Antioxidants

1.4.1 An introduction to antioxidants

Antioxidants can inhibit the onset of lipid oxidation with the ability to preserve PUFAs from deterioration. Antioxidants have also become an important additive in foods, chemicals and cosmetics. Antioxidant mechanisms for preserving PUFAs involve retarding lipid oxidation, by interfering with propagation or initiation [125].

Antioxidants can be classed as synthetic or natural. Synthetic antioxidants are effective and relatively cheap. A worldwide trend has emerged in recent years where synthetic additives (antioxidants) have been reduced in foods. With the decline in use of synthetic antioxidants in food sources there has been a growth in the use of natural antioxidants. Antioxidants from plant sources have become considerably attractive due to their benefits as anticarcinogenic agents and inhibitors of biologically harmful reactions in the body [125]. Diets which tend to be rich in plant antioxidants, consumed from fruits and vegetables have been associated with lower risks of coronary heart disease and cancer [125]. The inclusion of 5-a-day fruit and vegetables allows the body to gain important nutrients, which include natural antioxidants. The World Health Organisation have advised the intake of 500 g/day of fruits and vegetables [138]. There is reported evidence that the inclusion of vitamins E, C and β-carotene can reduce the risk of cancer, cataracts and cardiovascular disease [138].

Fruits and vegetables vary in their antioxidant content and levels, determining the antioxidant levels in fruit can be conducted using the ABTS radical cation assay. The assay screens the antioxidant activity. Mono-cation $ABTS^{\cdot+}$ is generated from the oxidation of ABTS with potassium persulfate, and is reduced in the presence of hydrogen donating antioxidants [139]. The assay is applicable for lipophilic and

hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids and plasma antioxidants [139].

1.4.2 Antioxidant mechanism

The chain breaking mechanism of antioxidants inhibits or retards lipid oxidation. The antioxidant interferes with either chain propagation or initiation. This is achieved through hydrogen atom transfer and the antioxidant readily donating hydrogen atoms to lipid alkyl, alkoxyl and peroxyl radicals.

An effective chain breaking antioxidant must have the ability to produce a relatively stable radical ($A^{\cdot}$). These radicals will then react slowly with the lipid substrate LH, Equation 3, but react quickly with peroxyl radical ($LOO^{\cdot}$), Equation 9. The antioxidant radical can then react again with the peroxyl radical to form stable peroxides, Equation 11. The antioxidant radical can also dimerize with another antioxidant radical to form A-A, equation 12 [125].

For the active antioxidant to effectively break the free radical chain, the antioxidant must produce phenoxyl radicals. The radical's unpaired electrons are delocalised around the aromatic structure and stabilized by the resonance energy. The antioxidant radicals of hindered phenols are stabilized by electron delocalisation of their phenoxyl structures. The effectiveness these antioxidants are directly related to the resonance stabilisation of its phenoxy radical [125].

Equations 1 to 12. The mechanism of antioxidants [125].

1.4.3 Synthetic and natural antioxidants

Synthetic antioxidants are applicable to food products as they are stable and can withstand processing conditions. Compared to natural antioxidants, synthetic antioxidants may be more effective when used at lower concentrations [140]. Synthetic antioxidants are relatively inexpensive and impart little effect to the flavour, colour and aroma of the final product [125]. Synthetic antioxidants used for food applications worldwide include BHA, BHT, PG and ascorbyl palmitate, Figure 25 [125].

Flavonoids are natural polyphenolic antioxidant compounds which are diverse in chemical structure and characteristics. Over 4000 classes of flavonoids have been identified with the major classes including flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones [141]. Flavonoids are found in seeds, bark and flowers of plants. Fruits rich in flavonoids are often seen to have a red or blue colour, hence its presence in berries and grapes [142]. Flavonoids in plants, provides protection against ultra violet radiation and pathogens. In humans flavonoids have demonstrated cardio protective effects [142].

The structure of flavonoids consists of two benzene rings combined with an oxygen containing pyrene ring. Different substitutions and further hydroxylation of the subsisted rings can yield a variety of antioxidants, Figure 26 [125]. The metal chelating properties of flavonoids indicates a greater inhibition of metal ion-induced oxidation than peroxyl radical oxidation [125, 143].

Figure 25. Synthetic antioxidants

Synthetic antioxidants used in food sources in low concentrations due to their effectiveness [125].

Rosemary extracts provide a source of natural antioxidants and at present are commercially available in food sources. The two main antioxidant components are phenolic diterpene, carnosic acid and carnosol. Carnosic acid consists of three six-membered rings including a dihydric phenolic ring. Carnosol is a derivative of carnosic acid containing a lactone ring, Figure 27 [125, 144]. Rosemarinic acid is the least effective fraction of rosemary extract, in bulk corn oil and oil-water emulsion it was the least effective extract [144].

Carotenoids are natural pigments and 600 different compounds have been identified with β-carotene and lycopene being the most common, Figure 28 [145]. The conjugated double bond system of carotenoids determines its light absorbing properties which contributes to the antioxidant properties [146]. The antioxidant activity of carotenoids is widely considered to occur through singlet oxygen quenching, and peroxyl radical scavenging.

Figure 26. Flavonoids and hydroxylation products

Flavonoids of the hydroxylation products provide a wide variety of antioxidants [125].

Figure 27. Rosemary extracts

Rosemary extracts include the Carnosic acid, Carnosol and Rosemarinic acid [125, 144].

Figure 28. Carotenoids.

β-carotene and lycopene conjugated double bond system determines their light absorbing properties which contributes to the antioxidant properties [145].

1.5 Vitamin E

1.5.1 Vitamin E, tocopherols and tocotrienols

Oxidation of fats and oils is a process of degradation, through a free radical mechanism. The pathways, rates of reaction and mechanism of degradation have been well documented and illustrated in earlier sections. It can be summarised that peroxidation is greatly affected by other chemical species in the oil as well as the physical conditions the oil is subjected too [147].

Vitamin E is a popular natural antioxidant, although it covers a combination of tocopherols and tocotrienols, all which have been well recognised for their inhibition of oxidation [147]. Tocopherols are present in the seed of plants, but can also be found in the leaves and other green parts of the higher plants. Tocotrienols are not found in the green parts of the plant, they are situated in the bran and germ parts of plant seeds. The antioxidant activity of tocopherols and tocotrienols is due to their ability to donate their phenolic hydrogens to lipid free radicals.

The basic structure of both tocopherols and tocotrienols are similar, and both consist of a chroman head (two rings one phenolic one heterocyclic) and a phytyl chain, Table 8. Tocopherols have a saturated phytyl chain, with varying numbers of methyl substituent's situated along the chain and around the phenolic ring. Tocotrienols also have the same chroman head with the varying methyl substituent's.

Table 8. Tocopherols and Tocotrienols

The structures and Chemical Names of the Tocopherols and Tocotrienols [147].

1.5.2 The effects of tocopherols on lipid peroxidation: Tocopherols as free radical scavengers.

The oxidation of PUFAs have three distinct phases, initiation, propagation, (includes chain branching and chain transfer) and termination [148]. The generation of radicals occurs in the initiation stage of oxidation. The radicals generated are alkyl radicals ($L^{\cdot}$) generated from PUFAs, Equation 1. The alkyl radicals formed are highly reactive and will combine with an available oxygen, at an intense rate, producing peroxy radicals ($LOO^{\cdot}$), Equation 2 [149].

A proposed explanation for the mechanism for antioxidant action of tocopherols, involves the tocopherol molecule (TOH) and the peroxy radical (susceptible to donating and receiving an electron respectively), move into close proximity to each other [150]. Their electron clouds begin to overlap and a transition state with a charge transfer is reached (LOO^{-}---TOH^{+}). Once the tocopherol molecule and the peroxy radical have reached a specific distance, proton tunnelling takes place. The chromanol molecule loses a hydrogen to a lipid peroxy, forming a chromanoxyl radical, Figure 29 [150]. The resultant chromanoxyl radical (TO.) formed, undergoes radical-radical coupling, with other radicals forming various adducts, Figure 30 [151-153].

Chromanols have been shown to be the most effective lipid antioxidants in nature. Firstly this is due to tocopherol phenolic compounds having special structural properties; a phytyl chains which make the lipid insoluble [147]. Secondly lipid peroxy radicals react faster with tocopherols, than their reactions with acyl lipids. One tocopherol molecule can protect 10^{3}-10^{8} PUFA molecules at low peroxide levels [154].

Tocopherols can also either react with alkoxyl radicals ($LO^{\cdot}$), which are formed from the propagation step, Figure 31, or can undergo self coupling to form dimers/trimers, Figure 32. [155].

In rare circumstances where oxygen is present in trace amounts and the hydroperoxides are present in minimal concentrations, tocopherols can then react direct with alkyl radicals ($L^{\cdot}$), Figure 33. The reaction leads to the formation of a tocopherol radical and the lipid substrate.

Figure 29. Formation of a chromanoxyl radical [125]

Figure 30. Formation of adducts [125]

Figure 31. Tocopherols reaction with alkoxyl radicals [125]

Figure 32. The formation of dimmers from tocopherol radicals [125]

1.5.3 Tocopherols as pro-oxidants

An effective antioxidant would produce stable radicals which do not react with stable molecules, such as molecular oxygen, lipid molecules and lipid hydroperoxides.

The above is not always achieved as antioxidants and/or their radicals can often undergo side reactions which can lead to the antioxidant acting as a pro-oxidant. The severity of such pro-oxidant reactions is due to many factors such as the structure of the antioxidant, temperature and concentration, [156, 157].

The pro-oxidant activity of α-tocopherol is related to its tocopheroxyl radical (α-$TO^{\cdot}$), [158]. The assumption being if $TO^{\cdot}$ was found in high concentrations, there would be an increase in the number of side reactions, which could initiate a reaction chain or even increase the rate of peroxidation, [158]. Suggested side reactions which may contribute to pro-oxidant effect of α-$TO^{\cdot}$ include, the reversible reaction of α-$TO^{\cdot}$ with unperoxidised lipids and hydroperoxides, leading to the formation of alkly and peroxy radicals [147]. Tocopherols also have the ability to recycle metal ions, which can act as pro-oxidants in Fenton and Fenton-like reactions [159].

1.5.4 Major factors affecting tocopherol antioxidant potency

Concentration

It has been reported that high levels of antioxidants, α-tocopherol, in vegetable oils can enhance pro-oxidant activity, [147, 160-164]. The pro-oxidant effects is often due to tocopheroxy radical (TO.), [158]. Tests have been carried out from 0-2000ppm, on oils stripped of tocopherols, iron, copper and pigments. The findings of the experiment found that tocopherols are not pro-oxidants themselves, but can act as a pro-oxidant synergistically. Tocopherols incorporated at high concentrations with

known pro-oxidants such as transition metals, lipid peroxides and other oxidising agents, can lead to an overall pro-oxidant effect on an oil [147].

Temperature

The order of tocopherol antioxidant efficiency at low to mild temperatures have been reported to be α>β>γ>δ, and the reverse order for higher temperatures [147, 165]. It has been shown that at higher temperatures and with high concentrations of tocopherol, there was less pro-oxidant effect of α-TOH [166]. This may be due to oils having a lower oxygen solubility at high temperatures. The autoxidative peroxide formation proceeds at lower rates and gradually becomes substituted by a polymerisation reaction. Polymerisation occurs due to the radicals cross linking to high molecular weight molecules. Increased temperature leads to a greater rate of hydroperoxide decomposition and a higher reactivity of transition metal ions. This leads to metal ions catalysing the rate of oxidation and reduction reactions [167]. Temperature effects on the anti/pro oxidant effects of tocopherol within an oil is greatly dependant on the chemical composition and the physical properties of the system [147].

Photoxoxidation

In the presence of light and a photosensitizer (chlorophyll), tocopherols act as free radical scavengers. Although tocopherols can react with singlet oxygen in similar conditions [147, 168]. Previous research has found that stripped oils with the higher levels of α-tocopherol had the best light stability but showed the least stability once aged in the dark at 60^{o}C [169].

The oxidation of PUFAs is accelerated by the exposure of light. Photoxoxidation is due to free radicals produced by ultraviolet light irradiation. These radicals can catalyse the decomposition of hydroperoxides and other peroxide containing species, carbonyl compounds and other complexes of unsaturated lipids [125]. The reaction proceeds via the free radical oxygen mechanism and can be inhibited by chain breaking antioxidants, or ultraviolet light deactivators [125].

Aims of PhD Research

The trading of speciality oils requires quality control measure to be in place, to ensure oils have not been degraded or adulterated. The quality of an oil will ultimately determine its trading possibilities and commercial application. The programme of research aims to firstly conduct quality control measures on a wide variety of speciality oils. Secondly it would be envisaged that the oils can be characterised and a database created to detail the lipid characteristics of the individual oils. The database will provide a future reference when comparing highly degraded and adulterated oils. The database will include commercial assays such as the peroxide and free fatty acid values. The inclusion of the lipid and fatty acid profile of individual oils will also give an insight into the quality of the oil. The stereochemical analysis of the TAGs may reveal underlying novel acylating activity which could insert a saturate at *sn*-2 position.

The deterioration of the oil quality through oxidation is of interest commercially, as the oils must undergo long periods of storage before use. Research carried out centred on oxidation of the oils will include developing and evaluating a suitable method which will accelerate oxidation. Determining oxidation through classical methods and inhibiting the rate of oxidation with the addition of antioxidants will also be conducted.

The understanding of oxidative thermostability of oils and methods to inhibit oxidation is of importance to Earthoil. The inhibition of oxidation with antioxidants is a desirable research venture.

The outcome of the research would be to attain a greater understanding of speciality oils through characterisation and the understanding of oxidative thermostability, with

an emphasis placed on the effectiveness of antioxidants. The data obtained from the characterisation of the oils can be relayed to Earthoil. In circumstance where oils have high free fatty acid and peroxide content, advice can be provided to reduce levels. The oxidative stability of oil was evaluated with the addition of tocopherols and pomegranate oil. The addition would allow for oils to be stabilised for longer periods during storage.

Chapter 2

Materials and Methods

2.1 Materials

All reagents and solvents (HPLC grade only) were purchased from Fisher Chemicals (Leics, U.K.) and Sigma-Aldrich (Dorset U.K.) collectively. Consumables for the Aglinet 6890 GC were purchased from Agilent Technologies (West Lothian, U.K.). Bleaching earths Tonsil 414 FF and 210 FF were supplied by Süd Chemie AG, (Moosburg, Germany). Fullers bleaching earth was supplied by Eurolabs (Leics, U.K.). Thin layer chromatography plates, Silica G60 were purchased from VWR Int, (Leics, U.K.).

All seed oils were supplied by Earthoil Plantations Ltd (Lichfield, U.K.), from organically grown crops where the oils were obtained by mechanical cold pressing at the site of origin and stored under commercial operative conditions.

2.2 Methods

2.2.1 Formation of fatty acid methyl esters

The oil (20 μl) was purged with nitrogen, followed by the addition of 2 ml 2.5% acidic dry methanol (sulphuric acid). The solution was refluxed at 70^{o}C for 1 hour, then removed and cooled to room temperature. The fatty acid methyl esters were extracted with 1.5 ml iso-hexane and 1.0 ml HPLC water. The whole was inverted

several times to ensure both phases have mixed. Once both phases had separated the fatty acid methyl ester upper phase was removed and placed into a 2.0 ml amber vial. Fatty acid methyl esters were analysed by GC-MS and GC-FID (Agilent 6890, MSD 5973), separated with a DB-23 column (50%-cyanopropyl)-methylpolysiloxane. Fatty acid methyl esters (1 µl) were separated by thermo gradient elution 180-240^{o}C, with a 1/50 split injection and a helium flow rate of 1ml/min. The inlet temperature of the GC was set at 250^{o}C with a constant head pressure (230 kPa).

The FID was set at 280^{o}C, the detector gases were hydrogen and air, with flow rates of 40 ml/min and 450 ml/min respectively. The Electron Ionisation Mass Spectrometer (Agilent MSD 5973), transfer line was set at was set at 250^{o}C, with a scan of 40 to 500 amu, threshold of a 100, MS quad at 150 ^{o}C and MS source at 230 ^{o}C.

2.2.2 Punicic acid fatty acid methyl ester

The preparation of punicic acid, fatty acid methyl esters differed from the standard derivitization method. The selected temperature for reflux was 40^{o}C for one hour. The lower temperature was selected to reduce bond migration [170].

2.2.3 Peroxide value

The peroxide value (PV) was determined following the Standard Operating Procedure V.4, Mylnefield Lipid Analysis [171].

The oil (5 g) was purged with nitrogen for 2 minutes, followed by the addition of acetic acid and chloroform (3:2 by vol; 30 ml). The addition of 0.5 ml saturated potassium iodide started the reaction which proceeded for 1 minute. The addition of

distilled water (30 ml) stopped the reaction. Starch indicator (5 ml) was added and titrated to colourless endpoint with 0.01N sodium thiosulphate.

Treatments were preformed in duplicate.

PV= (A-B) x N x 1000 /W

A= Titre of oil sample

B= Titre of blank

N= Normality of sodium thiosulphate

W= Mass of sample

2.2.4 Free fatty acid value

Acid value (free fatty acid value) was determined following AOCS Official Method Cd 3d-63 [172].

Phenolphthalein indicator (2 ml) was added to solvent system isopropyl alcohol and toluene (1:1 by volume; 125 ml), and neutralised with potassium hydroxide (0.1N) until a faint permanent pink colour was visible. The oil (2.5 g) was added to the neutralised solvent mixture, and mixed until it was completely dissolved. The solvent mixture was titrated with potassium hydroxide (0.1N) until the faint permanent pink colour returned. The assay was completed in duplicate.

FFA % Oleic acid = ((A-B) x N x 56.1 /W) /2.81

A= Titre of sample

B= Titre of blank

N= Normality of potassium hydroxide

W= Mass of sample

2.2.5 Conjugated diene [137]

The oil (100 mg) was added to a 100 ml volumetric flask followed by the addition of 75ml iso-octane. The flask was rotated and warmed until all the oil was completely dissolved; the solution was made up to volume with iso-octane. The fat solution was removed and furthered diluted to give a final concentration of 0.5 g/L. The absorbance was measured at 234 nm using a UV-Vis spectrophotometer Jenaway 6505, with the matching blank cell containing iso-octane. The conjugated diene value was reported as a percentage of conjugated dienoic acid.

C.D % = 0.84 $(A_s/bc-K_o)$

K_o = Absorptivity by acid (0.03) or ester (0.07) groups

A_s = Observed absorbance at 233 nm

b = Cell length (cm)

c = Concentration of sample in g/l

2.2.6 *p*-Anisidine

p-anisidine value (*p*-AV) was determined following AOCS Official Method Cd18-90 [173].

The oil (1.0 g) was added to a 25 ml volumetric flask followed by the addition of iso-octane, the whole was inverted several times. The absorbance of the solution was measured at 350 nm, reference cuvette contained iso-octane as a blank. The fat solution (5 ml) was pipetted into stoppered test tubes followed by the addition of *p*-anisidine reagent (1 ml). The whole was inverted several times; the procedure was repeated for iso-octane. After 10 minutes the absorbance of the fat solution was measured with the reference cuvette containing iso-octane *p*-anisidine solution as a blank.

p-AV= $25(1.2 \times A_2 - A_1) / M$

A_1= Absorbance of fat solution

A_2= Absorbance of *p*-anisidine fat solution

M= Mass of sample

2.2.7 Saponification value

Saponification value was determined following the AOCS Official Method Cd 3-25 [174]. The oil (2.0 g) was added to a 50 ml round bottom flask followed by the addition of ethanolic potassium hydroxide (25 ml). The solution was refluxed until all the sample was completely saponified (1 hour). The whole was cooled to room temperature, with avoidance of the solution becoming gel like. Phenolphthalein

indicator was added (1.0 ml) and titrated with hydrochloric acid (0.5M), until the pink colour disappeared. A blank determination was carried out in parallel.

SV= ((B-S) x M /W) x 56.1

B= Titre of blank

S= Titre of sample

M= Molarity of hydrochloric acid

W= Mass of sample

2.2.8 Thin layer chromatography

The sample (5-10 mg) was re-suspended in 100-200 µl chloroform, an aliquot (5 µl) was spotted onto an activated thin layer silica coated thin layer chromatography plate (Silica G60, 20x20 cm). The plate was developed with a polar or non polar solvent system. Once the plate had developed it was dried under nitrogen and stained gently with iodine vapour. The corresponding lipids were located and removed by spraying gently with water and scrapping the silica from the plate. The silica was placed into stoppered test tubes. The sample was covered with dry methanol (1.5 ml) followed by the addition of heptadecanoic acid internal standard (50µl, 1 $mmol^{-1}$). The samples were purged with nitrogen and stored overnight at $4^{o}C$.

The dry methanol was removed under nitrogen and the sample was methylated as given above.

Polar Solvent

Chloroform, methanol, acetic acid and water (85:15:7.5:2.5 by vol; 110 ml), Figure 34 [175].

Non Polar Solvent

Hexane, diethyl ether and acetic acid (70:30:1 by vol 101 ml), Figure 35 [175].

Figure 34. Non polar TLC separation.

Figure 34. Non polar TLC separation of oils, a, cholesterol esters; b, triacyglycerols; c, free fatty acids; d, 1,3-diacylglycerols; e, cholesterol; f, 1,2-diacylglycerols; g, monoacyclglycerols; h, phospholipids.

Figure 35. Polar TLC separation of stereochemistry products

Figure 35. Polar separation of phosphatidylcholine from stereochemical analysis of triacylglycerols; a, free fatty acids; b, phosphatidylcholine; c, lysophosphatidylcholine.

2.2.9 Oxidative stability

The method was conducted in triplicate for each of the oils.

The oil (2.5 g) was placed in a 50 ml conical flask and exposed to the atmosphere for the selected time period (0, 2, 4, 6, 24, 48, 120, 144, 168 hours). At the selected time period the peroxide value was conducted, *Method 1*.

Modifications to *Method 1* included *Method 2*, the oil (2.5 g) being placed in a Pyrex test tube, the peroxide and conjugated diene values determined. *Method 3* the oil (4.5 g) was placed in a 50 ml conical flask and the peroxide, conjugated and *p*-anisidine was determined. *Method 4* (4.0 g) of oil was placed in a 50 ml conical flask the peroxide and *p*-anisidine was determined at 18^{o}C and at 60^{o}C

2.2.10 Stereochemistry [175]

Purification of Triacylglycerols

The crude oil (200 mg) was dissolved in iso-hexane and added to a silica column (5cm x 0.5 cm). The triacylglycerols were eluted with washing the column with 5 column volumes (80 ml) of iso-hexane containing 5% dry diethyl ether. The eluent was evaporated under a steady stream of nitrogen, the corresponding triacylglycerols were re-suspended in 1.0 ml iso-hexane.

Preparation of Grignard Reagent

Magnesium turning (5 pieces) was added to 2 ml of dry diethyl ether followed by the addition of 1 iodine crystal. Bromoethane (0.2 ml) was added, the reaction was heated to 40^{o}C until the reaction began, after several minutes and once the grey precipitate was visible the diethyl ether was evaporated off under a steady stream of nitrogen.

The corresponding Grignard reagent, ethyl magnesium bromide, was diluted to the concentration 0.5M.

Hydrolysis of Triacylglycerols

The purified triacylglycerols (40 mg) was dissolved in 2 ml dry diethyl ether, followed by the addition of 0.5M Grignard reagent (1.0 ml). The mixture was shaken vigorously for 1 minute followed by the immediate addition of glacial acetic acid (0.05 ml) and distilled water (2 ml). The products were extracted with dry diethyl ether (3x10 ml). The extract was washed with 2% potassium bicarbonate solution (5 ml) and distilled water (5 ml).

The *α,β*-diacylglycerols which are required for the preparation of phosphatidylcholine derivatives were isolated by thin layer chromatography on boric acid impregnated plates. The plate was developed in hexane, diethyl ether solvent system 1:1 (100 ml), the isolated *α,β*-diacylglycerols were removed and extracted in 3x5 ml diethyl ether.

Preparation of Phosphatidylcholine Derivatives

To the *α,β*-diacylglycerols a mixture of chloroform, pyridine, phosphorus oxychloride (45.5/47.5/5 0.65 ml) was added. The solution was stored at 4^{o}C for an hour, and then at room temperature for an hour. To the mixture dry powdered choline chloride (200 mg) was added and stirred overnight at 30^{o}C. To the mixture distilled water (20 μl) was added followed by a further 30 minutes of stirring at 30^{o}C, the solvent was removed under evaporation with nitrogen. The products were extracted with chloroform, methanol, water and acetic acid (50:39:10:1 by vol;12 ml), and partitioned with 4M ammonia (4 ml). The products were re-extracted with fresh lower phase (2 ml).

The phosphatidylcholine derivatives were isolated through polar thin layer chromatography. The lipids were eluted from the silica gel with two washes with solvents methanol, chloroform (2:1 by vol; 6 ml). The first wash was for 18 hours and the second was for 2 hours. The solvent was evaporated off under nitrogen, and the lipid residue was re-suspended in chloroform (1 ml), and stored at -80^{o}C until the enzymatic hydrolysis.

Enzymatic Hydrolysis of Phosphatidylcholine

The phosphatidylcholine was dissolved in 2 ml diethyl ether, and sonicated for 10 mins at 0^{o}C with 0.01M Tris-HCl buffer (1 ml), pH 8.9. Phospholipase A_2 (Naja naja, calcium dependent) was added, the enzymatic hydrolysis was allowed to proceed at 25^{o}C for 90 minutes with vigorous mixing. The ether phase was evaporated off under nitrogen, and the products were extracted with acidified butan-1-ol; butan-1-ol, water, acetic acid (1:0.95:0.05 by vol; 1 ml), followed by extraction with butan-1-ol (1 ml). The extracts were combined and evaporated to dryness under a gentle stream of nitrogen. The lipids were re-suspended in chloroform (20 μl) and applied to an activated thin layer chromatography plate. The plate was developed in a polar solvent system; the procedure for thin layer chromatography is followed from this point on. The fatty acids were methylated at this stage and the stereochemistry was elucidated.

2.2.11 Bleaching

Method 1

The oil (1 g) was mixed with 0.33 sodium carbonate (18 ml), followed by the addition of adsorbent (Tonsil 4191FF, 2 mg). The solution was mixed for 5 minutes at 60^{o}C,

followed by the addition of calcium hydroxide (780 mg) and mixed for a further 10 minutes. The solution was filtered; the filtrate was mixed with 2% w/w bleaching earth, and mixed for 15 minutes at 100^{o}C. The mixture was filtered followed by the filtrate being centrifuged at 12 000 rpm. The oil was separated from the solid and analysed.

Method 2

The oil (1g) was mixed with 0.33 sodium carbonate (18ml), followed by the addition of adsorbent (Tonsil 4191FF, 2 mg). The solution was mixed for 5 minutes at 60^{o}C, followed by the addition of calcium hydroxide (780 mg) and mixed for a further 10 minutes. To the heated solution 2% w/w bleaching earth was added, and mixed for 15 minutes at 100^{o}C. The mixture was filtered followed by the filtrate being centrifuged at 12 000 rpm. The oil was separated from the solid and analysed.

2.2.12 Oil extraction

Bligh & Dyer [176]

The seeds (0.1-0.9 g) were homogenised in a pestle and mortar with a solution of chloroform, methanol and 0.15M acetic acid (10/20/7.5 v/v). The homogenate was transferred to stoppered measuring cylinder, with the pestle and mortar being rinsed in chloroform (10 ml) and added to the extract in the measuring cylinder. Distilled water was added (10 ml) and the measuring cylinder was inverted several times to ensure mixing of both phases. Once the phase had separated the lipid containing lower phase was removed, and the chloroform was removed under nitrogen. The corresponding lipids were re-suspended in chloroform (1ml) and stored at -80^{o}C, until used.

Hexane [177]

The seeds (1.0 g) were homogenised with 20 ml hexane, the homogenate was stirred for 2 hours at 4^{o}C. The hexane was removed through filtration and the sample was extracted a further two times. The extracts were pooled together and evaporated with a rotary evaporator at temperature below 30^{o}C. The oil was re-suspended in 1.0 ml hexane, purged with nitrogen and stored at -80^{o}C until analysis.

2.2.13 Punicic acid extraction [170, 178]

Pomegranate oil (10 g) was saponified with 150ml 0.3M KOH/ethanol solution under nitrogen protection for 24 hours at 25^{o}C. The mixture was acidified to pH 1.0 using 1M sulphuric acid. The upper layer containing the free fatty acids was extracted with hexane (20 ml x 3). The crude free fatty acids were obtained by the evaporation of the hexane at temperatures below 30^{o}C, with a rotary evaporator. The punicic acid was purified by recrystallisation twice from 90% ethanol at -20^{o}C. The isolation of punicic acid was determined through GC-MS, following the FAME method given above section, 2.2.1.

2.2.14 Removal of natural occurring antioxidants and tocopherols [179, 180]

The oil (10 g) was placed in a stoppered conical flask containing activated alumina (Aluminium oxide, Brockmann grade 2) (5 g), blanketed with nitrogen and sealed. The mixture was stirred for 48 hours at 25^{o}C, the slurry was removed and centrifuged at 12 000 rpm. The oil was removed and the lower alumina phase was discarded. The antioxidant content of the oils were analysed by Eurofins Laboratories.

Chapter 3

Characterisation of plant seed oils

3.1 Introduction

The introduction of speciality plant seed oils into the market place has created a need for characterisation and quality control of these oils. The fatty acid profile, peroxide (PV) and free fatty acid value (FFA) are standard requirements used when trading oils. The data obtained can affect the price and potential applications of these oils. Oils which contain undesirable high amounts of peroxides and free fatty acids would be considered difficult to trade, as the applications of poor quality oils are limited.

The aim was to characterise a broad spectrum of speciality plant seed oils. Characterisation techniques would include determining the oil content in the seeds, lipid profiling of the oils, and stereochemistry of fatty acids esterified to the triacylglycerols (TAG). The oils will be characterised with assays such as peroxide and free fatty acid value.

The proposed outcome was to create a database containing the characteristic of plant seed oils and to classify the oils according to the major acyl component of the TAGs. The database would be used in the future to asses other oils from the same plant, to determine its quality.

The outcome of the research carried out lead to the formation of a database, which allowed for trends to be distinguished in oils. The database also contained PV and FFA values which are key determinants of the overall quality of an oil. Stereochemical analyses are also included for some of the oils.

3.2 Results

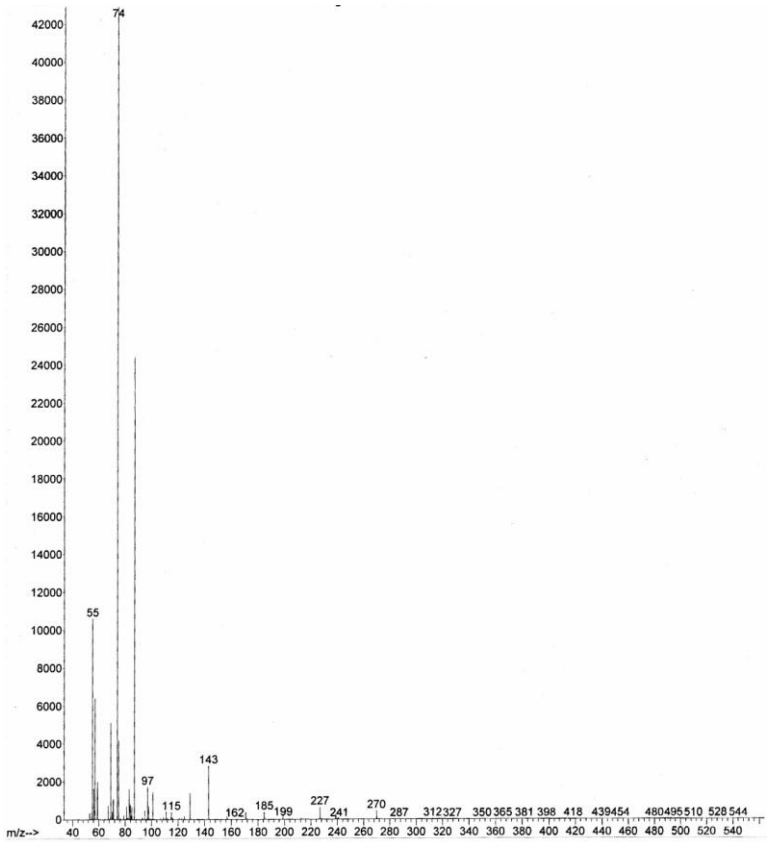
Initially the peaks of the GC chromatograms were identified by electron ionisation mass spectrometry. The key ions were identified through key fragments, and compared with reference spectra provided by the AOCS.

Table 9. Fragmentation ions of fatty acid methyl esters.

Fatty acid	**Library %**	**Fatty acid *m/z***	**Methoxyl ion *m/z* 32**	**McLafferty ion *m/z* 74**	**α ion Δ^9**	**α ion Δ^6**
16:0	94	270	239/241	227	n.d.	n.d.
16:1	99	268	236	194	236	n.d.
18:0	91	298	267	224	n.d.	n.d.
18:1	93	296	264	222	n.d.	n.d.
18:2	91	294	263	220	n.d.	n.d.
α -18:3	90	292	n.d.	n.d.	235	n.d.
γ-18:3	90	292	n.d.	n.d.	n.d.	194
18:4	82	289	n.d.	n.d.	n.d.	194
20:0	72	326	294	252	n.d.	n.d.
20:1	47	324	291	249	n.d.	n.d.
18:1 OH	91	312	n.d.	n.d.	n.d.	n.d.

The table collates the data obtained from mass spectra of fatty acid methyl esters of various seed oils. The ions represent the fragments detected in oil sample.

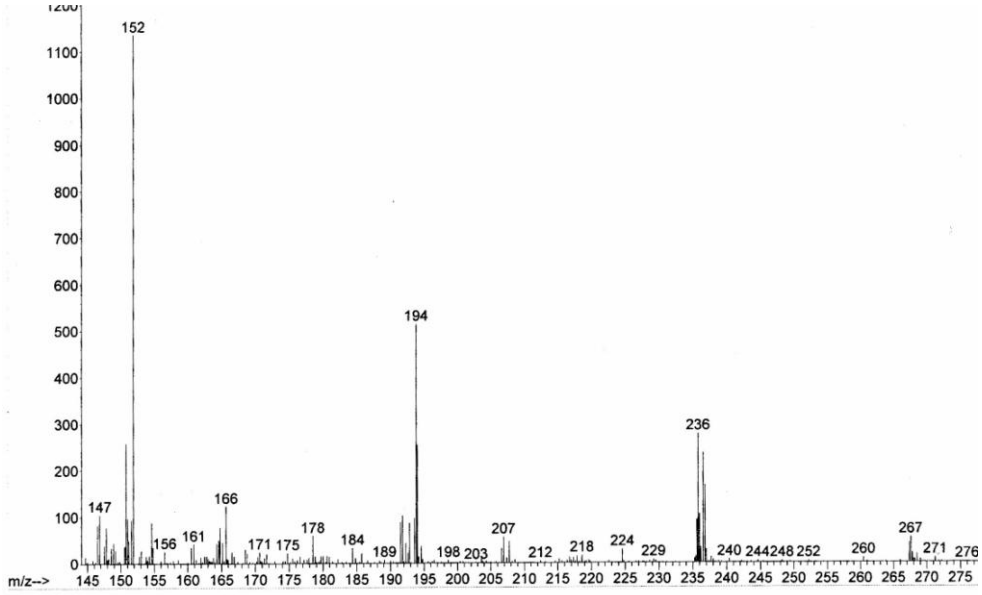
Figure 36. Mass spectra of methyl palmitic acid: sample and reference.



Methyl palmitic acid *m/z* 270 of a seed oil analysed. Key fragments include the loss of the methoxyl ion, *m/z* 239 and the loss of the McLafferty ion, *m/z* 227.

Mass spectra of methyl palmitic acid provided by AOCS archive, Lipid Library [181].

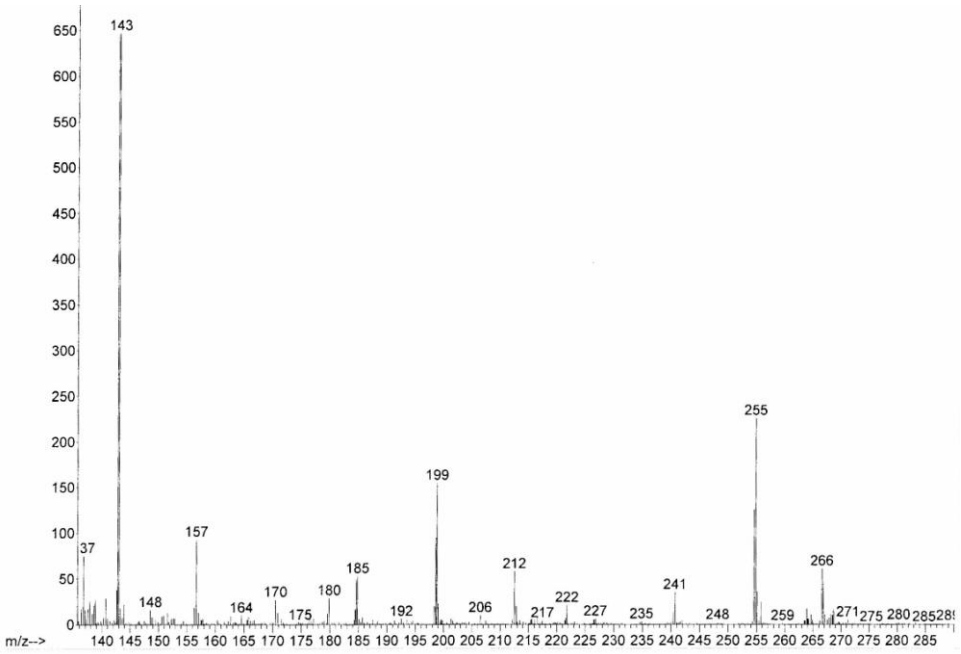
Figure 37. Mass spectra of methyl palmitoleic acid: sample and reference



Methyl palmitoleic acid *m/z* 268 of a seed oil analysed. Key fragments include the loss of the methoxyl ion, *m/z* 236 and the loss of the McLafferty ion, *m/z* 194.

Mass spectra of methyl palmitoleic acid provided by AOCS archives, Lipid Library [182].

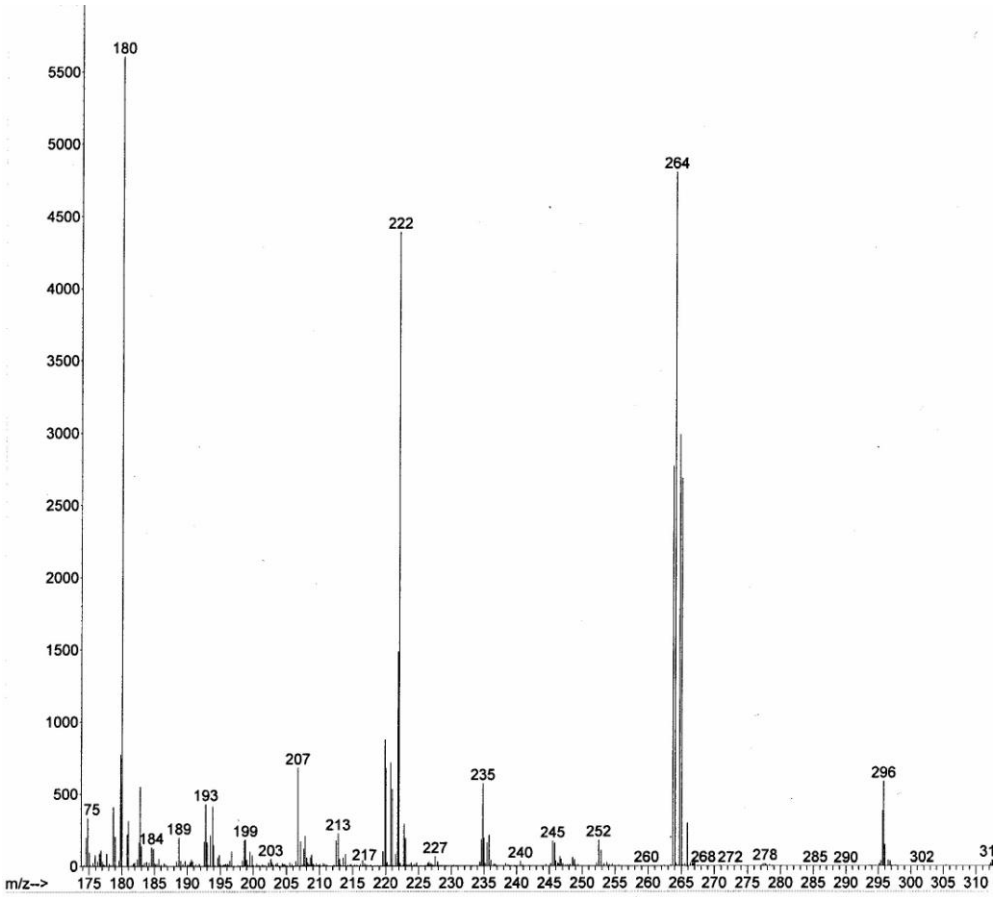
Figure 38. Mass spectra of methyl stearic acid: sample and reference



Methyl stearic acid *m/z* 298 of a seed oil analysed. Key fragments include the loss of the methoxyl ion, *m/z* 266/267 and the loss of the McLafferty ion, *m/z* 224.

Mass spectra of methyl stearic acid provided by the AOCS archives, lipid library [183].

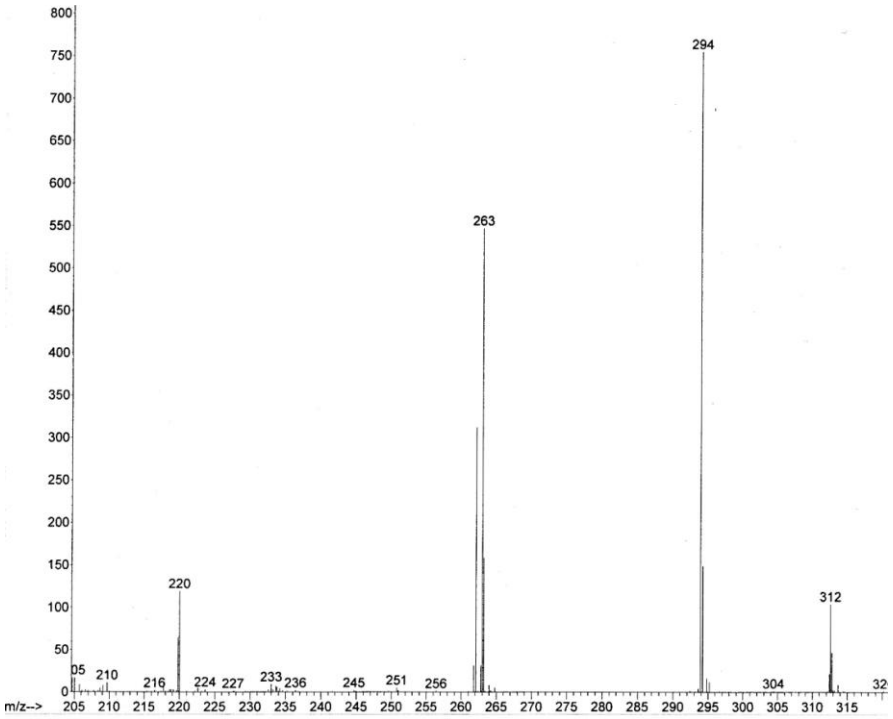
Figure 39. Mass spectra of methyl oleic acid: sample and reference



The molecular ion at *m/z* 296 is the methyl oleate of a seed oil analysed. The ion *m/z* 264 represents the loss of methoxyl group plus a hydrogen atom ($[M-32]^+$).

Mass spectra of methyl oleic acid provided by lipid library [184].

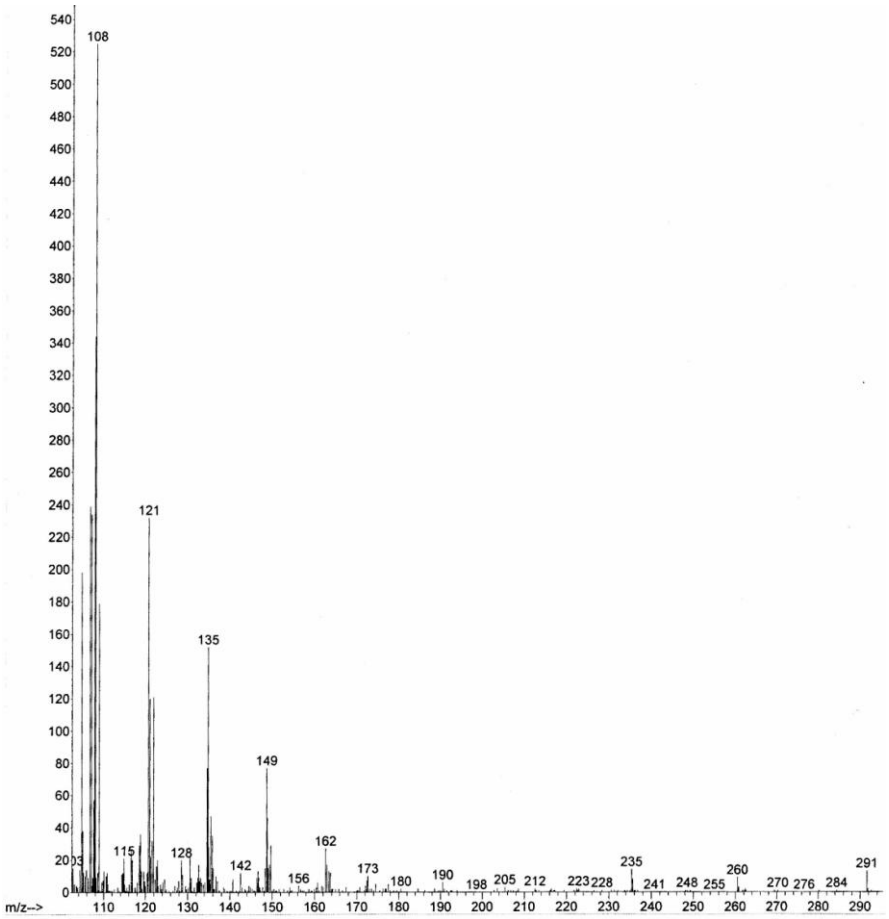
Figure 40. Mass spectra of methyl linoleic acid: sample and reference



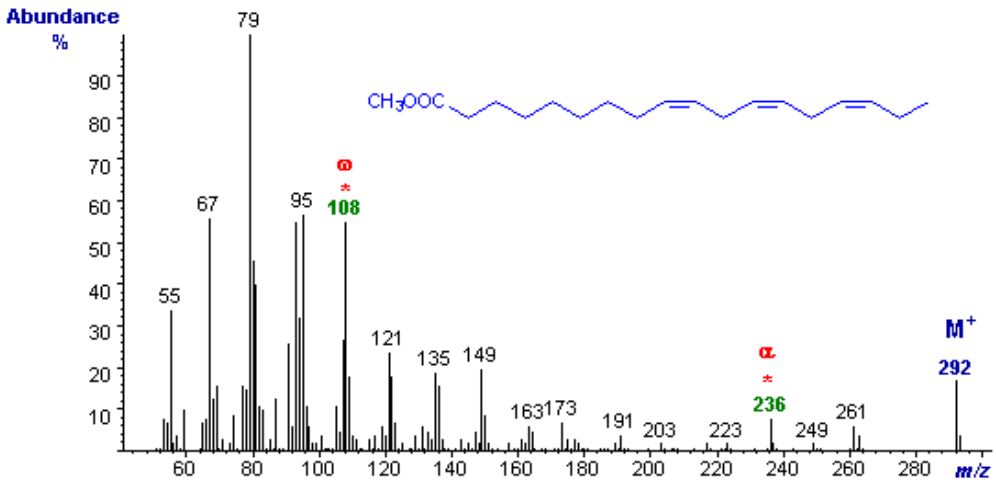
The ion 294 represents the methyl linolenic acid of a seed oil analysed, with the ion at *m/z* 220 the loss of the McLafferty ion. The ion *m/z* 236 is the alpha ion $\Delta^{9,12}$.

Mass spectra of methyl linoleic acid provided by lipid library [185].

Figure 41. Mass spectra of methyl α-linolenic acid: sample and reference

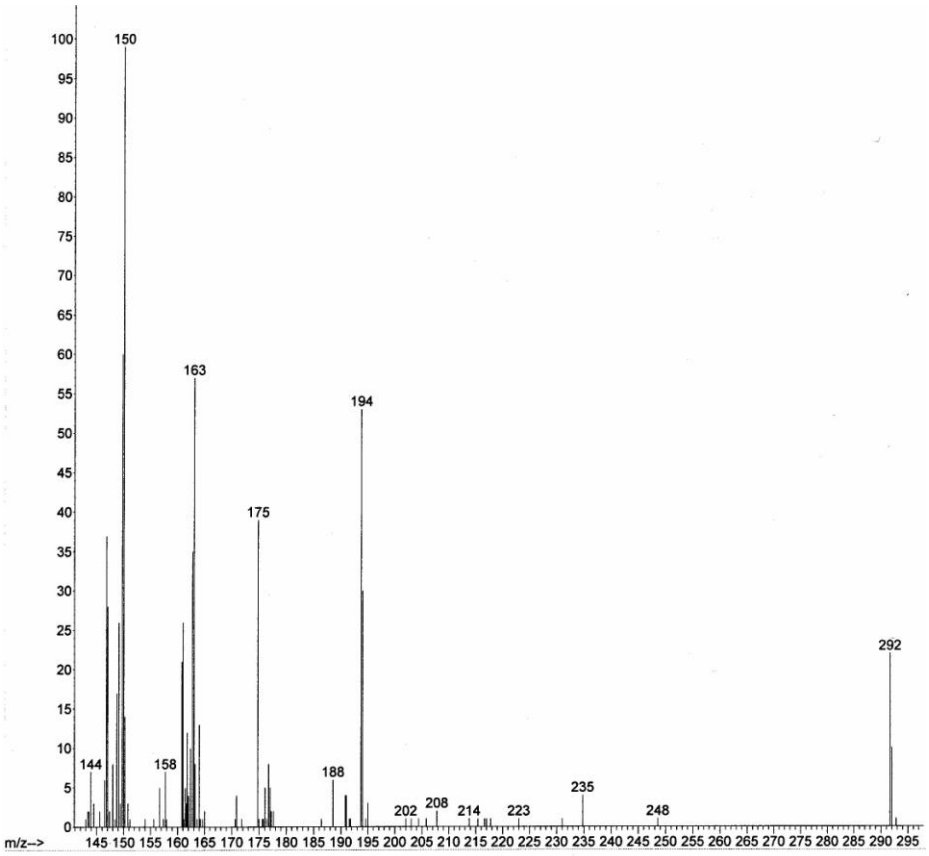


The ion 291 represents the methyl α-linolenic acid of a seed oil analysed. The ion *m/z* 236 is the alpha ion $\Delta^{9,12}$.



Mass spectra of methyl α-linolenic acid provided by lipid library [186].

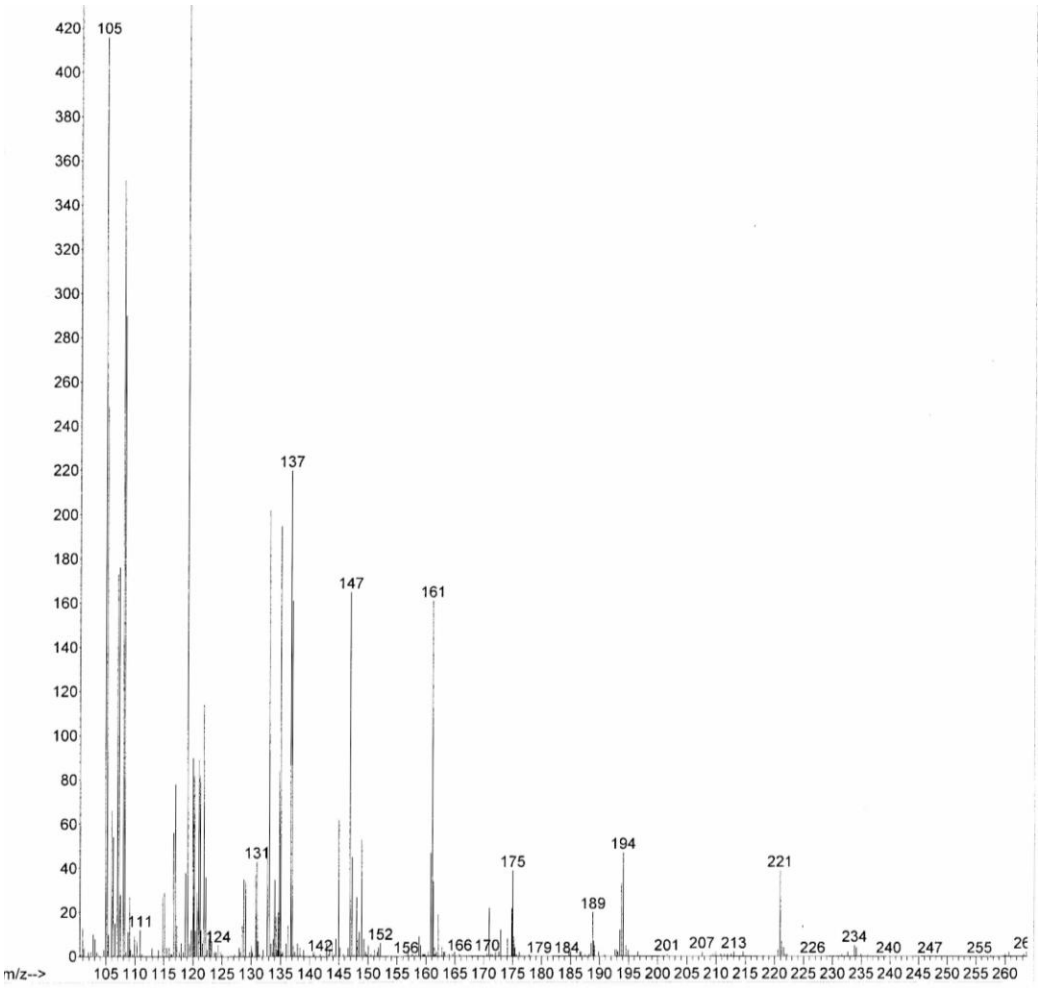
Figure 42. Mass spectra of γ-linolenic acid: sample and reference



The ion 294 represents the methyl γ-linolenic acid of a seed oil analysed.

Mass spectra of methyl γ-linolenic acid [187].

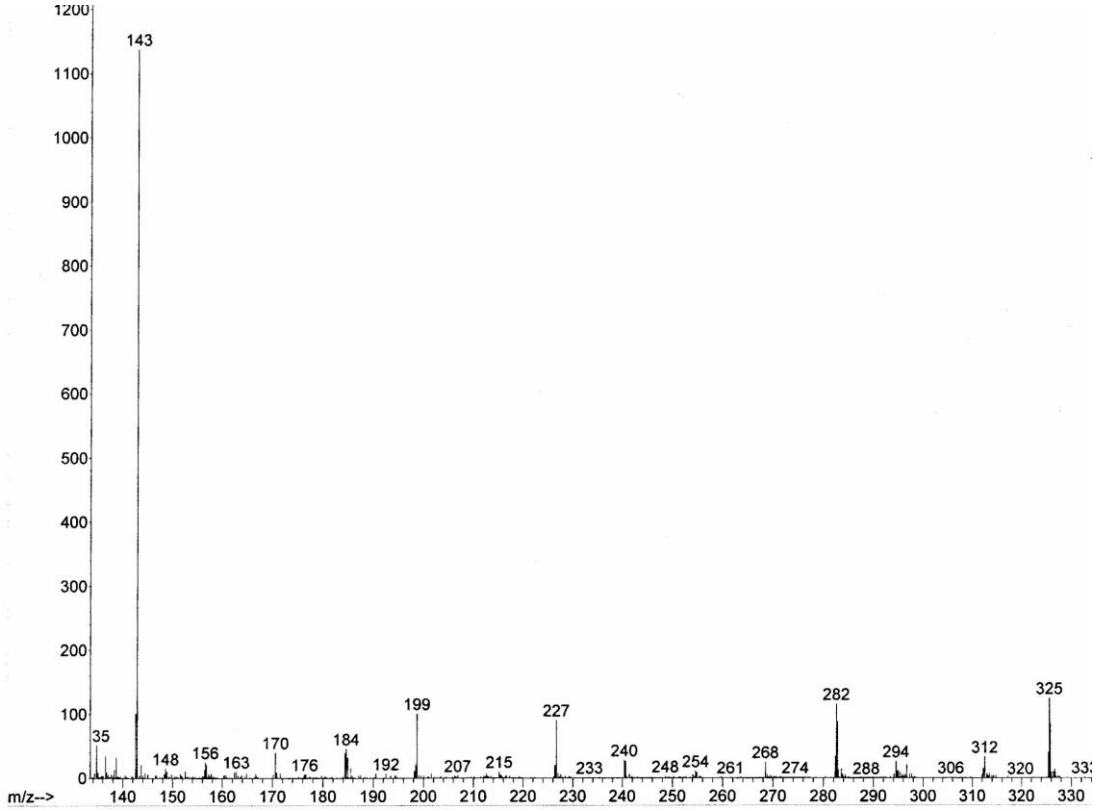
Figure 43. Mass spectra of methyl stearidonic acid: sample and reference



The ion 290 represents the methyl stearidonic acid of a seed oil analysed.

Mass spectra of methyl stearidonic acid [188].

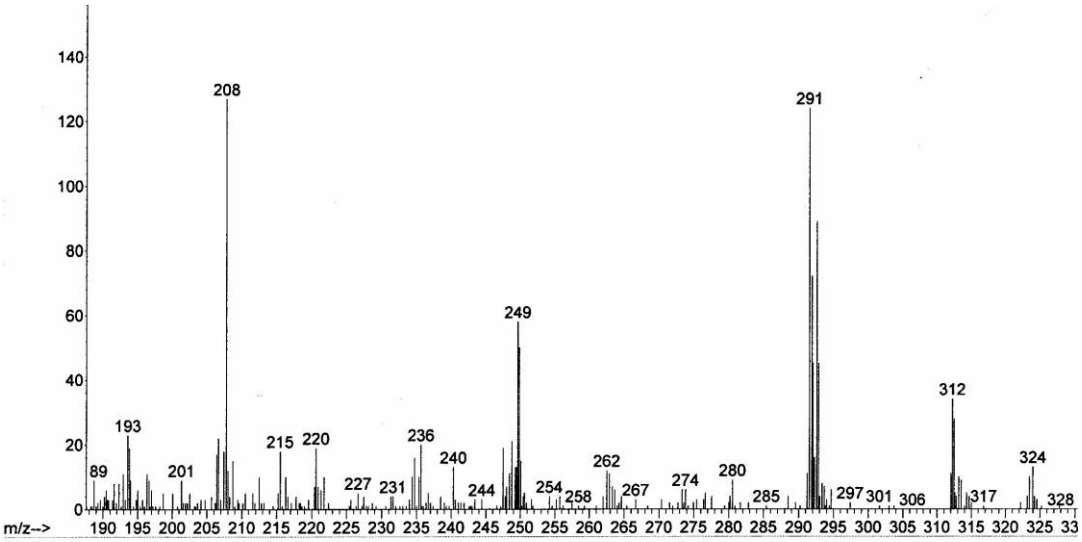
Figure 44. Mass spectra of methyl eicosanoic acid: sample and reference



The ion 326 represents the methyl eicosanoate acid of a seed oil analysed.

Mass spectra of methyl eicosanoic acid [189].

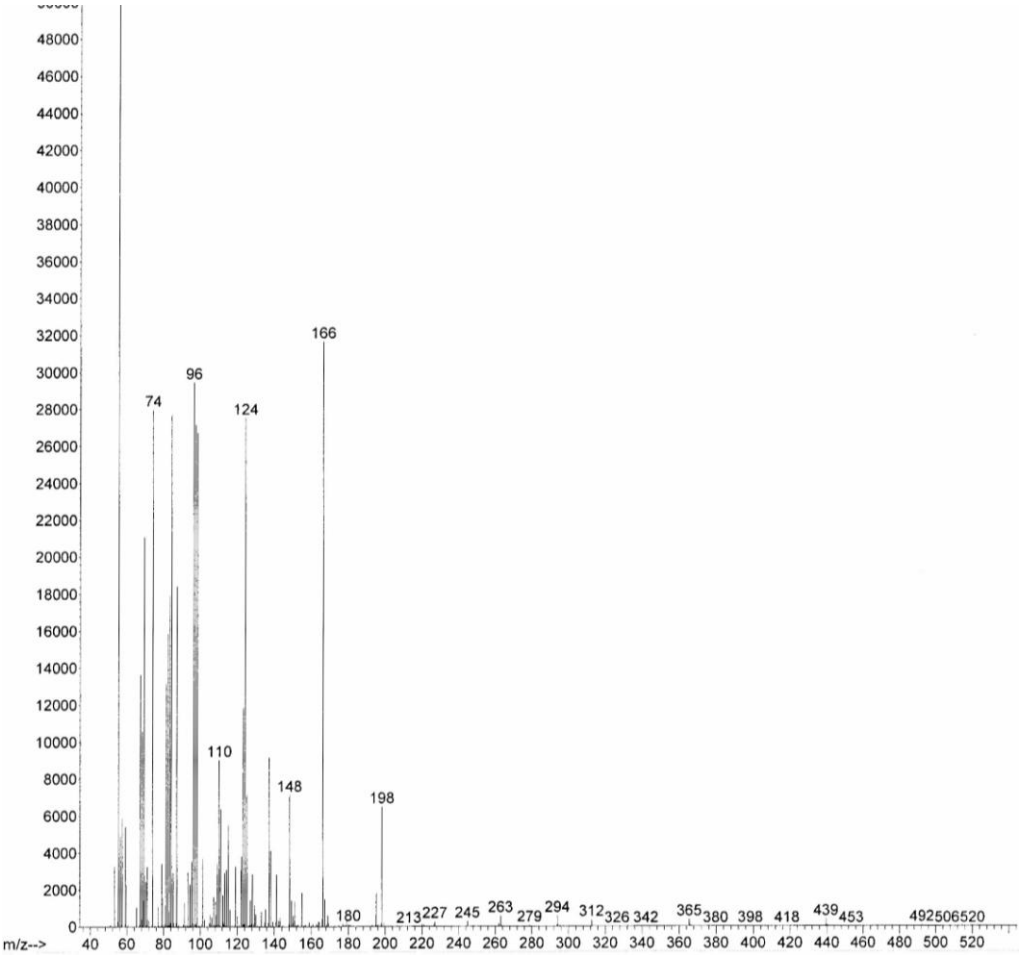
Figure 45. Mass spectra of methyl eicosenoic acid: sample and reference



The ion 292 represents the methyl eicosenoate acid of a seed oil analysed.

Mass spectra of methyl eicosenoic acid [190].

Figure 46. Mass spectra of methyl ricinoleic acid: sample and reference



The ion 312 represents the methyl ricinoleic acid of castor oil analysed.

Mass spectra of methyl ricinoleic acid [191].

Table 10. Oleic acid rich oils.

Oil	<C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	>C20:0	ΣSat	ΣMUFA	ΣPUFA	M/P[1]	PV	FFA
Camellia sinensis		9.3		1.8	80.4	8.6			11.1	80.4	8.6	9.3	7.0 ±3.6	0.9 ±0.5
		±0.5		±0.3	±1.5	±1.1							(*n* = 7)	(*n* = 7)
Moringa		7.3	2.1	4.9	80.0	1.1		4.7 ±2.4	14.3	84.6	1.1	76.9	1.4 ±0.9	0.7 ±0.5
		±0.4	±0.8	±1.3	±3.2	±0.8							(*n* = 9)	(*n* = 9)
Olive		11.8	0.5	3.0	77.9	7.0			14.8	78.4	7.0	11.2	13.8 ±12.3	0.2 ±0.2
		±0.4	±0.6	±0.1	±1.3	±0.2							(*n* = 3)	(*n* = 3)
Marula		11.6		5.2	74.9	8.3			16.8	74.9	8.3	9.0	1.0 ±1.1	2.6 ±0.5
		±2.5		±1.9	±3.3	±1.4							(*n* = 5)	(*n* = 5)
Sweet Almond		6.5	0.6	1.2	71.9	19.9			7.7	72.5	19.9	3.6	4.5 ±5.9	0.4 ±0.2
		±0.2	±0.2	±0.6	±4.5	±3.9							(*n* = 14)	(0.4 *n* = 14)
Hazelnut		6.6		1.4	69.2	22.8			8.0	69.2	22.8	3.0	6.7 ±4.1	1.4 ±0.8
		±1.1		±0.5	±8.1	±7.2							(*n* = 3)	(*n* = 3)
Rapeseed		4.8		0.8	68.2	19.6	6.7		5.6	68.2	26.3	2.6	40.4 ±57.8	0.5 ±0.4
		±0.7		±0.1	±1.6	±1.0	±0.2						(*n* =3)	(0.5 *n* = 3)
Apricot		5.6	0.8	1.0	66.1	26.6			6.6	66.9	26.6	2.5	4.0 ±5.5	0.4 ±0.3

		±0.8	±0.2	±0.2	±3.8	±3.8							(*n* = 12)	(*n* = 12)
Oil	**<C14:0**	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**>C20:0**	**ΣSat**	**ΣMUFA**	**ΣPUFA**	**M/P[1]**	**PV**	**FFA**
Avocado		19.0	8.0	0.3	60.7	11.6	0.4		19.3	68.7	11.6	5.9	6.0 ±4.7	1.3 ±1.0
		±3.6	±2.5	±0.2	±7.3	±2.6	±0.3						(*n* = 13)	(*n* = 13)
Shea Butter		5.0		27.4	59.6	7.7		0.4 ±0.5	32.8	59.6	7.7	7.7		
Nilotica		±0.2		±2.3	±2.2	±0.3								
Macadamia	0.8 ±0.3	9.0	24.3	2.8	56.9	2.3		3.5 ±1.6	14.4	83.3	2.3	36.2	2.2 ±3.1	0.7 ±0.8
		±0.6	±1.5	±0.4	±2.0	±0.4							(*n* = 28)	(*n* = 28)
Shea Butter		4.7		32.9	54.4	7.4		0.6 ±0.5	38.2	54.4	7.4	7.4	4.7 ±3.3	3.6 ±3.2
		±0.6		±6.8	±6.3	±0.7							(*n* = 21)	(*n* = 21)
Andiroba		29.1	1.1	7.6	49.2	12.3		0.8 ±0.4	37.5	50.3	12.3	4.1	15.2 ±11.9	0.9 ±0.4
		±0.3	±0.0	±0.6	±1.1	±0.2							(*n* = 4)	(*n* = 4)
Ground Nut		13.4		5.1	48.2	31.0		2.2 ±0.4	19.0	48.7	31.0	1.6		
		±0.8		±1.2	±0.4	±1.0								
Cherry*		9.4	0.7	42.1	47.5	0.3			51.1	48.2	0.3	160.7		
Neem		19.4		17.0	45.2	18.5			36.4	45.2	18.5	2.4	0.9 ±0.3	2.9 ±1.2
		±1.0		±1.8	±1.4	±1.1							(*n* = 3)	(*n* =3)

Oil	<C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	>C20:0	ΣSat	ΣMUFA	ΣPUFA	M/P[1]	PV	FFA
Calophyllum		14.4	0.1	11.2	42.3	31.8	0.2		25.6	42.4	32.0	1.3	1.3 ±1.2	
		±0.4	±0.1	±3.8	±3.3	±2.3	±0.3						(*n* = 8)	
Palm	1.3 ±0.9	42.0		3.4	42.1	11.2			46.7	42.1	11.2	3.8	5.2 ±7.4	0.1 ±0.1
		±3.7		±0.9	±2.9	±1.6							(*n* = 6)	(*n* = 6)
Yangu		19.1		4.6	42.1	33.5	1.1		23.7	42.1	34.6	1.2	8.5 ±12.2	2.1 ±2.5
		±0.6		±0.3	±1.1	±0.9	±0.1						(*n* = 5)	(*n* = 5)
Rice Bran*	0.2	18.2	0.2	1.4	39.0	38.2	1.6	1.2	21.0	39.8	39.8	1.0		
Acerola Cherry*		24.2	1.8	14.6	38.4	16.8	1.8	2.4	41.2	38.4	18.6	2.1		
Baobab		25.8		4.2	37.0	31.7	1.3		30.0	37.0	33.0	1.1	1.3 ±1.9	0.4 ±0.3
		±1.1		±1.3	±1.4	±2.0	±1.1						(*n* = 11)	(*n* = 11)
Cocoa Butter		28.1		32.0	34.8	5.1			60.1	34.8	5.1	6.8		
		±1.5		±1.8	±1.7	±1.1								
Sea Buckthorn Pulp*		37.1	28.2	1.0	27.9	4.6	1.2		38.1	56.1	5.8	9.7		

Values are a mean of ±SD for 20 samples, nd = not detected, *Represents oils which are not a mean, n=1. PV are values in Meq/kg. %FFA is a percent of oleic acid.
[1]MUFA/PUFA ratio.

Table 11. Linoleic acid rich oils.

Oil	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	>C20:0	ΣSat	ΣMUFA	ΣPUFA	M/P[1]	PV	FFA
Passion Seed	9.1 ±0.5		1.6 ±0.6	11.7 ±0.8	77.7 ±1.5			10.7	11.7	77.7	0.2	4.1 ±1.1 (*n* = 6)	0.4 ±0.4 (*n* = 6)
Guava	8.7 ±1.1		3.2 ±1.6	10.7 ±3.2	77.6 ±2.6			11.9	10.7	77.6	0.1		
Evening Primrose	6.3 ±0.2		1.6 ±0.3	5.6 ±0.7	76.3 ±1.1	10.3 ±0.7		7.9	5.6	86.6	<0.1	13.7 ±18.9 (*n* = 20)	0.4 ±0.2 (*n* = 20)
Grape	7.8 ±0.5		3.5 ±0.9	14.6 ±0.6	74.1 ±1.2			11.3	14.6	74.1	0.2	13.5 ±2.9 (*n* = 6)	0.7 ±0.1 (*n* = 6)
Poppy*	10.0		1.1	17.0	71.2	0.7		11.1	17.0	71.9	0.2	6.0	0.7
Melon*	10.6		4.8	19.2	65.4	0.7		15.4	19.2	66.1	0.3		
Sunflower	6.2 ±0.3		3.3 ±1.4	26.5 ±3.1	64.0 ±2.8			9.5	26.5	64.0	0.4	7.5 ±10.1 (*n* = 12)	0.9 ±1.3 (*n* = 12)
Wheat germ	17.1 ±0.7		4.1 ±6.7	8.9 ±6.7	62.5 ±1.0	7.5 ±0.3		21.2	8.9	70	0.1	13.0 ±16.9 (*n* = 2)	2.7 ±0.2 (*n* = 2)
Oil	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**>C20:0**	**ΣSat**	**ΣMUFA**	**ΣPUFA**	**M/P[1]**	**PV**	**FFA**
Pumpkin	14.2 ±0.4		4.0 ±1.4	21.7 ±0.2	60.2 ±1.3			18.2	21.7	60.2	0.4	20.4 ±16.7	1.3 ±0.1

												(*n* = 3)	(*n* = 3)
Black cumin	13.0 ±0.8		2.5 ±0.9	23.6 ±1.8	60.1 ±1.7	0.1 ±0.2	0.7 ±1.2	15.5	23.6	60.9	0.4	36.0 ±17.8	2.7 ±0.6
												(*n* = 6)	(*n* = 6)
Hemp	6.2 ±0.3		2.3 ±0.1	11.2 ±0.7	57.7 ±0.6	22.2 ±0.8	0.6 ±0.5	8.5	11.2	80.5	0.1	17.7 ±19.9	1.0 ±1.0
												(*n* = 6)	(*n* = 6)
Soybean	11.0 ±0.8		4.0 ±0.4	22.6 ±1.3	55.4 ±0.6	7.2 ±1.1		15.0	22.6	62.6	0.4	8.0 ±7.8	1.2 ±0.5
												(*n* = 2)	(*n* = 2)
Raspberry	2.9 ±0.2		0.7 ±0.3	11.6 ±0.7	55.2 ±1.1	29.6 ±1.3		3.6	11.6	84.8	0.1	46.6 ±39.2	3.0 ±2.5
												(*n* = 3)	(*n* = 3)
Cotton*	20.4	0.6	2.1	22.4	54.5			22.5	23.0	54.5	0.4		
Sesame	9.7 ±0.8		4.2 ±1.4	37.7 ±1.8	48.0 ±1.4	0.4 ±0.6		13.9	37.7	48.4	0.8	3.0 ±3.3	1.2 ±1.7
												(*n* = 18)	(*n* = 18)
Manketti	10.3 ±1.3		6.2 ±0.4	16.4 ±1.5	46.9 ±4.2	20.2[a] ±7.2		16.5	16.4	67.1	0.2	8.4 ±6.9	1.4 ±1.4
												(*n* = 4)	(*n* = 4)
Oil	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**>C20:0**	**ΣSat**	**ΣMUFA**	**ΣPUFA**	**M/P[1]**	**PV**	**FFA**
Strawberry	4.0 ±1.1		0.9 ±0.2	15.4 ±2.5	46.1 ±8.0	33.8 ±4.7		4.9	15.4	79.9	0.2	53.9	2.3

[a] Conjugates

Rosehip	3.3 ±0.3		1.3 ±0.3	14.0 ±1.4	44.2 ±1.3	37.3 ±1.4		4.6	14.0	81.5	0.2	27.2 ±28.9 (*n* = 15)	0.5 ±0.5 (*n* = 15)
P Grapefruit	15.8 ±5.8		3.2 ±0.8	31.3 ±7.7	45.4 ±7.9	4.3 ±1.0		19.0	31.3	49.7	0.6	11.7 ±27.3 (*n* = 15)	0.2 ±0.3 (*n* = 15)
Kakui	6.6 ±0.5		2.3 ±0.6	21.9 ±0.6	41.2 ±1.3	28.0 ±1.5		8.9	21.9	69.2	0.3	4.6 ±3.8 (*n* = 4)	2.0 ±2.1 (*n* = 4)
Borage	10.8 ±0.3		3.9 ±0.4	17.1 ±0.8	40.6 ±0.5	24.2 ±0.9	3.4 ±0.3	14.7	20.5	64.8	0.3	5.7 ±3.2 (*n* = 19)	1.2 ±1.6 (*n* = 19)
Yuzu*	20.9	0.6	3.5	33.2	39.8	2.0		24.4	33.8	41.8	0.8		
Lime	26.3 ±2.7	0.7 ±1.0	4.2 ±0.6	23.9 ±3.4	35.1 ±1.7	10.0 ±1.7		30.5	24.6	45.1	0.5	6.8 ±6.8 (*n*=4)	0.6 ±0.5 (0.6 *n*=4)
Sea Buckthorn seed	9.9 ±2.2	1.5 ±0.6	1.8 ±0.6	19.3 ±3.5	35.0 ±3.5	32.2 ±2.8							

Values are a mean of ±SD for 20 samples, nd = not detected, *Represents oils which are not a mean, n=1. PV are values in Meq/kg. %FFA is a percent of oleic acid.
[1]MUFA/PUFA ratio.

Table 12. Linolenic acid rich oils

Oil	C16:0	C18:0	C18:1	C18:2	C18:3	ΣSat	ΣMUFA	ΣPUFA	M/P[1]	Perox	FFA
Pomegranate	4.0 ±1.0	2.0 ±0.7	7.5 ±3.8	9.6 ±3.3	76.9[b] ±7.7	6.0	7.5	86.5	<0.1	3.7 ±1.9 (*n*=20)	2.0 ±1.2 (*n*=20)
Kiwi*	5.8	2.3	10.9	14.6	66.4	8.1	10.9	81	0.1	8.0	3.1
Brown Flax	5.0 ±0.6	2.7 ±0.5	16.5 ±2.3	15.2 ±1.2	60.6 ±2.9	7.7	16.5	75.8	0.2	5.7 ±6.4 (*n* = 8)	0.5 ±0.4 (*n* = 8)

Values are a mean of ±SD for 10 samples, nd = not detected, *Represents oils which are not a mean, n=1. PV are values in Meq/kg. %FFA is a percent of oleic acid.
[1]MUFA/PUFA ratio.

[b] Conjugated linolenic acid, Punicic acid accounting for 70%

Table 13. Speciality oils rich in short chain saturates

Oil	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	ΣSat	ΣMUFA	ΣPUFA	M/P[1]	Perox	FFA
Babacu*		6.3	45.2	17.8	9.7	2.9	15.8	2.3	81.9	15.8	2.3	6.9		
Coconut	7.6 ±1.2	6.9 ±0.6	51.9±1.9	17.6±1.0	7.4 ±0.7	2.7 ±0.5	4.8 ±1.0	1.0 ±0.3	94.1	4.8	1.0	4.8	0.3 ±0.2	0.2 ±0.2
													(*n* = 20)	(*n* = 20)

Values are a mean of ±SD for 10 samples, nd = not detected, *Represents oils which are not a mean, n=1. PV are values in Meq/kg. %FFA is a percent of oleic acid.
[1]MUFA/PUFA ratio.

Table 14. Ricinoleic acid rich oil

Oil	C16:0	C18:0	C18:1	C18:2	C18:3	18:1(OH)	ΣSat	ΣMUFA	ΣPUFA	M/P[1]	Perox	FFA
Castor	1.5 ±0.5	1.5 ±0.5	4.4 ±2.2	6.1 ±2.4	0.6 ±0.2	85.8 ±5.3	3.0	90.2	6.7	13.5	3.9 ±1.8	0.2 ±0.1
Oil											(*n* = 14)	(*n* = 14)

Values are a mean of ±SD for 10 samples, nd = not detected, *Represents oils which are not a mean, n=1. PV are values in Meq/kg. %FFA is a percent of oleic acid.
[1]MUFA/PUFA ratio.

Table 15. Oil Content of plant seeds

Seed	% Lipid	Range	% Lipid (Literature)
Acerola Cherry	1.8	n.d.	n.d.
Almond (Kernel)	49.6	n.d.	42-60 [90]
Apricots (Bitter)	12.9	n.d.	16 [3]
Ground Nuts*	54.8 ±9.1	44.3-59.9	40-50 [3]
Guava*	13.9 ±1.7	12.7-15.1	16 [192]
Hazelnuts	57.0	n.d.	55-63 [3]
Pomegranate	13.0	n.d.	6-19 [193]
Rosehip	8.9	n.d.	8.2 [194]
Sesame*	43.4 ±10.3	33.7-49.1	50-60 [3]

*Values are a mean of ±SD for 5 samples, nd = not detected. Literature values, [3, 90, 192-194]. Oils selected by Earthoil, to determine oil content of potential seeds for purchase.

Table 16. Lipid classes of oils.

Oil	Lipid Class	Fatty Acid							
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	>C20:0	Lipid %
Baobab									
	PL	33.8		15.5	30.2	20.5			0.3
	MG	47.1		12.2	25.4	15.3			0.1
	DG	21.2		5.2	46.1	25.9	1.6		3.2
	UFA	34.4		10.4	34.9	19.4	0.9		2.5
	TG	23.5		0.2	43.7	30.4	2.2		92.5
	SE	23.5		5.1	35.4	36.0			1.4
Brown Flax									
	PL	26.1		23.9	15.2	12.5	22.3		0.2
	MG	8.3		3.6	19.2	33.7	35.2		2.4
	DG	6.5		5.0	29.6	19.9	39.0		7.1
	UFA	11.9		3.7	30.7	26.3	27.4		1.5
	TG	5.2		3.3	15.8	15.3	60.5		87.7
	SE	4.6		13.9	24.3	31.0	26.2		1.1
Grape									
	PL	13.0		8.8	14.7	36.0	27.5		0.1
	MG	26.9		18.7	54.4				0.2
	DG	17.2		7.0	27.7	47.8	0.3		3
	UFA	19.4		7.3	22.6	1.9	48.8		4.3
	TG	13.2		0.2	15.0	71.4	0.3		92.2
	SE	13.9		21.2	15.6	49.3			0.2

Oil	Lipid Class	Fatty Acid							
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	>C20:0	Lipid %
Guava									
	PL	12.6		8.1	14.2	65.1			3.2
	MG	9.8		6.1	12.2	71.9			7.3
	DG	10.2		6.6	12.7	70.5			14.6
	UFA	15.4		9.0	20.1	55.5			11.9
	TG	9.7		5.6	12.1	72.6			61.7
	SE	10.3		6.0	13.5	70.2			1.3
Macadamia									
	PL	34.2		28.8	31.7	5.3			0.2
	MG	37.8		27.3	23.1	11.8			0.2
	DG	13.7	10.3	6.6	64.6	3.0		1.8	2.1
	UFA	27.0	5.3	17.4	35.1	15.2			0.9
	TG	8.7	17.4	3.8	63.3	0.5		6.5	96
	SE	20.6	11.9	12.9	50.0	4.6			0.6
Manketti									
	PL	14.6		16.4	22.5	46.5			0.5
	MG	14.9		14.3	22.8	48.0			0.8
	DG	11.5		10.8	22.2	49.0	6.5		8.8
	UFA	13.2		10.8	22.7	51.2	2.1		16.1
	TG	12.7		10.2	22.7	48.7	5.7		72.5
	SE	13.0		9.8	19.7	55.8	1.7		1.3

Oil	Lipid Class	Fatty Acid							
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	>C20:0	Lipid %
Moringa									
	PL	35.7		42.3	22.0				0.5
	MG	43.4		31.7	24.9				0.5
	DG	12.9		13.5	72.0	1.6			3.4
	UFA	17.7		19.0	63.3				4.2
	TG	7.5		8.2	83.7	0.6			89.7
	SE	21.3		16.8	55.2	6.7			1.7
Pomegranate									
	PL	16.8		14.3	25.0	29.3	14.6		1.2
	MG	16.4		8.3	22.3	47.5	5.5		1.1
	DG	11.8		8.1	21.7	39.0	19.4		7.0
	UFA	23.6		13.4	36.6	23.5	2.9		5.0
	TG	6.4		4.5	15.3	14.3	59.5		82.2
	SE	13.2		4.7	20.7	45.3	16.1		3.5
Poppy									
	PL	22.2		6.9	17.3	53.6			0.7
	MG	14.1		5.5	17.0	63.4			2.3
	DG	12.9		5.1	20.9	61.1			3.1
	UFA	14.4		5.4	17.8	62.4			3.9
	TG	9.4		3.4	21.4	65.8			89.2
	SE	9.2		3.3	21.0	66.5			0.8

Oil	Lipid Class	Fatty Acid							
		C16:0	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**>C20:0**	**Lipid %**
Sweet Almond									
	PL	32.4		26.4	30.1	11.1			0.3
	MG	28.3		18.6	43.4	9.7			0.7
	DG	10.2		5.7	70.2	13.9			3.5
	UFA	20.9		11.0	54.5	13.6			2
	TG	7.7		1.8	78.8	11.7			92
	SE	22.4		11.7	52.6	13.3			1.5
Wheat Germ									
	PL	22.2		1.6	10.3	59.9	6.0		2
	MG	48.9		0.0	0.0	51.1	0.0		0.4
	DG	24.8		0.9	14.0	55.4	4.9		5.6
	UFA	32.3		1.5	20.4	42.3	3.5		6.9
	TG	21.0		0.6	16.6	56.0	5.8		77.9
	SE	7.8		1.1	11.9	74.2	5.0		7.2

Table 17. Free Fatty Acid Content Determined Through AOCS Official Method and Thin Layer Chromatography.

	Free Fatty acid % Oleic acid	
	AOCS	**TLC**
Baobab	0.1-1.0 (0.4)	0.8
Brown Flax	0.1-1.4 (1.9)	0.5
Grape	0.7-0.8 (0.7)	1.0
Macadamia	0.1-2.4 (2.2)	0.3
Manketti	0.1-2.7 (1.4)	3.7
Moringa	0.1-1.3 (0.7)	2.7
Pomegranate	0.2-4.1 (0.5)	1.8
Poppy	0.7	0.7
Sweet Almond	0.2-0.6 (0.4)	1.1
Wheat Germ	2.6-2.8 (2.7)	2.9

Table 18. Stereochemistry of fatty acids esterified to triacylglycerols.

Oil	Position	FA						
		C16:0	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**>C20:0**
Calophyllum	TG	14.0	n.d.	12.3	39.0	34.7	n.d.	n.d.
	Sn-1	35.6	n.d.	30.2	23.7	10.4	n.d.	n.d.
	Sn-2	12.0	n.d.	4.7	20.5	62.8	n.d.	n.d.
	Sn-3	5.6	n.d.	1.9	65.0	27.5	n.d.	n.d.
Cherry	TG	6.0	0.5	1.8	40.7	51.1	n.d.	n.d.
	Sn-1	30.8	n.d.	31.4	25.2	12.6	n.d.	n.d.
	Sn-2	36.2	n.d.	27.2	25.9	10.7	n.d.	n.d.
	Sn-3	21.8	0.2	20.2	30.5	27.3	n.d.	n.d.
Coffee*	TG	6.0	n.d.	2.2	11.2	59.4	20.9	n.d.
	Sn-1	69.2	n.d.	15.6	5.2	10.0	n.d.	n.d.
	Sn-2	13.8	n.d.	6.1	14.2	66.0	n.d.	n.d.
	Sn-3	63.9	n.d.	16.7	13.2	6.2	n.d.	n.d.
Marula	TG	12.5	n.d.	7.3	74.2	5.5	n.d.	0.6
	Sn-1	18.9	n.d.	10.0	70.4	0.4	n.d.	0.2
	Sn-2	10.0	n.d.	5.0	84.5	0.5	n.d.	0.2
	Sn-3	8.4	n.d.	6.9	67.7	15.6	n.d.	1.3
Pomegranate*	TG	7.6	n.d.	3.6	10.8	11.1	67.0	n.d.
	Sn-1	23.6	n.d.	13.5	17.7	42.3	2.9	n.d.

	Sn-2	10.2	n.d.	2.9	6.8	74.5	5.6	n.d.
	Sn-3	2.1	n.d.	5.0	14.6	69.7	8.6	n.d.
Poppy	TG	7.8	n.d.	2.2	16.9	72.7	0.5	n.d.
	Sn-1	39.6	n.d.	12.9	20.0	27.5	0.0	n.d.
	Sn-2	20.9	n.d.	13.1	26.1	39.9	0.0	n.d.
	Sn-3	6.7	n.d.	7.1	52.9	33.4	0.0	n.d.
Sea Buckthorn	TG	35.0	26.8	0.8	33.2	3.7	0.5	n.d.
	Sn-1	38.7	25.8	0.1	28.1	7.4	0.1	n.d.
	Sn-2	22.8	17.8	7.3	40.3	11.6	0.2	n.d.
	Sn-3	33.6	28.4	4.8	24.1	8.1	1.0	n.d.

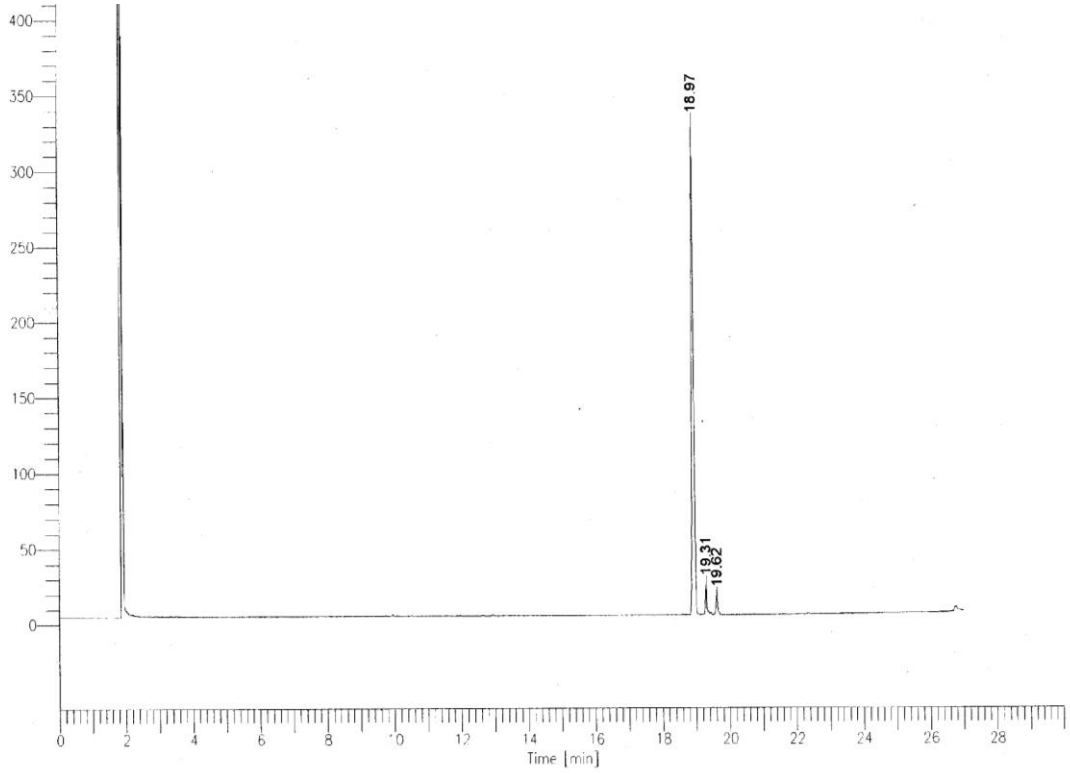
nd = not detected. * Indicates errorness results.

Table 19. Methylation of punicic acid

Temperature / ^{o}C	**% Punicic acid**
40	95.7 ±1.6
60	87.3 ±5.2
80	70.5 ±5.8

Values are a mean of ±SD for 2 samples. Punicic acid salts extracted from pomegranate oil The salts were methylated at various temperatures to evaluate the optimum temperature for low content of punicic acid isomers.

Figure 45. Punicic acid isolated from Pomegranate oil



The isolation of punicic acid from pomegranate oil. The potassium salt was methylated and separated using GC-MS. At 40^{o}C isomers appears in small quantities.

3.3 Results and Discussion

The oil samples analysed have been grouped to reflect their degree of fatty acid unsaturation. The fatty acid content of the oil was analysed through GC-MS and GC-FID. The mass spectral data confirmed that the key fragmented ions represented the associated fatty acid.

3.3.1 Mass spectral data.

The mass spectral data was obtained for the majority of the fatty acids analysed. The mass spectrometer was an electron ionisation (EI). The methyl esters were subjected to an electron impact, the resulting charged aliphatic chain being broken into a determinate number of fragments [195].

The derivitisation of the fatty acids leads to a methyl ester being formed at the carboxylic end of the fatty acid. The data can be interpreted with the fragments formed from the loss of the methoxyl (*m/z* $[32]^+$) and the McLafferty (*m/z* $[74]^+$) ions. The McLafferty ion rearrangement ion is important in determining esters derivatives of most fatty acids. The McLafferty rearrangement is one of the most widely occurring ions, and has been studied in depth in the process of mass spectrometry. The McLafferty ion is an important ion for identification purposes. The presence of double bonds also gives specific fragments which can be identified on the spectra. The common fragments from PUFA are the alpha (α) and the omega (ω) ions, which occur at either side of the double bond. These specific fragments can be used to determine the double bond position, and how many double bonds are present in the fatty acid methyl ester.

The fragmented key ions of all the fatty acids analysed are displayed in Table 9. The key ions for all the fatty acids is the methoxyl ion *m/z* 43^{+}, and the McLafferty ion *m/z* 74^{+}. The introduction of double bonds in fatty acids, give rise to fragments which can account for the double bond. The fragmented ion *m/z* 108 is present from the fragmentation of methyl oleic acid (Figure 39). The fragment arises from the cleavage which takes place between carbons 5 and 6, with the addition of a hydrogen [184].

The fragmented ions of linolenic acid was used to distinguish between the α and γ form of the fatty acid. Methyl linolenic acid molecular ion *m/z* 292 was present in the α and γ fatty acid (Figure 41 & 42). α-linolenic acid fragments gave two key ions *m/z* 235 and 108. The ion *m/z* 235 indicates the alpha ion of the $\Delta^{9,12}$ bond was present [186]. The ion *m/z* 108 is characteristic for methyl esters of PUFAs with the n-3 moiety, which was present in the spectra for α-linolenic acid [186]. γ-linolenic acid gave key fragment ions *m/z* 194 and 150. The ion *m/z* 194 represents the fragment of the alpha ion of $\Delta^{6,9}$ bond, which was present in γ-linolenic spectra [187]. The ion *m/z* 150 is a characteristic ion of methyl esters from PUFAs with the n-6 moiety, this ion was present in the γ-linolenic spectra [187].

The key ions of the spectra become important when the methyl ester fatty acid ion becomes difficult to detect due to low abundance. The ion *m/z* 289 represents methyl stearidonic acid (Figure 43), and is characteristically undistinguishable. The ion *m/z* 108 was present in the spectra of methyl stearidonic acid. The ion represents the n-3 structure of methyl stearidonic acid. The alpha ion for $\Delta^{6,9}$ was also visible at *m/z* 194. Fatty acids which have four or more double bonds, fragment to give a cyclic tropylium ion *m/z* 91 [188]. The ion *m/z* 91 was present in the spectra of methyl stearidonic acid. The mass

spectral library also confirmed that the fragments represented stearidionic acid, giving a library match of 82%.

Methyl ricinoleic acid can be distinguished by the ion *m/z* 312. The ion *m/z* 312 was not detected in the spectra (Figure 46). Fragmented ions which arise from the loss of water were detected in the high mass region, *m/z* 294. The ion *m/z* 198 was also detected, which indicates the cleavage between carbons 11 and 12. The ion at *m/z* 166 was present in the spectra which represents the further loss of methanol from the carboxyl group [191]. The spectra obtained for methyl ricinoleic acid was in agreement with the spectra obtained from the AOCS archives [191], and a library match of 91%.

3.3.2 Oleic acid rich oils (Table 10). The oleic content of *Camellia sinensis* and Moringa oil reached 80% of the oils content. Moringa oil contained 84.6% monounsaturated fatty acids (MUFAs), and *Camellia sinensis* containing 80.4% MUFAs. Macadamia and Sea buckthorn oil both contained palmitoleic acid which exceeded 20% of the total fatty acid content. Palmitoleic acid is not observed in a large quantities in plant oils [96, 103]. Macadamia oil contained significant amounts of palmitoleic acid, 24.3%. The levels analysed coincided with reported levels [96]. Sea buckthorn pulp has been reported to contain 39-12% palmitoleic acid [104]. The study also showed that oils obtained from the *rhamnoides* species contained higher amounts of palmitoleic acid compared to the *sinensis* species [104]. The oil did not have a recorded FFA and PV due to the colour of the oil. The dark orange colour of sea buckthorn is attributed to the *β*-carotene content.

The levels of β-carotene in sea buckthorn oil has been reported to be approximately 15-55% of the total carotenoid content of the soft parts of the berry [103].

The presence of long chain fatty acids, >C20 was observed in Moringa, Shea butter, Macadamia, Andiroba, Ground nut, Acerola cherry and Rice bran oils. Macadamia oils fatty acid content contained C20:0 and C20:1, which accounted for 3.5% of the oils content. Macadamia oil has been reported to contain 3-4% of C20:0 and C20:1 [96]. The study also revealed Macadamia oil to contain trace amounts of C22:0, C22:1 and C24:0, which was not detected in Earthoil samples [96]. Moringa oil also contained a significant amount of C20:0 and C20:1 fatty acids, 4.7%. Moringa has been reported to contain 4.6-6.0% C20:0 and C20:1 [119]. The study also reported a substantial levels of C22:0, 5.7-6.0% [119].

The oleic acid rich oils showed a range of MUFA/PUFA (M/P) ratio from 76.9 (Moringa) to 1.1 (Baobab). The M/P ratio of Baobab oil coincides with published values of approximately 1.2 [196]. Moringa oil M/P ratio can vary depending on geographical location. Moringa cultivated in various regions of Pakistani showed a M/P ratio of 41.4-85.6 [119]. The Moringa sub species *Moringa pterygosperma* had a M/P ratio of 108 [197]. The change in geographical location and species can have an effect of the fatty acid content of Moringa oil.

The PV and FFA content of the oils gave an insight to the quality of the oils. Rapeseed oil displayed the largest range in peroxide content 4.3-107 $meqkg^{-1}$ (with a mean of 40.4 $meqkg^{-1}$) for oleic rich oils. Rapeseed oils high peroxide value may be attributed to the oil being exposed to oxygen for prolonged periods. A study showed Rapeseed oil exposed to the atmosphere for 16 days at $40^{o}C$, had a peroxide content of $166meqkg^{-1}$ [198].

Avocado oil exhibited the widest FFA content 2.5-7.8% (1.5%) for oleic rich oils, this may be attributed to the oils enzymatic activity, whereby lipases cleave fatty acids from lipid molecules.

3.3.3 Linoleic acid rich oils (Table 11). Passion seed oil (M/P, 0.2) had a linoleic content of 77.7%, which was the highest of all linoleic rich oils. The reported levels of linoleic acid in Passion seed oil vary from 74-77%, with a given M/P ratio of 0.1-0.2 [199, 200]. Evening primrose oil (EPO) (M/P, <0.1) comprised of 88.6% PUFAs. The high PUFA content was attributed to the linoleic acid content, and a significant content of γ-linolenic acid. The levels of γ-linolenic acid content of oils supplied by Earthoil coincide with literature values of 8-9% [201, 202]. The reported PUFA levels for EPO ranged from 80-82 % [201, 202]. The literature showed EPO to contain trace levels of C14:0 and C20:0 to C24:0 fatty acids, which were not detected in the oils supplied by Earthoil [201, 202]. Blackcurrant oil (M/P, 0.2) contained 3.1% stearidonic acid (C18:4 $\Delta^{6,9,12,15}$) and 14.8% γ-linolenic. The reported levels of stearidonic acid and γ-linolenic are given as, approximately 3% and 13% respectively [194, 203, 204]. The importance of stearidonic acid and γ-linolenic in human diets allow for the biosynthesis of EPA and DHA. Borage oil (M/P, 0.3) also contained γ-linolenic, 24%. The literature values for the content of γ-linolenic in Borage oil coincide with the levels analysed here [205].

Black cumin (M/P, 0.4) has been reported to contain moderate levels of C20:2, 2.4% [206, 207]. The levels analysed in three samples supplied by Earthoil was approximately

2.4% ±0.2. The long chain fatty acid has not been observed in all Black cumin samples analysed, and may be a result of a differing sub species.

The M/P ratio for linoleic rich oils ranged from <0.1 (EPO) to 0.8 (Sesame). Peroxide value and free fatty acids varied depending on oils. Evening Primrose oil showed the largest range for peroxide content 2.2-91.5 $meqkg^{-1}$ (13.7 $meqkg^{-1}$) of all the linoleic rich oils. Reported peroxide levels in EPO typically range from 1.5-6 $meqkg^{-1}$ [201, 202], with an absolute range of <1-12 $meqkg^{-1}$ [201]. The average PV for EPO oils supplied by Earthoil did not fall within the reported literature range. Borage showed the largest range in free fatty acid content, 0.1-7.9% (1.2%) of all the linoleic rich oils.

3.3.4 Linolenic acid rich oils (Table 12). Linolenic rich oils analysed were limited to Flaxseed, Kiwi and Pomegranate oil. Flaxseed and Kiwi oil both contained α-linolenic acid. Pomegranate oil (M/P, <0.1) contained 76.9% conjugated linolenic acid isomers. The abundant isomer was punicic, with an approximate content of 70% of the oils fatty acid content. Kiwi oil (M/P, 0.1) did not contain long chain fatty acids. The literature however indicates trace amounts of long chain fatty acids C20: and C20:1 [3, 199]. The peroxide content for pomegranate and kiwi oil was below the accepted 10 $meqkg^{-1}$. The FFA for both oils did exceed the accepted industry levels of 2% oleic acid.

3.3.5 Saturated fats (Table 13). Coconut (M/P, 4.8) and Babacu (M/P, 6.9) oils contained short chain saturated fatty acids. Coconut oil contained 94.1% saturates, with

C12:0 accounting for 50% of the total fatty acid composition. The low PUFA content of coconut oil does not make it susceptible to oxidation. The peroxide values ranged from 0.1 to 0.8 meqkg^{-1}.

3.3.6 Castor oil (Table 14). Castor oil (M/P, 13.5) contained approximately 85% ricinoleic acid. The reported levels of ricinoleic acid in castor oil coincide with the data obtained in this current chapter [3, 31]. The oil displayed low levels of FFA and PV.

3.3.7 Oil content of seeds (Table 15). Oil content of a selected variety of seeds showed nut based seeds contained the largest amounts of oil. Hazelnuts contained the greatest content of oil 57%, whilst Acerola cherry seeds contained the lowest content of oil 1.8%. The oil content of the seeds was in accordance with the literature values, with Acerola cherry having not been previously reported [3, 90, 192-194].

3.3.8 Lipid classes of oils (Table 16). The majority of plant seed oils contain approximately 80% TAGs, with the remainder 20% accounting for minor lipid constituents [4]. Manketti and Guava seed oils contained less than 80% TAGs. Manketti contained 72.5% and Guava contained 61.7%, TAGs. Both oils had increased levels of free fatty acids and diacylglycerols, which may be the result of hydrolysis of the TAGs from processing and/or lipases activity. Oils that contain large amounts of free fatty acids

become difficult to trade. When values for free fatty acids were high Earthoil was informed. Advice was given on modifying processing and handling conditions, which would include reducing friction caused during cold pressing, and nitrogen blanketing during extracting oil.

The free fatty acids levels obtained from thin layer chromatography (TLC) can be compared to the %FFA method used by industry, AOCS official method, Table 17. The FFA content from the TLC was expressed as a percentage of oleic.

Manketti and Moringa oil FFA content deduced from TLC, did not compare to the AOCS method for %FFA. The remainder of the oils analysed through TLC did fall within the given AOCS range for the specific oil. Poppy seed oil, which was from the same batch, displayed identical FFA, using both methods.

The TLC method to determine the free fatty acid would be considered to give greater accuracy. Due to the free fatty acids being quantified by GC-MS. This does not rule out possible error, which can be caused through the loss of silica gel from the TLC scrapings. The AOCS method is dependant on titration, which can be affected by various parameters such as human error.

3.3.9 Stereochemical analysis (Table 18). The stereochemical analysis of the fatty acids esterified to position sn-*1* and sn-*2* allow for the fatty acid at position sn-*3* to be determined. The data obtained from the analysis establishes possible metabolic pathways for the incorporation of fatty acids to TAGs. The oils selected have limited literature published on their stereochemistry.

Calophyllum oils TAGs contained almost equal quantities of oleic and linoleic acid. The *sn*-1 position contained above 60% saturates. Classically the *sn*-2 position would be esterified with an unsaturated fatty acid. Calophyllum oil contained above 60% linoleic acid at position *sn*-2. Position *sn*-3 was esterified with 65% oleic acid, followed by 27% linoleic acid. Calophyllum oil stereochemical distribution of fatty acids on the glycerol backbone indicates that the TAGs are assembled according to the classical model.

The TAGs of Coffee seed oil contained 20% linolenic acid, although this was not detected at any of the positions. This maybe due to poor recovery from the TLC plate, or small losses throughout the determination of the stereochemistry of the oil. The *sn*-1 position contained 70% palmitic acid, and 15% stearic acid. The *sn*-2 position was enriched with PUFAs, with linoleic acid accounting for 65% of its esterified fatty acid. The *sn*-3 position was esterified with over 80% saturated fatty acids.

Poppy seed oil was rich in linoleic acid, which accounted for 72% of the TAG composition. The *sn*-1 position was enriched with palmitic acid, although significant levels of linoleic acid (27%) was detected. The *sn*-2 position contained 40% linoleic acid and 26% oleic acid. At the *sn*-3 position oleic acid accounted for over 50% of its distribution. Poppy seed oil showed fatty acid distribution similar to Calophyllum and Coffee seed oil.

Sea buckthorn oil can be distinguished by its high palmitoleic acid content. The TAGs contained 26% palmitoleic acid, which was found at high levels at *sn*-1 and *sn*-3, 25% and 28% respectively. The *sn*-1 position was dominated with palmitic, palmitoleic and stearic acids. Oleic acid accounted for 40% of the fatty acid composition esterified to position *sn*-2. Palmitic and palmitoleic acid were also found at notable levels at position

sn-2. The *sn*-3 position was similar to the *sn*-1 position with palmitic, palmitoleic and stearic acids, being the most abundant fatty acids.

Cherry, Marula and Pomegranate oils, showed unusual distribution of fatty acids on the glycerol backbone. This maybe due to poor recovery of the individual lipid classes from the TLC plate, when calculating positions against the TAG fatty acids, an uneven distribution is displayed. Therefore these results can not be considered in this current study. The data has been provided to highlight the difficulties with the method used to evaluate the fatty distribution of fatty acid on the glycerol backbone. The data should agree within 5% when using the two calculations provided by Christie. Recent developments in this area of lipid chemistry has seen new methods being developed. The TAGs stereochemistry can be determined through LC-MS, with a chiral phase columns.

3.3.10 Punicic acid methylation (Table 19). During the methylation of Pomegranate oil it was observed that the punicic acid content of the oil was low compared to literature values [208]. Punicic acid was isolated from the Pomegranate oil and methylated at various temperatures. The methylation of punicic acid at 40^{o}C, gave a punicic acid content of 94% (Figure 45). The chromatogram shows three peaks, the main peak, was identified as punicic acid, the two minor peaks were identified as isomers of punicic acid. Literature suggest that the high temperature forces the conjugated linolenic acid to isomerise [208]. The study found that methylating at 40^{o}C was effective in reducing isomerisation of conjugated linolenic acid. [208].

3.4 Conclusions

The fatty acid composition, together with the PV and FFA content gave a reasonable indication into the quality of the oil. The quality of the oil was based on low peroxide and FFA values 10 $meqkg^{-1}$ and 2% respectably. The fatty acid profile gave an indication the quality of the oil, in terms of adulteration and extreme degradation through oxidation. The large amount of data collected from the analysis of the oils, allowed for the creation of basic database which contained information regarding the oils fatty acid profile, PV and FFA. The database was used in future analysis to compare the quality of oils analysed, and to determine if oils have been improperly handled during extraction. The database also allows Earthoil to track the quality of the oils from the point of extraction to processing.

The stereochemical analysis of Calophyllum, Coffee, Poppy and Sea Buckthorn, have not previously been undertaken and no literature values exist for these commodities. The oils all contained mainly saturates at position *sn*-1, a MUFA or PUFA at position *sn*-2, and a saturate or MUFA at position *sn*-3. This distribution of acyl groups is consistent with the general distribution patterns found in oils with similar fatty acid composition. The results obtained have highlighted potential problems with the well established method, including poor quantification of minor fatty acids, which can be lost throughout the experimental stages of the method.

Chapter 4

Evaluating the oxidative stability of plant seed oils

4.1 Introduction

The previous chapter characterised the oils in accordance of their fatty acid composition. The fatty acid composition influences the oxidative stability of an oil. The increased content of PUFAs should theoretically make the more oil susceptible to oxidation.

The current chapter aims to evaluate the oxidative stability of a selection of oils varying in fatty acid composition and antioxidant content. An oil which can be easily oxidised would require correct preventative measure to reduce oxidation. The oxidative stability of an oil can be affected by extraction and storage conditions. Storage of oils for long periods of time, without correct preventative measures towards oxidation can cause the oil to degrade.

Whilst evaluating the oxidative stability and the thermo oxidative stability of the selected oils, it has also been proposed to supplement the oils with readily available and effective antioxidant, α-tocopherol (α-TOC). The addition of α-TOC may help increase the host oils oxidative stability. The addition of α-TOC could, however, potentially lead to pro oxidant activity, which would decrease the oils oxidative stability. It is the purpose of this chapter to evaluate the anti/pro oxidant effects of adding α–TOC to a range of speciality oils varying in their degree of unsaturation. In order to evaluate this effect it is also necessary to establish the best experimental approach to evaluate lipid oxidation.

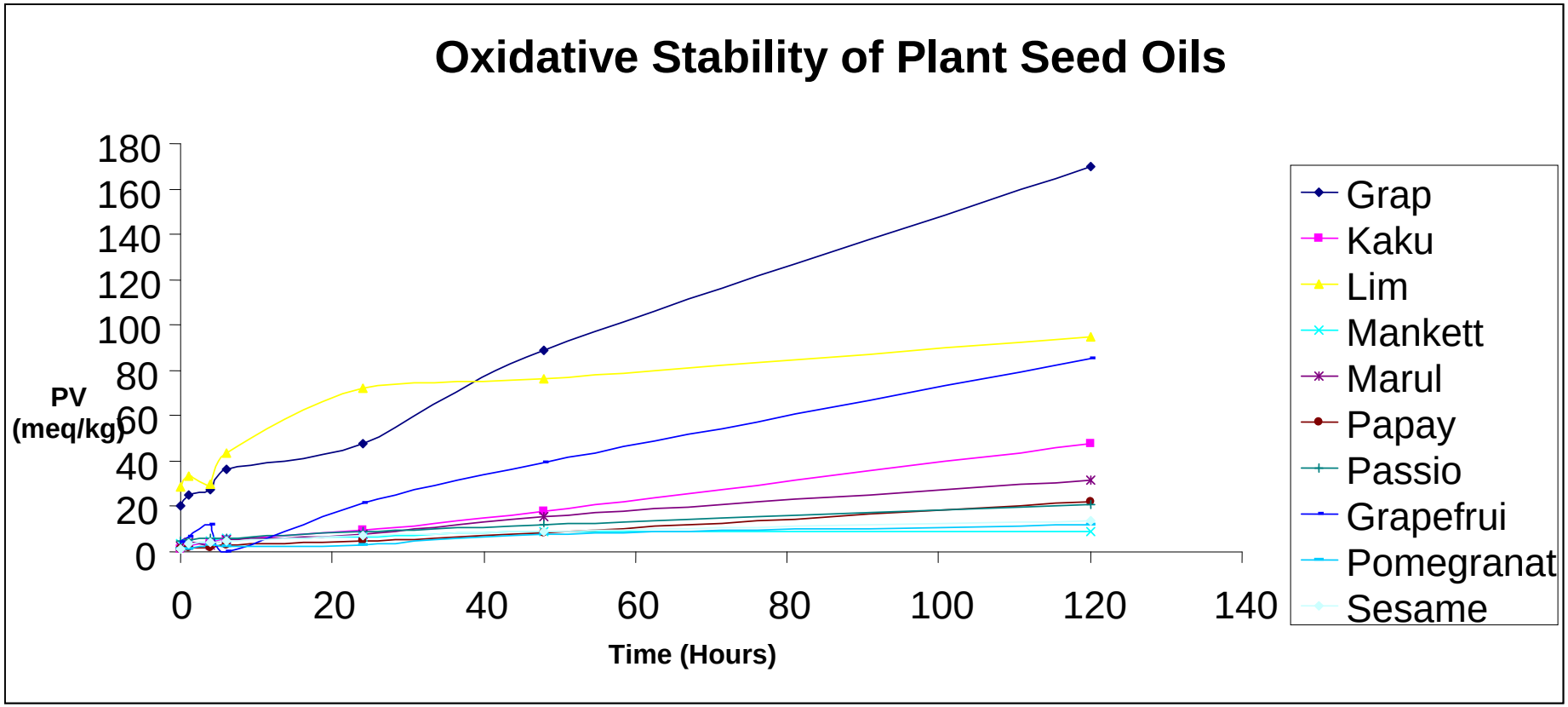
4.2 Results

Table 20. Oxidative stability of plant seed oils

	PV meqkg^{-1}						
Time (Hours)	**0**	**1**	**4**	**6**	**24**	**48**	**120**
Grape	20.2 ±0.6	24.9 ±1.5	27.3 ±1.1	36.6 ±0.5	47.6 ±1.4	89.0 ±0.7	170.1 ±8.0
Kakui	1.1 ±0.1	2.1 ±0.1	4.3 ±0.2	5.1 ±0.7	9.6 ±1.7	18.1 ±1.7	47.4 ±8.9
Lime	28.7 ±0.0	33.2 ±3.0	29.9 ±0.3	43.5 ±0.3	72.1 ±1.0	76.2 ±0.9	94.5 ±2.1
Manketti	3.5 ±0.2	3.8 ±0.0	4.5 ±0.1	6.0 ±0.6	6.7 ±0.0	8.8 ±0.3	9.1 ±1.7
Marula	2.1 ±0.8	3.9 ±1.2	3.0 ±0.3	5.1 ±0.2	7.7 ±0.4	15.5 ±0.6	31.6 ±0.1
Moringa	2.7 ±0.8	nd	nd	nd	3.2 ±0.4	15.8 ±0.9	32.9 ±2.6
Papaya	1.2 ±0.0	1.6 ±0.0	2.0 ±0.0	2.7 ±0.2	4.8 ±0.0	8.4 ±0.0	21.9 ±2.9
Passion	4.9 ±0.4	5.1 ±0.1	±6.2 ±0.3	6.0 ±0.1	8.9 ±0.5	11.9 ±0.0	20.8 ±1.1
Grapefruit	4.0 ±0.0	6.4 ±0.0	12.2 ±0.9	nd	21.5 ±0.5	39.1 ±0.5	85.1 ±2.7
Pomegranate	1.1 ±0.1	1.2 ±0.0	2.8 ±0.0	2.4 ±0.0	3.2 ±0.3	7.8 ±0.8	11.8 ±0.1
Sesame	1.0 ±0.0	3.6 ±0.3	4.2 ±0.3	4.5 ±0.1	6.9 ±1.3	8.8 ±1.1	13.5 ±0.3

PV are values in meqkg^{-1}, analyses was conducted at 18^{o}C. Results represent the mean ±SD for triplicate analyses. Nd = not detected.

Graph 1. Oxidative stability of plant seed oils



Oxidative stability time course of plant seed oils.

Table 21. Peroxide accumulation of MUFA rich oils.

Oil	Saturates	MUFA	PUFA	MUFA/PUFA	PV Increase ($meqkg^{-1}$)
Moringa	14.3	84.6	1.1	76.9	30.2
Marula	16.8	74.9	9.0	8.3	29.5
Papaya	20.2	74.9	4.9	15.3	20.7

PV are values in $meqkg^{-1}$, storage and analyses conducted at $18^{o}C$. The PV accumulation represents 120 hour minus 0 hour.

Table 22. Peroxide accumulation of PUFA rich oils.

Oil	Saturates	MUFA	PUFA	MUFA/PUFA	PV Increase ($meqkg^{-1}$)
Passion	10.7	11.7	77.7	0.2	15.9
Grape	11.3	14.6	74.1	0.2	49.9
Kakui	8.9	21.9	69.2	0.3	46.3
P. Grapefruit	19.0	31.3	49.7	0.6	81.1
Sesame	13.9	37.7	48.4	0.8	12.5
Manketti	16.5	16.4	67.1*[1]	0.2	5.6
Lime	30.5	24.6	45.1	0.5	65.8
Pomegranate	6.0	7.5	86.5*[2]	<0.1	10.7

PV are values in $meqkg^{-1}$, storage and analyses conducted at $18^{o}C$. The PV accumulation represents 120 hour minus 0 hour. *[1] Manketti contained 20.2% conjugated linolenic acid, *[2] Pomegranate contained 76.9% conjugated linolenic acid.

Table 23. Oxidative stability of Brown Flax and Passion Seed Oil

	Brown Flax				Passion Seed			
Time (Hours)	**0**	**24**	**48**	**120**	**0**	**24**	**48**	**120**
Peroxide meqkg^{-1}	3.6 ±0.6	5.1 ±0.1	8.8 ±1.37	9.6 ±0.4	3.6 ±0.1	13.5 ±1.1	16.7 ±0.6	28.1 ±3.6
C.D. %	2.4 ±0.4	2.4 ±0.1	3.1 ±0.1	2.9 ±0.1	0.2 ±0.1	0.2 ±0.1	0.2 ±0.1	0.3 ±0.1
***p*-Anisidine**	0.00 ±0.00	3.1 ±1.1	1.9 ±0.8	2.00 ±1.3	1.2 ±0.3	1.1 ±0.1	2.5 ±0.4	3.5 ±3.3

Oxidative stability of brown flax and passion seed oil, assays conducted in 50ml conical flasks with a surface area of 13.9 cm^2. Results represent the mean ±SD for triplicate analysis. C.D %, refers to conjugated diene percentage in oil.

Table 24. Oxidation of oleic rich oils at 18°C and 60°C

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
Baobab						
18°C						
Control	1.2 ±0.7	nd	8.5 ±0.2	0.6 ±0.3	9.9 ±1.7	1.5 ±0.1
+ α-TOC	0.7 ±0.2	nd	12.2 ±0.3	nd	16.0 ±0.5	0.5 ±0.1
60°C						
Control	1.2 ±0.7	nd	1.3 ±0.2	nd	3.2 ±1.1	2.0 ±0.4
+ α-TOC	0.7 ±0.2	nd	2.5 ±0.2	nd	3.6 ±0.0	1.6 ±0.9
Macadamia						
18°C						
Control	0.0 ±0.0	nd	3.6 ±0.4	nd	5.2 ±0.0	nd
+ α-TOC	0.0 ±0.0	nd	3.9 ±0.1	nd	5.3 ±0.2	nd
60 °C						
Control	0.0 ±0.0	nd	0.3 ±0.1	nd	0.3 ±0.1	nd
+ α-TOC	0.0 ±0.0	nd	0.4 ±0.0	nd	0.5 ±2.0	nd

Moringa						
18 °C						
Control	2.7 ±0.8	nd	15.8 ±0.9	1.5 ±0.8	32.9 ±2.6	nd
+ α-TOC	2.1 ±0.3	0.6 ±0.3	13.6 ±1.0	nd	19.1 ±7.0	0.8 ±0.5
60 °C						
Control	2.7 ±0.8	nd	8.0 ±1.0	Nd	4.3 ±0.8	nd
+ α-TOC	2.3 ±0.2	nd	8.4 ±0.4	Nd	5.6 ±0.4	1.7 ±0.9
Yangu*						
18 °C						
Control	15.9 ±0.1	4.5 ±0.3	25.5 ±1.1	5.3 ±0.2	27.2 ±1.0	5.3 ±0.5
+ α-TOC	29.9 ±0.7	0.4 ±0.3	31.7 ±0.7	nd	37.6 ±0.6	2.6 ±0.3
60 °C						
Control	15.9 ±0.1	4.8 ±0.3	39.9 ±1.6	6.3 ±0.4	69.6 ±1.2	7.1 ±0.4
+ α-TOC	30.3 ±3.1	3.6 ±0.8	76.5 ±7.4	5.9 ±0.4	150.1 ±29.2	8.1 ±2.7

PV are values in meqkg^{-1} oil. *p*-A.V. are unitless. Results represent the mean ±SD for triplicate analyses. Poly = polymerization. nd = not detected. *Data of same batches with varying time zero values. Concentration of α-TOC was 750 ppm.

Table 25. Oxidation of linoleic acid rich oils at 18°C and 60°C

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
EPO						
18 °C						
Control	9.6 ±0.7	3.1 ±0.3	38.8 ±2.4	3.4 ±0.2	62.8 ±0.7	2.9 ±0.2
+ α-TOC	9.3 ±0.3	3.1 ±0.3	33.9 ±0.4	4.2 ±0.8	56.7 ±3.2	3.2 ±0.8
Grape						
18 °C						
Control	5.8 ±0.5	2.4 ±0.2	36.0 ±1.6	2.8 ±0.2	66.6 ±3.3	2.9 ±1.4
+ α-TOC	5.3 ±0.5	2.4 ±0.1	35.4 ±1.1	2.9 ±0.2	65.7 ±3.8	2.4 ±0.2
Manketti*						
18 °C						
Control	13.1 ±0.7	16.9 ±2.7	17.2 ±1.4	19.3 ±0.2	14.5 ±2.0	17.5 ±0.6
+ α-TOC	7.7 ±1.1	16.0 ±0.5	15.6 ±0.8	9.3 ±0.5	11.4 ±1.0	19.8 ±1.2

60 °C						
Control	13.1 ±0.7	16.9 ±2.7	50.3 ±8.6	50.2 ±5.7	Poly	Poly
+ α-TOC	7.7 ±1.1	16.0 ±0.5	101 ±21.0	23.5 ±2.5	186.8 ±16.1	70.2 ±4.2
Rosehip						
18 °C						
Control	37.7 ±0.3	18.1 ±0.5	51.0 ±0.1	18.1 ±0.5	54.8 ±0.1	16.6 ±0.4
+ α-TOC	37.6 ±0.2	18.2 ±0.3	50.8 ±0.2	18.3 ±0.1	58.8 ±2.3	17.2 ±0.4
Wheat Germ						
18 °C						
Control	8.6 ±0.1	3.4 ±0.5	12.7 ±0.4	7.3 ±1.1	24.7 ±0.3	13.7 ±0.4
+ α-TOC	8.1 ±0.2	4.4 ±0.1	11.3 ±0.2	5.7 ±0.5	17.5 ±0.4	nd
60 °C						
Control	8.6 ±0.2	3.4 ±0.5	77.7 ±1.1	5.2 ±1.2	228.3 ±1.3	10.8 ±2.2
+ α-TOC	8.1 ±0.2	4.4 ±0.1	94.7 ±9.7	5.9 ±1.3	280.8 ±11.1	15.1 ±3.2

PV are values in meqkg^{-1} oil. *p*-A.V. are unitless. Results represent the mean ±SD for triplicate analyses. Poly = polymerization. nd = not detected. *Data of same batches with varying time zero values. Concentration of α-TOC was 750 ppm.

Table 26. Oxidation of linolenic rich oils at 18°C and 60°C

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
Brown Flax*						
18°C						
Control	15.6 ±0.4	0.8 ±0.3	18.6 ±0.4	Nd	20.0 ±0.7	0.9 ±0.8
+ α-TOC	15.6 ±0.4	0.8 ±0.3	18.3 ±0.7	1.0 ±0.2	19.1 ±0.4	1.3 ±0.3
60 °C						
Control	15.6 ±0.4	0.8 ±0.3	602.7 ±5.9	20.0 ±0.3	poly	Poly
+ α-TOC	15.6 ±0.4	0.8 ±0.3	234.4 ±29.2	9.8 ±1.1	583.9 ±10.9	20.9 ±2.1
Pomegranate*						
18 °C						
Control	7.3 ±0.2	14.3 ±0.2	7.7 ±1.1	19.4 ±0.3	8.7 ±1.1	22.3 ±0.4
+ α-TOC	7.3 ±0.2	14.3 ±0.2	8.0 ±0.7	21.0 ±0.8	10.4 ±0.7	24.2 ±1.9
60 °C						

Control	7.3 ±0.2	14.3 ±0.2	56.6 ±2.5	21.4 ±1.6	poly	Poly
+ α-TOC	7.3 ±0.2	14.3 ±0.2	94.2 ±8.3	25.2 ±1.6	poly	Poly

PV are values in meqkg^{-1} oil. p-A.V. are unitless. Results represent the mean ±SD for triplicate analyses. Poly = polymerization. nd = not detected *Result provided by MSc Students working on same research project. Concentration of α-TOC was 750 ppm.

4.3 Results and Discussion

The datasets have been grouped according to the major fatty acid component in the oil prior to evaluating their oxidative stability. Initially, oils were screened for peroxides (PV) in order to get a general indication of their likely stability. However, in order to follow the breakdown of peroxides we later employed the *p*-anisidine test to monitor the production of non volatile carbonyl compounds.

4.3.1 Oxidative stability of plant seed oils (Table 20 and Graph 1). Initial studies carried out on various plant seed oils, solely relied on the peroxide value to determine the extent of oxidation. The peroxide values displayed in Table 20 and Graph 1, track the peroxide value from the initial exposure of oxidation up to 120 hours of exposure. The data can vary depending on oil, for instance the starting PV at time point zero for pomegranate is 1.1 $meqkg^{-1}$, whereas lime has a starting peroxide of 28.7 $meqkg^{-1}$. The accumulation of the peroxide content gives a more in-depth insight into the level of oxidation of each oil, and proceeding tables display the data in such format. Interestingly the table does indicate that all oils had a differing starting PV, and for clarification purposes, oils were analysed directly from the bottles supplied by Earthoil. Oils such as Grape and Lime, displayed a high initial PV, which indicates the oil, has already been exposed to oxygen and that peroxide accumulation is active.

4.3.2 Peroxide accumulation in MUFA rich oils (Table 21). Moringa oil's (M/P, 76.9) peroxide accumulation through the 120 hour time period at 18^{o}C was 30 meqkg^{-1}. Moringa oil has been reported to have remarkable oxidative stability. The oxidative stability was attributed to its low PUFA content and high abundance of flavone myricetin, a powerful antioxidant [3]. The tocopherol content of Moringa oil has been reported to be dependant on cultivar [119]. The tocopherol content has been reported to range from 247-289 ppm [119]. Moringa oils high oxidative stability makes it an ideal oil which can been blended to less stable oils, to increase its oxidative stability [121].

Marula (M/P, 8.3) oil's oxidative stability was similar to Moringa oil, with an accumulation of 29.5 meqkg^{-1}at 18^{o}C. A comparative study of Marula and Moringa oil, reported Marula oil to be less stable than Moringa oil, when using the OSI Rancimat to determine the oxidative stability of the oil [197]. The data obtained in this current chapter showed the oils to have similar oxidative stability, even though their MUFA/PUFA ratio was largely different. The peroxide content for both oils being similar indicated that both oils were accumulating peroxides. Another scenario could be one of the oils was accumulating peroxides and other breaking down peroxides. The oxidative stability of the oils can not be fully determined based on the peroxide content of both oil's. The need to measure secondary oxidation products would give an insight to the oxidation state of the oil's.

Papaya oil (M/P, 15.3) showed the lowest accumulation in peroxide. Based on the peroxide content, papaya oil would be considered to be the most stable oil. Although this is not a complete conclusion as only the primary oxidation products have been measured.

The MUFA rich oils low peroxide content maybe de to the high levels of MUFA's which are not easily oxidisable and other parameters such as antioxidant content.

4.3.3 Peroxide accumulation in PUFA rich oils (Table 22). Pink grapefruit (M/P, 0.6) exhibited the greatest accumulation in peroxide content over 120 hours at 18^{o}C was 81 meqkg^{-1}. Passion seed oil (M/P, 0.2) had a low accumulation in peroxide content throughout the 120 hour period, 15.9 meqkg^{-1}. The oils low peroxide accumulation may be due to the presence of natural occurring tocopherols reported as 465 ppm [199].

Sesame oils (M/P, 0.8) peroxide accumulation was relatively low, 12 meqkg^{-1}. The low peroxide accumulation may be attributed to the oils tocopherol content of 400-700 ppm [3]. The low peroxide content may also be due to the other antioxidant species present in the oil, including ligans such as sesamin, sesamolin, sesamolin [3]. Grape seed oil has been associated with high levels of antioxidants. Grape seed oil (M/P, 0.2) has been reported to contain rich quantities of flavonoids, phenolics and stilbenes [109]. The peroxide accumulation of grape seed oil was 50 meqkg^{-1}. The peroxide content for the oil was relatively high, and considering its antioxidant properties it would be expected be more stable. Grape seed oil has be used as a blending oil ideal for use with unstable oils such as sunflower oil [209]. The antioxidant content makes it an oil which increases the stability of oils with low stability [209].

Lime and Grape seed oils both exhibited high starting peroxide values, 28 meqkg^{-1} and 20 meqkg^{-1} respectively, (Table 20). The high starting peroxide values indicated that the oils have been oxidised prior to evaluating their oxidative stability.

Manketti (M/P, 0.2) and Pomegranate (M/P, <0.1) oils both contained conjugated linolenic acid. Manketti contained approximately 20% conjugates with α-eleostearic acid being the dominant isomer. Pomegranate contained approximately 76% conjugates of which 80% was punicic acid. The peroxide accumulation of Manketti oil was the lowest exhibited by all the PUFA rich oils, 5.6 meqkg^{-1}. Pomegranate's peroxide accumulation throughout the 120 hour period was 10.7 meqkg^{-1}. The high oxidative stability of both oils may be due to the antioxidant of the oils. Pomegranate oil has been reported to contain 161-170 ppm α-tocopherol and 80-92 ppm γ-tocopherol [118]. The antioxidant content of Manketti oil has been reported to contain 233 ppm tocopherols. [210]. When comparing the tocopherol content of Manketti and pomegranate oil, to Passion seed oil (465 ppm), it is evident that the low tocopherol content of Manketti and Pomegranate may not be the only contributing factor to their oxidative stability, and the conjugated fatty acid content may need to be considered.

4.3.4 Assays to evaluate oxidation (Table 23). The peroxide value can not be used solely to determine the oxidative stability of oils. The above data for the peroxide value can sometimes be misleading, and does not always give a true indication of the state of oxidation. The values obtained do not give an indication as to whether the peroxide content was from the formation or breakdown of peroxides. To asses the oxidative stability of an oil, measuring the secondary oxidation products would give a more informed indication of the current state of oxidation. The non volatile aldehyde compounds formed from the breakdown of hydroperoxides can be measured using the *p*-

anisidine assay. The *p*-anisidine (*p*-A.V) assay, which primarily measures the aldehydes, principally 2-alkenals and 2,4-dienals [211]. The peroxide value also measure minor secondary products such as epoxides, therefore the conjugated diene was used to measure the formation of the hydroperoxides.

Passion seed oil (M/P, 0.2), was previously analysed, the data showed it to have a low peroxide accumulation in Table 20. When the oil was further examined, the peroxide accumulation was low, 24.5 meqkg^{-1} (Table 23). The conjugated diene content remained constant through the 120 hour period. The non volatile aldehyde (NVA) content increased steadily. The oxidative stability of Passion seed oil is relatively high during a 120 hour period, which was indicated by the peroxide and NVA content.

Brown flax oil (M/P, 0.2) accumulated a peroxide content of 6.0 meqkg^{-1}. The conjugated diene content remained relatively constant. The NVA content increased to a maximum 2. The oils oxidative stability was remarkably high. Brown flax is rich in linolenic acid, which would render the oil susceptible to oxidation. The tocopherol content of Brown flax seed oil has been reported to be approximately 39.5-50 mg/100g [3, 212]. The oils stability maybe due to the oils optimum levels of tocopherols. There have been studies were the natural occurring tocopherol content has been too concentrated, leading to a pro oxidant effect. This was observed in Soybean oil, the removal of a proportion of the tocopherols improved the oils oxidative stability [180].

The addition of *p*-anisidine assay added an extra dimension to the interpretation of the oils oxidative stability. The following oils analysed, were assayed using the *p*-anisidine assay, as it gave a greater understanding of the oils secondary oxidation behaviour during oxidation.

4.3.5 Oxidation of oils rich in oleic acid (Table 24). Baobab oil (M/P, 1.1) remained relatively stable at 18^{o}C accumulating low levels of peroxides, and NVAs, 9.9 meqkg^{-1} and l.5 respectively. The addition of 750 ppm α-TOC increased the PVs by 38%, although lowering the NVAs. At 60^{o}C Baobab oil PVs were lowered with no significant change in the NVAs. The addition of α-TOC had no effect on the oxidative stability at 60^{o}C; the PVs remained similar to Baobab control. The increased temperature may have increased the rate of oxidation, leading to the formation of volatile compounds, hence the low accumulation of NVAs.

Macadamia oil, (M/P, 36.2) remained relatively stable through the 120 hour time period. The PV reached 5.2 meqkg^{-1}, and the NVAs were undetectable. The addition of α-TOC had no effect on the oxidative stability of the oil; there was no significant change in the peroxide and the NVA content. At 60^{o}C the levels of peroxides detected were proportionally lower than for all treatments at 18^{o}C. The addition of α-TOC had no effect on the oxidative stability. The increased rate of oxidation with increasing temperature again suggests that oxidation proceeds through the formation of volatile compounds.

Macadamia has been reported to contain low levels of peroxides 0.6-3.6 meqkg^{-1} when fresh, which is consistent with the data obtained here [96]. It has been reported that the tocopherol and phenolic content of Macadamia oil is considerably low compared to other nut oils [213]. Increased oxidative stability with increased levels of antioxidants was observed by some cultivars in the study, the data set for this chapter showed 750 ppm α-TOC to have no effect on the oxidative stability of Macadamia oil [96]. This maybe due to the levels of the α-TOC being to low to have a significant effect on the oxidative stability.

Yangu oil (M/P, 1.2) at 18^{o}C, PV was markedly increased over 120 hours with an accumulation of 11.3 meqkg^{-1}, and NVAs levels at 5.3. It must be noted that Yangu oil which was supplemented with 750 ppm α-TOC had an increased peroxide content at time zero. Therefore comparison of the data will be based on the overall accumulation of peroxides and NVA in each individual oils. The addition of 750 ppm α-TOC decreased the peroxide accumulation to 7.7 meqkg^{-1}, and increased the NVA content by 1.4. The incubation of Yangu oil at 60^{o}C increased the PV to 69.6 meqkg^{-1} and the NVAs to 7.1. The addition of α-TOC at elevated temperatures increased the peroxide content by 66 meqkg^{-1} to give an overall accumulation of 120 meqkg^{-1}. There was no significant change in the NVA content with the addition of 750 ppm α-TOC, suggesting volatile compounds may have been formed through hot oxidation.

The PV of Moringa oil (M/P, 76.9) exposed to the atmosphere increased over 120 hours to 33 meqkg^{-1}. The addition of α-tocopherol reduced the peroxide content by over 40%. Moringa oil incubated at 60^{o}C showed PVs that were significantly lower than the PVs at 18^{o}C. At 48 hours a reduction of 38-50% and at 120 hours 70-87% reduction in PVs, this maybe due to increased levels of oxidation. The incubation of Moringa oil with α-TOC at 60^{o}C showed an increase in the non-volatile aldehydes. The PV for the addition of α-TOC to Moringa oil at elevated temperature remained unchanged. Oxidation rate of oils generally increases with temperature, the lower PVs at 60^{o}C indicates the oil has rapidly decomposed to volatile compounds as the levels of NVAs were low.

A comparative study of two oleic rich oils, Moringa and Marula, where both oils contained approximately 70% oleic acid, showed Moringa to be more stable [197]. The study used an accelerated oxidation method (AOCS Cd 12b-92). Moringa oil with the

addition of 1000 ppm of tocopherols had an oil stability index (OSI) value of 133h, compared with Marula 37h [197]. The difference in the OSI values confirmed the oils were not comparable in terms of their ability to resist oxidation. The authors suggested the 7% PUFA content of Marula oil compared to the 1% PUFA content of moringa oil was an important factor [197]. The oleic rich oils that were analysed in the current chapter vary in PUFA content and their antioxidant content. The variation of PUFA and antioxidant content may indicate contrast in levels of oxidation.

Low levels of peroxides and NVAs detected in Baobab, Macadamia and Moringa oils exposed to oxidation at 60^{o}C suggests that volatile compounds are generated at elevated temperatures. Decomposition of oleic acid (18:1n9) occurs from the cleavage of alkoxyl radical formed from the hydroperoxides autoxidation. The homolytic cleavage of either side of the alkoxyl intermediate from 8 hydroperoxide forms decanal and 2-undeacanal. Nonanol is formed from either side of the 9- or 10-hydroperoxide, octane is formed from 10-hydroperoxide and octanol and heptane is formed from the 11-hydroperoxide [125]. Determining the presence of volatiles would require GC headspace analysis.

In summary the addition of α-tocopherol did not give the desired effect of increasing the oxidative stability of the oleic rich oils. A positive effect was observed in Moringa and Yangu oil at room temperature.

4.3.6 Oxidation of oils rich in linoleic acid (Table 25). Five linoleic rich oils were examined for their oxidative stability, namely, Evening primrose oil (EPO), Grape, Manketti, Rosehip and Wheat germ.

Evening primrose oil, EPO (M/P, <0.1) was shown to be relatively unstable with a PV of 62.8 meqkg^{-1} and NVAs content of 2.9. The addition of α-TOC had no marked effect on the oxidative stability at 18^{o}C. Contradictory reports have shown that the addition of a mixture of α,γ tocopherol and phosphatidylethanolime (PE) increased the oxidative stability of EPO [205, 214]. Grape oil (M/P, 0.2), showed similarities to EPO, the PV and NVAs was relatively elevated. The addition of 750 ppm α-TOC showed no effects on the PV and NVA levels. The low oxidative stability of Grape seed oil is in contrast with its reported rich content of phenolic compounds and flavonoids [109]. The peroxide content of Grape seed oil was shown to be high in the previous chapter. The oxidative stability of Grape seed from the current data indicates a low oxidative stability.

Manketti oil (M/P, 0.2) contained 20% conjugated linolenic acid and showed a low peroxide accumulation of 1.4 meqkg^{-1}.The NVA content was recorded at 17.5. It must be noted that Manketti oil which was supplemented with α-TOC had a decreased peroxide content at time zero. Therefore comparison of the data will be based on the overall accumulation of peroxides and NVA in each individual oils. The addition of α-TOC increased the peroxide content by 2meqkg^{-1}. The addition of α-TOC had no significant effect on the oxidative stability at 18^{o}C. The oil's oxidative stability at 18^{o}C showed degradation due to the high NVA levels. This suggests that the slow oxidation at room temperature may yield NVA as opposed to volatile compounds. The incubation of Manketti oil at 60^{o}C polymerized beyond 48 hours, which may be due to the heat which lowers the oil's solubility leading to polymerization [147]. The addition of α-TOC did not increase the oil's oxidative stability. The PV was recorded as 186.8 meqkg^{-1}. The

addition of α-TOC increased the NVA content to 70.2. The addition of α-TOC did not enhance manketti oil's oxidative stability, and showed to have adverse effects.

Wheat germ oil (M/P, 0.2) increased steadily in peroxide content with the final PV of 25 $meqkg^{-1}$ at 18^oC. The NVA level was recorded as 13.7 at 120 hours. The addition of α-TOC reduced the PV by 29% and the NVAs became undetectable at the end of the time course. At 60^oC the peroxide content surpassed 200 $meqkg^{-1}$ and the NVAs was above 10. The addition of α-TOC increased the PV by 23%, indicating a proxidants effect. The addition of α-TOC increased the oxidative stability at 18^oC but had adverse effects at 60^oC.

Wheat germ has been reported to be rich in tocopherols, 1790-3200 mg/kg [215]. Wheat germ antioxidant extracts were added to various stripped oils at 50^oC, and it was found to reduce the hydroperoxide content of the oils [216]. The antioxidant unsaponifiable matter has been reported to provide stability to rapeseed oil during storage at 60^oC [215]. The addition of α-TOC did increase the oxidative stability of Wheat germ oil at 18^oC, but failed to enhance the oils thermo oxidative stability.

All the 18:2 n6 oils showed different oxidative properties with the addition of α-TOC at 18^oC and 60^oC. The data indicates that the oils' oxidative stability is complexed and that the oil's natural antioxidant content may play an underlying role.

4.3.7 Oxidation of oils rich in linolenic acid (Table 26). Brown flaxseed oil (M/P, 0.2) oxidised at a slow rate at 18^oC. The addition of α-TOC had no marked effect on the oxidative stability. At 60^oC the oil showed an increase in peroxide content at 48hrs (>600

meqkg^{-1}), the oil polymerized by 120 hours. The addition of α-TOC reduced the peroxide content by 60% at 48 hours. The reduction in the peroxide and NVA content delayed the onset of polymerisation by approximately 12-24 hours. The current data set has showed Brown flaxseed oil to be stable. The addition of α-TOC has been shown to increase the oils thermo-oxidative stability.

Brown flaxseed oil was analysed earlier in the chapter, and was found to be stable. The data obtained from the current data set, has also shown Brown flaxseed to be stable. The oils high linolenic acid content may be a direct reflection of the oils antioxidant activity toward protection of the PUFAs.

Pomegranate oil (M/P, <0.1) contains approximately 77% conjugated linolenic acid, with punicic acid being the predominant isomer. The peroxide content of PO increased by 16% over the 120 hour period with the NVAs steadily increasing. The addition of α-TOC had no effect on the oxidative stability of PO at 18^{o}C. At 60^{o}C the oil's PV and NVA increased until 48 hours, thereafter the oil polymerised. The addition of 750 ppm α-TOC at 60^{o}C acted as a proxidants increasing the PV by 40% and the NVAs by 15%, at 48 hour, at 120 hours the oil is fully polymerised.

The reasonable oxidative stability at 18^{o}C may be due to the antioxidant content. PO has been reported to contain 2630-2900 mg/Kg tocopherols [118], and 7.8-72.1 mg/g phenolic compounds [217].

The oxidation of conjugated fatty acid differs from non conjugated fatty acids, the mechanism and kinetics of conjugated fatty acid oxidation is still not fully understood [218-220]. The pathway for the oxidation of conjugated fatty acids does have elements of the pathway for non conjugated fatty acid hydroperoxide formation [218], although

breakdown products formed include dimers and polymers [220]. Under mild oxidation conditions (30^{o}C in the dark) conjugated C18:2 was more stable than non conjugated 18:2 [220]. However recent studies have shown conflicting interest, comparison of C18:2, C18:3 and their conjugated forms showed stability in the order of 18:2>18:3>conj18:2>conj18:3 [221].

4.4 Conclusions

The time course studies on a wide range of oil's varying in their degree of unsaturation, and their subsequent oxidative stability differed depending on supplementation with α-tocopherol.

The oxidative stability of a variety of oils has been analysed and it can be concluded that the fatty acid content is a determinant factor. The antioxidant content of the oil also plays a role in the oxidative stability mechanism. Supplementing oil's with 750 ppm α-TOC did not enhance the oxidative stability of the majority of the oil's analysed. Positive results were obtained for Moringa, Yangu, Wheat germ and Brown flaxseed oils. In the case of these oils it maybe that the addition of 750 ppm α-TOC was an optimum concentration to influence the delay of the oxidation. The addition of 750 ppm α-TOC at 60^{o}C to Manketti and Pomegranate oil, delayed the onset of polymerisation. Manketti and Pomegranate oil both polymerised at 120 hours with out the addition of α-TOC. The addition of 750 ppm α-TOC may delay the onset of oxidation, although there maybe interaction between the free radicals generated from the conjugated fatty acids and the α-TOC. The addition of 750 ppm α-TOC allowed the oil to remain viscous at 120 hours at elevated temperatures.

Chapter 5

Preliminary blending of Pomegranate oil

5.1 Introduction

The oxidative stability of plant seed oil's was evaluated in the previous chapter. The addition of α-tocopherol did not increase the oxidative stability of the majority of the oils assayed.

It has been proposed that unstable oil's can be blended with stable oil's to increase the oxidative stability of the unstable oils. Sunflower oil has been blended with minor amounts of Moringa oil, which led to a reduction in the peroxide content of Sunflower oil [121].

Pomegranate oil showed to have a relatively high oxidative stability, which was observed in chapter 4. The reason for the high oxidative stability is not fully known, but the antioxidant content and possibly the conjugated fatty acid content may be a contributing factor. The oxidative stability may be attributed to the high content of conjugated linolenic acid. Conjugated linolenic acid may have antioxidant properties similar to carotenoids. The oil is readily available as it is a commercial implicit product of Earthoil. Therefore it would be an ideal oil for blending with unstable oils.

A current patent prevents Moringa oil being supplemented with Vitamin E to increase its oxidative stability. It is envisaged that Pomegranate oil or other plant seed oil's, when blended with Moringa oil could increase its oxidative stability. Pomegranate has been selected as a preference as it has previously shown to have a relatively high oxidative

stability. Earthoil also produces a large quantity of Pomegranate oil, which makes it commercially viable for there purposes.

The aim of the current chapter is to determine the effectiveness of Pomegranate blends with Moringa and Borage oils. It would be envisaged that Pomegranate oil may have positive effects on the oxidative stability of Moringa and Borage, due to its antioxidant properties. The antioxidant properties may be due to the oils conjugated linolenic acid, punicic acid, which may have similar properties to lycopene and other carotenoids.

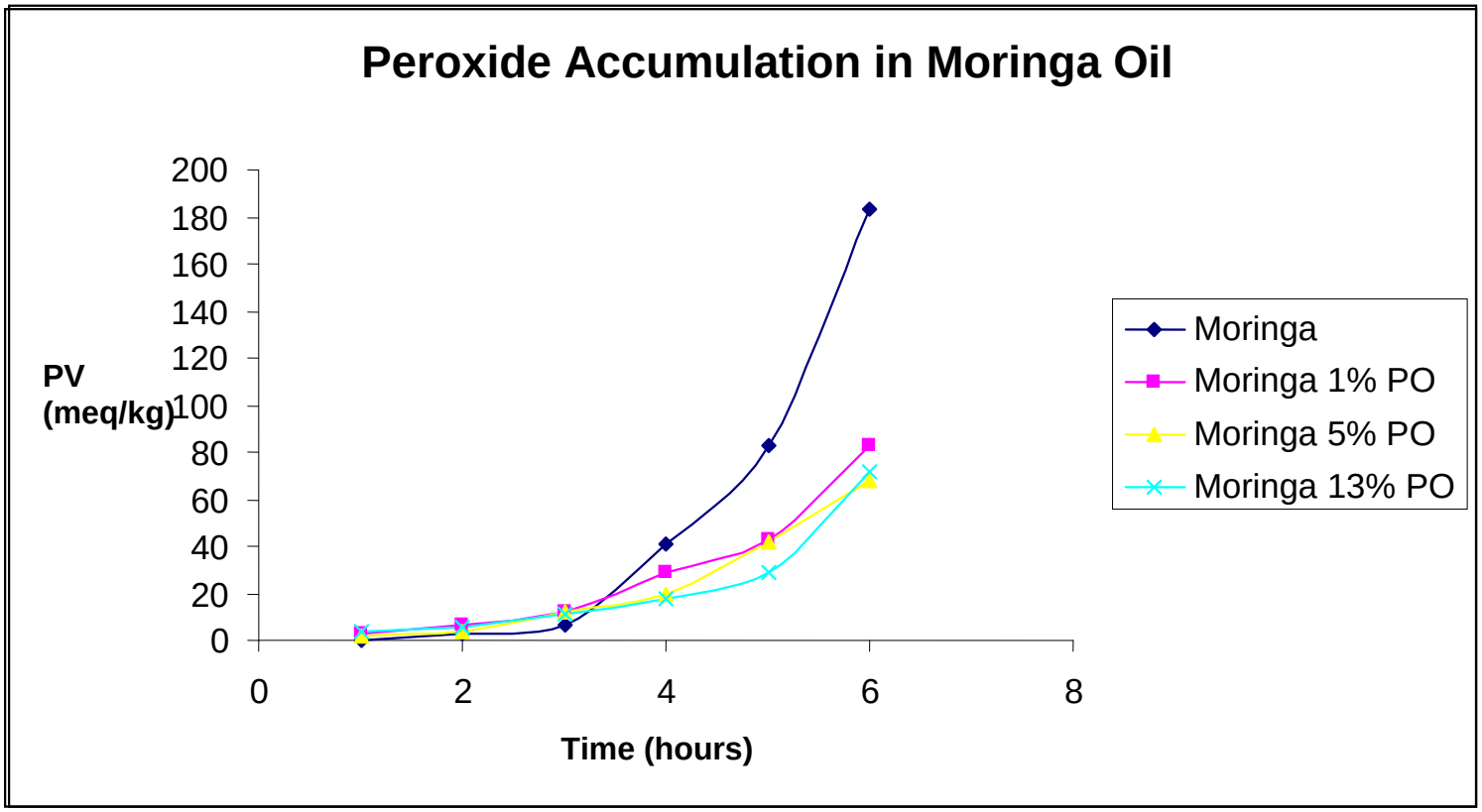
5.2 Results

Table 27. Peroxide accumulation in Moringa Oil

	PV meqkg^{-1}						
	0hr	**1hr**	**4hr**	**24hr**	**48hr**	**120hr**	**Accumulation***
Moringa	0.4 ±0.0	3.0 ±0.3	6.6 ±0.3	40.7 ±2.5	82.8 ±11.4	182.8 ±9.0	182.4
Moringa 1% PO	3.0 ±0.1	6.1 ±0.1	12.1 ±0.0	28.4 ±0.2	43.2 ±0.1	82.5 ±0.3	79.5
Moringa 5% PO	1.4 ±0.3	4.0 ±0.6	12.1 ±0.3	19.9 ±3.4	42.3 ±3.6	68.0 ±1.4	66.6
Moringa 13% PO	3.3 ±0.1	5.9 ±0.1	11.6 ±0.3	17.7 ±0.6	28.5 ±4.2	71.7 ±5.1	68.4

Peroxide accumulation in moringa oil over a 120 hour time period. Moringa oil additives, pomegranate oil (PO). Assays were conducted in 50ml conical flasks with a surface area of 13.9 cm^2. Results represent the mean ±SD for triplicate analysis. * Represents the peroxide accumulation from time point zero. All zero hour samples differ due to assays prepared on different days. The accumulative peroxide has been determined to offset the zero hour differences.

Graph 2. Peroxide accumulation in Moringa Oil



Graph 2..Peroxide accumulation in moringa oil over a 120 hour time period. Moringa oil additives, pomegranate oil (PO). Assays were conducted in 50ml conical flasks with a surface area of 13.9 cm^2. Results represent the mean ±SD for triplicate analysis.

Table 28. Moringa peroxide accumulation with the addition of α-TOC and PO.

	Peroxide meqkg^{-1}					
Antioxidant	**0ppm**	**50ppm**	**100ppm**	**500ppm**	**1000ppm**	**5000ppm**
Moringa	26.4 ±0.9	nd	nd	nd	nd	nd
Moringa PO	27.3 ±1.3	nd	17.2 ±1.3	18.6 ±1.6	19.9 ±1.8	26.3 ±1.8
Moringa α–TOC	27.3 ±1.3	nd	15.4 ±1.3	17.0 ±2.2	12.7 ±0.14	10.2 ±1.9

Peroxide accumulation in moringa oil over a 120 hour time period. Moringa oil additives, pomegranate oil (PO), and α-tocopherol (α-TOC) at various concentrations.. Assays conducted in 50ml conical flasks with a surface area of 13.9 cm^2. Results represent the mean ±SD for triplicate analysis.

Table 29. Peroxide and non volatile aldehyde accumulation in Borage oil treated with 1% PO and 750ppm α-TOC.

	Time (h)					
	0		**48**		**120**	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
Borage						
Control	8.0 ±0.0	0.9 ±0.6	43.3 ±1.9	1.5 ±0.7	87.1 ±8.3	0.8 ±0.7
+ 1% PO	7.3 ±0.2	3.3 ±0.2	10.2 ±0.5	2.7 ±1.0	11.8 ±0.2	2.9 ±0.7
+ α-TOC	7.2 ±0.0	1.2 ±0.7	23.8 ±2.2	1.8 ±0.6	43.7 ±3.9	1.1 ±0.5
60 °C						
Control	8.0 ±0.0	0.9 ±0.6	32.2 ±0.6	6.1 ±1.0	107.0 ±6.7	22.6 ±2.2
+ 1% PO	7.3 ±0.2	3.3 ±0.2	47.5 ±2.8	11.1 ±0.8	187.7 ±81.5	63.7 ±5.5
+ α-TOC	7.2 ±0.0	1.2 ±0.7	125.1 ±3.8	18.7 ±1.8	304.0 ±56.2	61.7 ±1.3

Peroxide values of Borage oil over a 120 hour time period. Borage oil additives, pomegranate oil (PO) and α-tocopherol (α-TOC). Assays conducted in 50ml conical flasks with a surface area of 13.9 cm^2. Results represent the mean ±SD for triplicate analysis. The analysis was carried out at 18°C.

5.3 Results and Discussion

Pomegranate oil (PO) was blended into Moringa firstly as a percentage weight and secondly as parts per million (ppm). Moringa and Borage oil were both host oils for the addition of PO.

5.3.1 Percentage addition of pomegranate oil (Table 27). Pomegranate oil was added as a percentage weight to Moringa oil. The addition of PO ranged from 1 to 13%. The previous chapter's data showed Moringa oil to be relatively stable, with a low accumulation in peroxides and non volatile aldehydes. Moringa oil analysed in this current chapter was freshly extracted from the seed on the day of analysis. In this instance the accumulation of peroxide reached a maximum of 182 $meqkg^{-1}$. It can only be assumed that the high peroxide content was due to contamination of the oil at some point during the extraction. Although the contamination was a set back, the oil did provide consistent results with the addition of PO oil.

The presence of PO blended to the heavily oxidised Moringa oil reduced the peroxide content significantly. The greatest reduction in peroxide was observed with the addition of 13% followed by 5% and finally 1%. The addition of 1% would be an ideal supplementation as it would provide a significant reduction in the peroxide content, without altering the fatty acid content by a significant amount. The addition of 1% PO would also be more cost effective when considering long term usage of the oil.

5.3.2 Pomegranate and α-tocopherol (Table 28). The addition of PO to Moringa oil was compared with α-tocopherol. In this instance PO and α-tocopherol were blended at ppm.

Moringa oil peroxide content appears to be in agreement with the peroxide content of the pervious chapter. The peroxide content of Moringa oil was furthered reduced with the addition of α-TOC and PO. The optimum supplementation of α-TOC addition to Moringa oil was 5000 ppm, whilst PO optimum supplementation to Moringa oil was 100 ppm.

Comparing the data obtained from the percentage weight addition to the ppm addition of PO to Moringa oil, percentage addition was more effective. The addition of 5 and 13% PO to Moringa oil, reduced the peroxide content by 63%. The addition of 1% PO oil to Moringa oil reduced the peroxide content by 56%. The addition of 100ppm PO reduced the peroxide content by 35%.

The addition of PO to Moringa oil at various concentrations has provided positive data. The data shows that highly oxidised and relatively stable Moringa oil can be stabilised by the addition of PO. The addition of PO and α-TOC can be expressed as ppm or as a percentage weight, the concentration remains the same in both aspects.

5.3.3 Borage oil blended with 1% PO (Table 29). Borage oil (M/P, 0.3) was shown to be unstable at 18^{o}C over 120 hours, with a PV of 87.1 meqkg^{-1}, and NVA content of 8.30. The addition of α-TOC and PO greatly improved the oxidative stability. α-TOC reduced the PV by 50% and the NVAs by 53%, whilst PO reduced the PVs by 85% and the NVAs by 98%. At 60^{o}C the PV of Borage oil was 107.2 meqkg^{-1} and the NVAs was 22.6. The

addition of α-TOC and PO at elevated temperatures showed negative effects on Borage oil increasing the peroxide content and NVA content by up to 3-fold. The addition of α-TOC and PO may have caused a proxidant effect, by increasing the rate of oxidation.

Borage oil has been subjected to blending in a published study. The addition of virgin Olive oil to Borage oil did not increase the Borage oils oxidative stability [222]. The data obtained for PO addition to Borage oil indicates an increased oxidative stability. The thermo-oxidative stability was reduced by the addition of PO.

5.3.4 Radical scavenging properties of punicic acid.

The blending of PO with Moringa and Borage oil showed positive effects, which may be attributed to the tocopherol and phenolic compounds in the oil. The tocopherol content of Pomegranate oil used for blending was determined through an external analytical institution, Eurofins. The tocopherol content of the PO was 43 mg/kg. Reported levels of tocopherols and phenolics have been given as 2630-2900 mg/Kg tocopherols [118], and 7.8-72.1 mg/g [217] respectively. To fully evaluate PO stabilising properties, individual components would need to be isolated and analysed. Each component would need to be isolated and analysed to determine the component which is responsible for PO's antioxidant properties.

Pomegranate oil contains conjugated fatty acids which may have stabilising properties. The effects of conjugated fatty acids in biological systems have shown positive effects [116, 223, 224], but negative effects have also been observed [225, 226]. The

concentration of the conjugated fatty acids may play a critical role in the oxidative stability of the oils.

Pomegranate oil has a variety of conjugated linolenic acids, with punicic acid accounting for over 70% of the conjugates. The conjugated double bond system may have antioxidant capabilities similar to that displayed by carotenoids such as *β*-carotene and lycopene. Lycopene and *β*-carotene have the ability to quench singlet oxygen, $^1O_2$, species and have peroxyl radical scavenging properties. This may be exhibited by the conjugated system of punicic acid (PA) [146, 227].

The antioxidant capability of punicic acid may be due to light filtering, $^1O_2$ quenching, sensitizer inactivation and free radical scavenging. Firstly the singlet oxygen quenching $^1O_2$ could occur by energy transfer from the singlet $^1O_2$ to punicic acid, without the generation of oxidised products, similar to carotenoids, Equation 12 & 13 [228].

It has been reported that carotenoids inactivated the excited sensitizers; this is achieved by absorbing the energy from the sensitizer. The excited carotenoid returns to its ground by the transfer of energy to the oil [228]. The punicic acid antioxidant mechanism is modelled on carotenoids, the excitation of PA may also occur. It has been reported that *β*-carotene has great difficulty in donating its hydrogen to a alkyl or peroxyl radical of PUFAs [229]. Although *β*-carotene can donate hydrogen to the hydroxyl radical, giving rise to a carotene radical, which is relatively stable due to the delocalisation of unpaired electrons through its conjugated polyene system, Equation 14 [229].

The punicic acid radical may have the ability to react with peroxyl radicals and form non radical products. This would only occur in an environment with a low presence of oxygen, Equation 15 [228].

A lipid peroxy radical may have the ability to add to punicic acid, giving rise to a punicic acid peroxy radical (LOO-PA$^{\cdot}$). This would be likely in an oxygen rich atmosphere, Equation 16. The punicic peroxy radical could then react with the $^{3}O_{2}$ followed by the reaction with lipid molecules, Equation 17. The corresponding alkyl radical propagates the chain reaction of lipid oxidation, Equation 18 [228].

The proposed mechanism of punicic acid antioxidant ability was based on the mechanism of carotenoids [227-230]. Further research into punicic acid antioxidant mechanism would need to be carried for a fuller understanding. Research is ongoing in the field of conjugated fatty acids with regards to their beneficial health properties, the research into punicic acid is limited, hence the lack of literature available on this conjugated fatty acid.

The theory that punicic acid may have stabilising properties is only a proposal at this point. It would require extensive research, beginning with the isolation of punicic acid and assaying it in a variety of oils.

An alternative evaluation of the efficacy of pomegranate oil/punicic acid would be to screen a variety of oils with the addition of pomegranate oil. If positive results were to be obtained it would be a strong justification for further research to be conducted into punicic acid.

Proposed radical mechanism for punicic acid antioxidant abilities [227-230].

5.4 Conclusions

The addition of Pomegranate oil has shown positive effects when blended with highly oxidised Moringa oil. Pomegranate oil also showed positive effects when added to moderately oxidised Moringa and Borage oils. The addition of 1% PO leads to a significant reduction in the peroxide content of both oils. It has been speculated that punicic acid may be the active component in pomegranate that helps stabilise oils. Punicic acid activity is a speculation, and it must be highlighted that all other antioxidant components have not been disregarded at this early stage of evaluation. To evaluate this proposal Pomegranate oils' efficacy will need to be further tested with oils varying in the degree of unsaturation.

Chapter 6

Screening Pomegranate oil for oxidation stabilising potential

6.1 Introduction

The preliminary blending of PO with Moringa and Borage oil, showed PO to reduce the peroxide content by significant amounts. To distinguish the component which acts as the stabilising component of Pomegranate oil, would require extensive research. The tocopherol content of PO oil used for blending in the previous chapter was 4.3 mg/100g and the same batch off Pomegranate oil was used for this current chapter.

The aim of this chapter was to evaluate the effectiveness of PO's stabilising properties. The oil was blended with a selection of oil's varying in fatty acid and antioxidant content. The oxidative stability and thermo-oxidative stability was evaluated, and a definitive conclusion was reached. A positive outcome from PO blends to plant seed oil's would lead to further research into punicic acid and other potential stabilising components of PO.

As previously discussed in earlier chapters, existing patents prohibits the use of vitamin E as a stabilising agent for a selection of oils. A positive outcome from the blending of PO with plant seed oils would have potential commercial gains. It would allow Earthoil to overcome patent restrictions, with the potential to licence PO blending with plant seed oil's.

6.2 Results

Table 30. Oxidation of oleic rich oils blended with 1% PO at 18^{o}C and 60^{o}C

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
Baobab						
18^{o}C						
Control	6.8 ±1.7	0.2 ±0.1	8.5 ±0.2	0.6 ±0.3	9.9 ±1.7	1.5 ±0.1
+ 1% PO	6.0 ±0.4	0.8 ±0.3	6.8 ±0.4	1.3 ±0.2	8.8 ±0.0	v
60^{o}C						
Control	1.2 ±0.7	nd	1.3 ±0.2	nd	3.2 ±1.1	2.0 ±0.4
+ 1% PO	0.9 ±0.2	1.9 ± 0.0	1.7 ±0.2	nd	4.8 ±1.7	3.6 ±0.7
Macadamia						
18^{o}C						
Control	nd	nd	3.6 ±0.4	nd	5.2 ±0.0	nd

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
+ 1% PO	0.2 ±0.0	0.4 ±0.3	1.9 ±0.1	2.8 ±0.5	8.1 ±0.5	1.9 ±0.3
60°C						
Control	nd	nd	0.3 ±0.1	nd	0.3 ±0.1	nd
+ 1% PO	0.2 ±0.0	0.4 ±0.1	0.6 ±0.2	2.1 ±0.9	0.9 ±0.2	2.3 ±0.6
Moringa						
18°C						
Control	2.7 ±0.8	nd	15.8 ±0.9	1.5 ±0.8	32.9 ±2.6	nd
+ 1% PO	2.1 ±0.2	nd	15.5 ±0.8	nd	29.6 ±1.4	nd
60°C						
Control	2.7 ±0.8	nd	8.0 ±1.0	nd	4.3 ±0.8	nd
+ 1% PO	2.4 ±0.4	nd	9.8 ±0.2	nd	5.0 ±0.2	2.1 ±0.4
Yangu*						
18°C						
Control	15.9 ±0.1	4.5 ±0.3	25.5 ±1.1	5.3 ±0.2	27.2 ±1.0	5.3 ±0.5

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
+ 1% PO	27.1 ±0.1	nd	30.4 ±0.4	0.0 ±0.0	33.9 ±0.8	2.8 ±0.2
60^{o}C						
Control	15.9 ±0.1	4.8 ±0.3	39.9 ±1.6	6.3 ±0.4	69.6 ±1.2	7.1 ±0.4
+ 1% PO	34.4 ±0.2	3.7 ±0.4	66.9 ±0.9	8.5 ±1.3	286.8 ±154.7	47.8 ±0.9

PV are values in meqkg^{-1} oil. p-A.V. are unitless. Results represent the mean ±SD for triplicate analyses. Poly = polymerization. nd = not detected. *Data of same batches with varying time zero values.

Table 31. Oxidation of linoleic rich oils blended with 1% PO at 18^{o}C and 60^{o}C

	Time (h)					
	0		**48**		**120**	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
EPO						
18^{o}C						
Control	9.6 ±0.7	3.1 ±0.3	38.8 ±2.4	3.4 ±0.2	62.8 ±0.7	2.9 ±0.2
+ 1% PO	9.8 ±0.3	3.5 ±1.4	37.6 ±0.4	5.5 ±1.2	58.5 ±2.0	4.8 ±1.9
Grape						
18^{o}C						
Control	5.8 ±0.5	2.4 ±0.2	36.0 ±1.6	2.8 ±0.2	66.6 ±3.3	2.9 ±1.4
+ 1% PO	5.8 ±0.8	2.9± 0.2	25.4 ±1.1	3.2 ±0.5	59.8 ±2.1	2.4 ±0.1
Manketti*						
18^{o}C						
Control	13.1 ±0.7	16.9 ±2.7	17.2 ±1.4	19.3 ±0.2	14.5 ±2.0	17.5 ±0.6

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
+ 1% PO	9.9 ±0.2	20.1 ±0.6	13.1 ±0.3	17.9 ±0.7	14.1 ±0.5	17.5 ±0.9
60°C						
Control	13.1 ±0.7	16.9 ±2.7	50.3 ±8.6	50.2 ±5.7	Poly	Poly
+ 1% PO	16.9 ±1.2	8.5 ±0.3	85.1 ±1.6	28.6 ±4.6	166.7 ±38.2	66.9 ±8.8
Rosehip						
18°C						
Control	37.7 ±0.3	18.1 ±0.5	51.0 ±0.1	18.1 ±0.5	54.8 ±0.1	16.6 ±0.4
+ 1% PO	37.4 ±0.4	17.4 ±0.4	49.5 ±0.4	17.9 ±0.8	53.3 ±0.5	17.0 ±0.2
Wheat Germ						
18°C						
Control	8.6 ±0.1	3.4 ±0.5	12.7 ±0.4	7.3 ±1.1	24.7 ±0.3	13.7 ±0.4
+ 1% PO	12.5 ±0.2	3.6 ±0.6	18.4 ±0.4	0.7 ±0.6	31.9 ±1.1	Nd
60°C						
Control	8.6 ±0.2	3.4 ±0.5	77.7 ±1.1	5.2 ±1.2	228.3 ±1.3	10.8 ±2.2

	Time (h)					
	0		48		120	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
+ 1% PO	12.5 ±0.2	3.6 ±2.3	93.2 ±4.0	7.1 ±1.4	201.4 ±19.6	13.5 ±2.4

PV are values in meqkg^{-1} oil. p-A.V. are unitless. Results represent the mean ±SD for triplicate analyses. Poly = polymerization. nd = not detected. *Data of same batches with varying time zero values.

Table 32. Oxidation of linolenic rich oils blended with 1% PO at 18^{o}C and 60^{o}C

	Time (h)					
	0		**48**		**120**	
	P.V.	***p*-A.V.**	**P.V.**	***p*-A.V.**	**P.V.**	***p*-A.V.**
Brown Flax*						
18^{o}C						
Control	15.6 ±0.4	0.8 ±0.3	18.6 ±0.4	nd	20.0 ±0.7	0.9 ±0.8
+ 1% PO	15.6 ±0.4	0.8 ±0.3	16.9 ±0.4	1.3 ±0.2	17.0 ±0.4	0.2 ±0.2
60^{o}C						
Control	15.6 ±0.4	0.8 ±0.3	602.7 ±5.9	20.0 ±0.3	poly	Poly
+ 1% PO	15.6 ±0.4	0.8 ±0.3	166.3 ±5.8	7.8 ±1.0	605.9 ±8.2	18.2 ±1.0
Pomegranate*						
18^{o}C						
Control	7.3 ±0.2	14.3 ±0.2	7.7 ±1.1	19.4 ±0.3	8.7 ±1.1	22.3 ±0.4
60^{o}C						
Control			56.6 ±2.5	21.4 ±1.6	poly	Poly

PV are values in meqkg^{-1} oil. p-A.V. are unitless. Results represent the mean ±SD for triplicate analyses. Poly = polymerization. nd = not detected *Result provided by MSc Students working on same research project

6.3 Results and Discussion

The oil's have been grouped according to their degree of sataturation. The data for the addition of 1% PO has been presented with the control data for the host oils, which was previously displayed in chapter 4. It must be noted that the control was carried out at with the current data, which was completed as one large study. The oxidative stability is determined by employing the peroxide and *p*-anisidine assays.

Pomegranate oil which was added to the oils contained 4.3 mg/100g of tocopherols.

6.3.1 Oleic acid rich oils (Table 30). Baobab oil (M/P, 1.1) remained relatively stable at 18^{o}C accumulating low levels of peroxides, and NVAs, 9.9 meqkg^{-1} and l.5 respectively. The addition of PO did not enhance the oil's oxidative stability. The peroxide and NVA content remained relatively constant. At 60^{o}C the PVs were lowered with no significant change in the NVAs. The addition of PO did not enhance Baobab's thermo-oxidative stability. The peroxides remained similar to the control, although an increase was observed in the NVAs. The increased temperature may have increased the rate of oxidation. This may have lead to the formation of volatile compounds, hence the low accumulation of NVAs in the control and PO blend.

Macadamia oil, (M/P, 36.2) remained relatively stable during the 120 hour time period. Macadamia oil control has been discussed in detail in chapter 4. The addition of PO at 18^{o}C increased the peroxide content by 55%. The NVAs which were undetectable in the control have been detected upon the addition of PO. At elevated temperatures Macadamia

oil showed remarkable stability. The addition of 1% PO increased the content of peroxides and NVAs. The increased rate of oxidation with increasing temperature again suggests that oxidation proceeds through the formation of volatile compounds. The addition of 1% PO did not enhance Macadamia oil's oxidative/thermo oxidative stabilities.

Yangu oil (M/P, 1.2) at 18^{o}C accumulated 11.3 meqkg^{-1} of peroxide. The NVA content at 120 hours was recorded as 5.3. It must be noted that Yangu oil which was supplemented with PO had an increased peroxide content at time zero. Therefore comparison of the data will be based on the overall accumulation of peroxides and NVA in each individual oils. The addition PO lowered the peroxide accumulation to 6.8 meqkg^{-1}. The addition of PO lowered the NVA content in Yangu oil. At 60^{o}C the peroxide content was increased, although the data was erroneous as the ±SD was above100 meqkg^{-1}. Yangu control had a *p*-anisidine value of 7.1, the addition of PO increased the *p*-anisidine value to 47.8, a 6 fold increase. The addition of PO to Yangu oil had no effect on the oxidative stability and negative effects on the thermo-oxidative stability. At the elevated temperature the negative effect maybe due to the proxidants effects of the PO, which has caused an increase in oxidation.

Moringa oil (M/P, 76.9) had a peroxide content of 33 meqkg^{-1}, with undetectable amounts of NVAs. The addition of PO at 18^{o}C had no effect on the oxidative stability of the oil. The peroxide content remained similar to the control, and low levels of NVAs was detected. At an elevated temperature of 60^{o}C Moringa oil's peroxide content was reduced and NVAs remained undetectable, this maybe due to the peroxides have been broken down into volatile compounds. The addition of PO at elevated temperatures had

no effect on the oxidative stability of Moringa oil. Therefore the addition of PO to Moringa oil did enhance the oil's oxidative stability.

The previous chapter showed on two separate occasions that PO stabilised Moringa oil. The addition of PO stabilised highly oxidised and moderately oxidised Moringa oil. The current data set has shown Pomegranate oil to have no effect on the oxidative stability of the oil. The confliction in the data obtained, highlights the complexity of Pomegranate oils stabilisation properties. The complexity maybe due to the concentration of the antioxidants in PO and the host, if the antioxidant content of PO and the host oil are optimised then the oil would become stabilised. If the antioxidant content of the oil is not optimised then a proxidant effect can be the outcome.

6.3.2 Linoleic acid rich oils (Table 31). Evening primrose oil (M/P, <0.1), was relatively unstable with a PV of 62.8 meqkg^{-1} and NVA content of 2.9. The addition of PO had no marked effect on the peroxide content and the NVAs content. Therefore PO blended with EPO did not have an effect on the oxidative stability.

Grape oil (M/P, 0.2) control was unstable at 18^{o}C. The peroxide content accumulation was 60 meqkg^{-1}. The NVAs reached a value of 2.4. The blending of PO with Grape seed oil had no effect on its oxidative stability. The peroxide and NVAs remained similar to the control.

The addition of PO to Manketti oil (M/P, 0.2) showed an increase in the peroxide accumulation at 18^{o}C. It must be noted that Manketti oil which was supplemented with PO had a decreased peroxide content at time zero. Therefore comparison of the data will

be based on the overall accumulation of peroxides and NVA in each individual oil. Manketti oil's control sample increased NVA content shows that the oil has degraded at 18^{o}C. This may indicate oxidation at low temperatures leads to the formation on non volatile compounds as opposed to volatile compounds.

The incubation of Manketti oil at 60^{o}C lead to its polymerisation beyond 48 hours. Polymerization of oils at elevated temperatures has been reported due to heat lowering the oils solubility leading to polymerization [147]. PO addition to Manketti oil at 60^{o}C increased the oil's oxidative stability, by delaying the onset of polymerisation. The addition of PO to Manketti had no significant effect on the oils oxidative stability at 18^{o}C. The addition of PO to Manketti oil at elevated temperature showed a positive effect by delaying the onset of polymerisation. The reason for the delay in oxidation maybe due to the antioxidant effects exhibited by PO.

Wheat germ oil (M/P, 0.2) peroxide content increased steadily at 18^{o}C, with a final value of 25 meqkg^{-1}. The NVA levels at 18^{o}C were relatively high, with the content of 13.7 at 120 hours. The blending of 1% PO to Wheat germ oil showed no significant effect on the PV, but did lead to a reduction in the NVA levels. The peroxide content of Wheat germ control at 60^{o}C surpassed 200 meqkg^{-1}. The NVA content of Wheat germ was recorded to be greater than 10. The blending of PO to Wheat germ oil at 60^{o}C reduced the PV by 10%, although no reduction was observed for the NVA levels. The addition of PO to Wheat germ oil did not have a significant effect on its oxidative stability at 18^{o}C or 60^{o}C.

6.3.3 Linolenic acid rich oils (Table 32). Brown flaxseed oil (M/P, 0.2) oxidised at a slow rate at 18^{o}C. The addition of PO did not have an effect on the peroxide or NVA content. Brown flaxseed oil oxidative stability at 60^{o}C can not be fully evaluated as the oil polymerises, suggesting poor oxidative stability. The addition of PO to Brown flaxseed oil at 60^{o}C reduced the oils PV by 72% at 48 hours, this lead to the preservation of the oil from polymerization. The oxidative stability of Brown flaxseed oil was not enhanced with the blending of 1% PO. The thermo-oxidative stability of Brown flax oil has shown positive results with the blending of 1% PO.

6.4 Conclusions

The previous chapter showed positive results when PO was blended with Moringa and Borage oil and this was the basis for the screening in this chapter. The blending of PO failed to prevent oxidation of a number of oil's based on assessments made using peroxides and anisidine values. The results obtained highlight the complexities in evaluating the effectiveness of Pomegranate oils stabilising properties. The progressive study does show that future studies would require the isolation of PO individual antioxidant components, and then evaluating the each component's antioxidant properties.

Chapter 7

Processing of oils

7.1 Introduction

Crude oils extracted from the seeds of the plants often require processing to remove non-glyceride impurities [44]. The processing procedure includes degumming, alkali refining, bleaching, hydrogenation and deodorisation, or deodorisation directly after bleaching [125]. Degumming of an oil removes the phosphatides (gums), a process that involves treating the oil with water at 50-70^{o}C. Post degumming the oils undergo alkali refining to neutralise the fatty acids, and convert the free fatty acids into insoluble soaps [44]. The soaps are removed from the oil prior to bleaching with acid treated clay (Earth). Bleaching often renders the oil colourless in appearance. Bleaching also has the added effect of removing impurities such as chelated pro oxidant metals, sulphur compounds, peroxides and traces of aldehydes and ketones [44, 231].

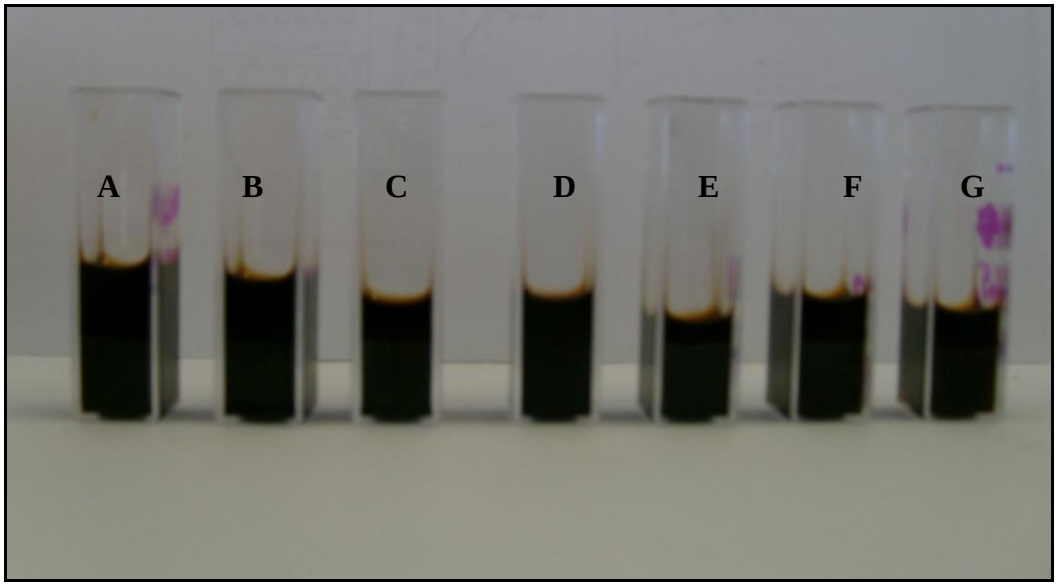
Alkali refining and bleaching of an oil can be time consuming and costly, reducing both the cost and processing time could be achieved if both steps could be combined. This would effectively encompass adding the bleaching earth directly to the oil/soap mixture. Earthoil has proposed a method whereby the refining and the bleaching would be combined to make one step process as opposed to two steps. Effective combinations both processes is the proposed aim of this current chapter. A small scale laboratory trial will be conducted, simulating commercial processing of oils. A positive outcome from the

trial would allow Earthoil to tailor build a processing plant which would be able to carry out the combined process of alkali refining and bleaching.

7.2 Results

Cotton seed is an oil with a distinctive black colour. The reduction in colour is a process which involves the removal of gossypol.

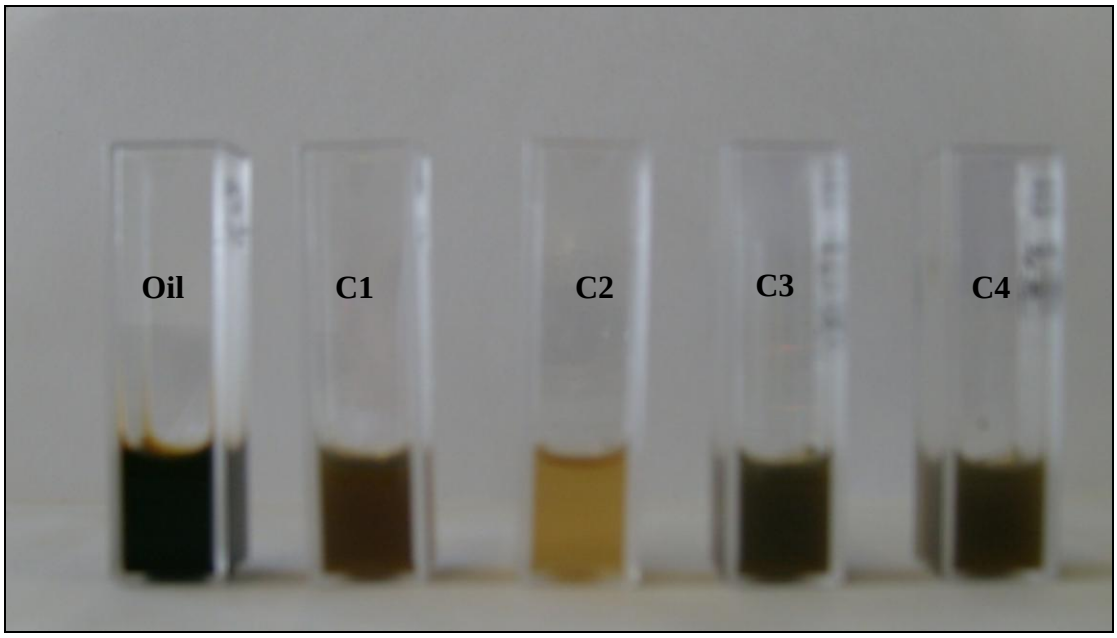
Figure 46. Colour reduction in cotton seed oil.



Cotton seed oil (D) treated with activated carbon to reduce the colour content of the oil prior to processing. Cotton seed oil was incubated with activated carbon under constant mixing. Samples A-C were incubated for 18 hours at 45^{o}C, with 400mg (A), 200mg (B) and 50mg (C) activated carbon. The oils were also incubated at 18 hours at 18^{o}C, with 400mg (G), 200mg (F) and 50mg (E) activated carbon.

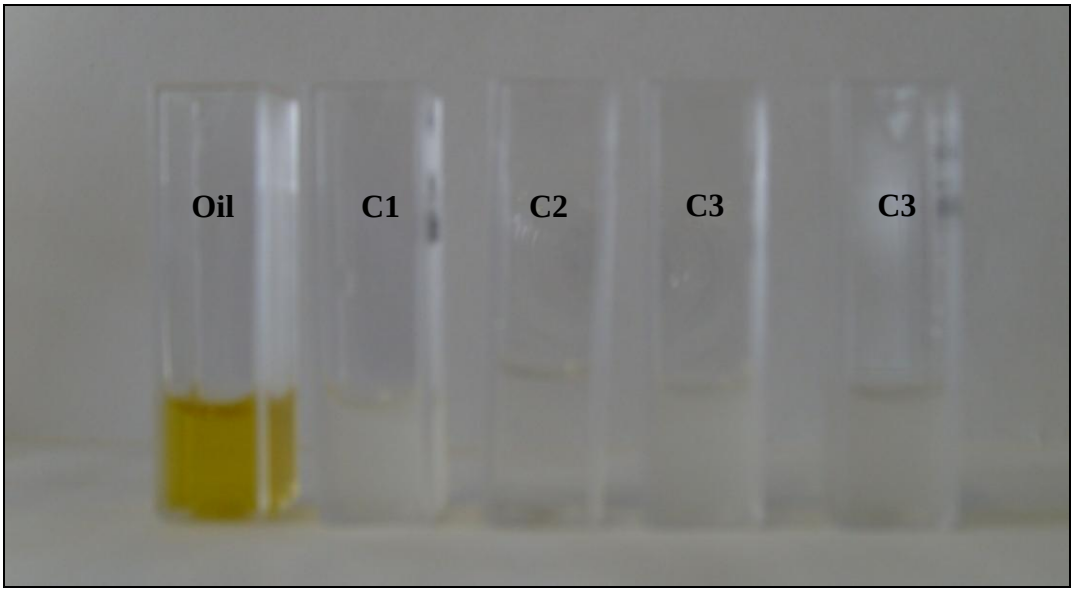
Alkali refining and bleaching of various oil's to remove colour and impurities. This is required by many consumers in the oil industry.

Figure 47. Alkali refining and bleaching of cotton seed oil



Cotton seed oil treated with Tonsil 419 FF adsorbent and acid activated Tonsil Optimum 210 FF bleaching earth.C1, alkali refined oil; C2, alkali refined and bleached; C3, combined alkali refining and bleaching with 2% earth; C4, combined alkali refining and bleaching with 10% earth.

Figure 48. Alkali refining and bleaching of Moringa oil



Moringa oil treated with Tonsil 419 FF adsorbent and acid activated Tonsil Optimum 210 FF bleaching earth.C1, alkali refined oil; C2, alkali refined and bleached; C3, combined alkali refining and bleaching with 2% earth; C4, combined alkali refining and bleaching with 10% earth

Table 33. The removal of Vitamin E from pomegranate and macadamia oil

	Vitamin E mg/100g	
Time	**Macadamia**	**Pomegranate**
Control (0 hrs)	<0.1	4.3
24 hrs	<0.1	1.6
48 hrs	<0.1	0.7

The removal of vitamin E from macadamia and pomegranate oil. the oils were extracted with alumina for 24 to 48 hours.

7.3 Results and Discussion

The effectiveness of bleaching can be determined through the colour removal of the oil. Two oils were selected, a dark coloured oil, Cotton seed, and a light coloured oil, Moringa.

7.3.1 Colour removal from cotton seed oil (Figure 46). Cotton seed oil visually was a dark coloured oil. The dark colour has been attributed to the gossypol content (0.12%) [232]. The removal of gossypol was required prior to the bleaching of the oil. The treatment of the oil with activated carbon and stirred at 18^{o}C and 45^{o}C. The colour removal was not observed in the oils, at both temperatures with varying concentrations of activated carbon. The complexity of colour removal of Cotton seed oil highlights the need to take caution when cold pressing the oil. Poor pressing conditions can lead to an increase in the oil's gossypol content.

7.3.2 Cotton seed oil (Figure 47). The dark brown colour of Cotton seed oil was greatly reduced when the alkali refining and bleaching were carried out as two separate processes (C2). The combined steps of alkali refining and bleaching did not prove to be successful at 2% (C3) and 10% (C4) bleaching earth addition. A colour reduction was observed when comparing C3 and C4 with the un-processed cotton seed oil, but on comparison with C2, combined processing was unsuccessful.

Cotton seed has been reported to be a difficult oil to bleach, due to the dark colour of the oil, which is attributed to the gossypol content [232]. A modification to the method would to double bleach the oil, which would effectively help reduce the colour of the oil. Due to time constraints of the project this was never attempted, although is a possible route to follow in future research.

7.3.3 Moringa oil (Figure 48). The yellow colour of Moringa oil was completely removed when alkali refining and bleaching was carried out as two separate processes (C2). The combined steps of alkali refining and bleaching proved to be successful at 2% (C3) and 10% (C4) bleaching earth addition. Comparison of the two step process (C2), to the combined process (C3&4), showed the combined process oils to be turbid, which may have arisen from filtering. To accurately measure the colour of each of the oils obtained through both processes, a Lovibond tintometer would be required to measure colour change.

Pumpkin seed oil was alkali refined and bleached separately with Tonsil Optimum 210 FF bleaching earth. The data obtained from the Lovibond tintometer showed the initial oil to have a reading of 10,9 Red and 15 Yellow units. The post processing readings were 5,0 Red and 3,2 Yellow units [231]. A similar study was carried out with Tomato seed oil, again Tonsil Optimum 210 FF bleaching earth was used, the Lovibond tintometer gave an initial reading of 22 Red and 10 Yellow units. The post processing readings were 10 Red 2 Yellow, again a significant reduction in colour [233].

In both studies the bleaching earth used was Tonsil Optimum 210 FF, which is an acidic bleaching earth. The importance of the bleaching earth can determine the efficiency of the colour removal [234].

A linear relationship has been reported based on the bleaching efficiency relationship towards the bleaching earths surface area and the acidity [234]. Earths which have been treated with an acid have increased void spaces in their particles. The increased voids lead to an increase in the total surface area. A greater surface area increases the contact of the bleaching earth with the oil, leading to increased bleaching activity [44].

Each individual plant is unique and demands its own optimal processing conditions [235]. Individual oils require specific bleaching earths, with varied temperature and bleaching earth contact time [235]. Acidity, moisture and the initial quality of the oil are parameters which would determine the processing pathway required to produce an oil which is marketable [235].

7.3.4 Tocopherol removal (Table 33). Several oils were processed to remove the tocopherols from the oil. The removal of the tocopherols can be achieved through column chromatography with activated alumina. A modification to method was proposed whereby the oil was incubated with activated alumina in an inert atmosphere such as nitrogen.

The removal of the tocopherols would allow for a blank oil to be assayed for its oxidative stability. The tocopherol content was analysed through HPLC at Eurofins laboratories,

UK. A range of samples were processed although the costly analysis only allowed for two oils be analysed. Macadamia and Pomegranate oils were selected for analysis.

Macadamia oil had an initial tocopherol content of <0.1 mg/100g. The incubation of Macadamia oil with alumina for 24 and 48 hours, gave a tocopherol content of <0.1 mg/100g. The tocopherol content of untreated and treated Macadamia oil was at the lower limits of detection. Macadamia has been reported to be a nut oil with low levels of tocopherols, which was displayed by the results obtained in this present study [213].

Untreated Pomegranate oil had a tocopherol content of 43 mg/100g. The reported levels of tocopherols in pomegranate oil has been recorded as 16-17 mg/100g α-tocopherol and 8-9 mg/100g γ-tocopherol [118]. The levels of tocopherols can be dependant on the cultivar and geographical location.

Pomegranate oil which was incubated with alumina for 24 and 48 hours showed a reduction in the tocopherol content, 63 and 84% respectively. Therefore plant seed oils which require tocopherol removal would require treating for a minimum 48 hours to effectively reduce the tocopherol content. Although as with all processing procedures of oils, contact time and alumina concentration would be dependant on the oil selected.

7.4 Conclusions

The effectiveness of combing the alkali refining and bleaching showed a positive outcome with Moringa oil. The combined processing did not prove to be successful with the dark coloured cotton seed oil. To accurately determine the effectiveness of combined refining a larger variety of oils would need to be studied, with various acidified bleaching earths.

The processing of oils would ultimately be dependant on the oil. The processing would be modified and adjusted based on the oils physical and chemical properties.

Chapter 8

General Conclusions

Research focused on evaluating the oxidative stability and characterisation of plant speciality oils supplied by the sponsor, Earthoil Plantations (Bury St Edmunds, Suffolk, UK).The fatty acid profiles, free fatty acid and peroxide content of the all speciality oils was collated into a database. Oils within the database were categorised based on their major fatty acid content. Using the database as a reference source it is now possible to compare the variation in composition from different batches harvested for example from different geographic locations or from closely related species. It is also possible to observe oils which characteristically had high peroxides or free fatty acid content. This can be used to inform extraction and subsequent processing protocols for such oils. The database also enabled Earthoil to asses the quality of oils they have processed for example Macadamia, Evening Primrose and Sweet Almond oil. If an oil had consistent high PV of FFA then advice was given to the company on how to overcome these problems. The reference source was also used to identify anomalies in specific batches and to allow the company to self evaluate how this may have occurred for that batch handling process. For example an oil that had a high peroxide, the feed back would have been to handle and store the oil in an inert atmosphere. Relaying information back to Earthoil on the quality of the oils was a major part of the interaction with the company.

A number of the speciality oils provided by Earthoil are very new commodities which have not yet gained a significant market niche. Oils such as Callophyllum, Poppy seed, Coffee seed and Sea Buckthorn oils, were stereochemically analysed for the first time.

The oils were enriched with a saturated and/or a monounsaturated fatty acids at positions *sn*-1 and *sn*-3. The *sn*-2 position of the four oils was esterified with a polyunsaturated and/or a monounsaturated fatty acid consistent with known acylation patterns in are well studied species

The oxidative stability of a selection of oils was evaluated at 18^{o}C and 60^{o}C. The oils were supplemented with α-tocopherol to enhance the oxidative stability. The addition of 750ppm of α-tocopherol at 18^{o}C increased the oxidative stability of Brown flax, Moringa, Wheat germ and Yangu oils. At 60^{o}C Brown flax, Manketti and Pomegranate oil all polymerised beyond 48 hours. The addition of 750ppm α-tocopherol delayed the onset of polymerisation by up to 48 hours in Brown flaxseed oil. The addition of α-tocopherol also showed proxidants effects at 18^{o}C with Baobab oil. At 60^{o}C, α-tocopherol addition showed proxidants effects in Yangu oil and Wheat germ oil. For both oils α-tocopherol increased the oxidative stability at 18^{o}C. Therefore the addition α-tocopherol to Yangu and Wheat germ oil must be at low temperatures, as the antioxidant begins to act as proxidants at elevated temperatures.

Pomegranate oil showed a high resistance to oxidation, and was blended into other speciality oils at 1%. Pomegranate oil increased the oxidative stability of Yangu oil at 18^{o}C. The addition of Pomegranate oil to Wheat germ oil at 60^{o}C, decreased the peroxide content by 10%. In Manketti and Brown flax seed oil at elevated temperatures, Pomegranate oil delayed the onset of polymerisation. Preliminary studies of Pomegranate oil blending to Moringa and Borage oil showed it to be more effective than α-tocopherol. The addition of 1% Pomegranate oil displayed pro oxidant activities in Macadamia and Wheat germ oil at 18^{o}C. At 60^{o}C the addition of 1% Pomegranate oil showed proxidants

activity in Baobab oil. The effectiveness of Pomegranate oil maybe due to it conjugated linolenic acid fatty acid, Punicic acid.

Future research for characterisation of the oils should be focused on employing a variety of assays to characterise the oils. Determinations of the antioxidant content using high throughput screening methods such as the ABTS radical cation assay would provide valuable data on the antioxidant potential of the oils which could then be used to further interpret the kinetics of oxidation currently assessed by PV and *p*-AV determinations. Additional determinations such as volatile production, using GC headspace analysis would be valuable and provide another parameter with which to assess oil oxidation. Further characterisation of parameters such as density, unsaponifiable matter and chlorophyll content could all be added to the database Stereochmical analysis provides valuable information on underlying acylation patterns and this could achieved at a faster pace using LC-MS based methods which has more rapid turn around times than the Brockerhoff procedures used here.

The future research directions for the oxidative of speciality oil should be focused on evaluating the antioxidant potential of punicic acid. This will require its isolation from triacylglycerols by hydrolysis and removal of any other potential components in the oils with antioxidant properties. Addition of purified punicic acid to other oils will allow evaluation of its potential antioxidant properties and if proven successful would allow processing protocols to be patented. This would be a highly favoured route as the use of natural antioxidants is favoured over synthetic additives because of worldwide health concerns.

References

1. Gurr, M.I., Harwood, J.L., and Frayn, K.N. (2002). Lipid Biochemistry, 5th Edition, (Oxford: Blackwell Science).
2. Nelson, D.L., and Cox, M.M. (2005). Lehninger Principles of Biochemistry, 4th Edition, (W. H. Freeman and Company).
3. Gunstone, F.D., Harwood, J.L., and Dijkstra, A.J. (2007). The Lipid Handbook, 3rd Edition, (CRC Press).
4. Stobart, A.K., and Griffiths, G. (2002). Fatty Acid Biosynthesis and Triacylglycerol Assembly. In Handbook of Food Enzymology, J.R. Whitaker, A.G.J. Voragen and D.W.S. Wong, eds. (New York: Marcel Dekker), pp. 125-145.
5. Ohlrogge, J.B., Kuhn, D.N., and Stumpf, P.K. (1979). Subcellular Localization of Acyl Carrier Protein in Leaf Protoplasts of *Spinacia oleracea*. Proceeding of The Natural Academy of Sciences *76*, 1194-1198.
6. Heldt, H.-W. (2005). Plant Biochemistry, 3rd Edition, (London: Elsevier Academic Press).
7. Konishi, T., Shinohara, K., Yamada, K., and Sasaki, Y. (1996). Acetyl-CoA Carboxylase in Higher Plants: Most Plants Other Than Gramineae Have Both the Prokaryotic and the Eukaryotic Forms of This Enzyme. Plant Cell Physiology *37*, 117-122.
8. Ohlrogge, J.B. (1994). Design of New Plant Products: Engineering of Fatty Acid Metabolism. Journal of Plant Physiology *105*, 821-826.
9. Thelen, J.J., and Ohlrogge, J.B. (2002). Metabolic Engineering of Fatty Acid Biosynthesis in Plants. Metabolic Engineering *4*, 12-21.
10. Jaworski, J.G., Post-Beittenmiller, D., and Ohlrogge, J.B. (1993). Acetyl-Acyl Carrier Protein is Not a Major Intermediate in Fatty Acid Biosynthesis in Spinach. European Journal of Biochemistry *213*, 981-987.

11. Ohlrogge, J.B., and Jaworski, J.G. (1997). Regulation of Fatty Acid Synthesis. Annual Reveiws Plant Physiology *48*, 109-136.
12. Hawkings, D.J., Kridl, J.C., and 743–752, P.J. (1998). Characterization of Acyl-ACP Thioesterases of Mangosteen (Garcinia mangostana) Seed and High Levels of Stearate Production in Transgenic Growth. The Plant Journal *13*, 743-752.
13. Knutzon, D.S., Bleibaum, J.L., Nelsen, J., Kridl, J.C., and Thompson, G.A. (1992). Isolation and Characterization of Two Safflower Oleoyl-Acyl Carrier Protein Thioesterase cDNA Clones. Journal of Plant Physiology *100*, 1751-1758.
14. Bourdin, B., Adenier, H., and Perrin, Y. (2007). Carnitine is Associated With Fatty Acid Metabolism in Plants. Journal of Plant Physiology and Biochemistry *45*, 926-931.
15. Rawsthorne, S. (2002). Carbon Flux and Fatty Acid Synthesis in Plants. Progress In Lipid Research *41*, 182-196.
16. Fox, B.G., Shanklin, J., Somerville, C., and Munck, E. (1993). Stearoyl-Acyl Carrier Protein A9 Desaturase from Ricinus communis is a Diiron-oxo Protein. Proceeding of the Natural Academy of Sciences *90*, 2486-2490.
17. Shanklin, J., and Cahoon, E.B. (1998). Desaturation and Related Modifications of Fatty Acids. Annual Review of Plant Physiology and Plant Molecular Biology *49*, 611-641.
18. Sayanova, O., Smith, M.A., Lapinskas, P., Stobart, A.K., Dobson, G., Christie, W.W., Shewry, P.R., and Napier, J.A. (1997). Expression of a Borage Desaturase cDNA Containing an N-terminal Cytochrome *b5* Domain Results in The Accumulation of High Levels of D6-Desaturated Fatty Acids in Transgenic Tobacco. Proceeding of the Natural Academy of Sciences *94*, 4211-4216.
19. Stymne, S., and Stobart, A.K. (1987). Triacylglycerol Biosynthesis. In The Biochemistry of Plants: A Comprehensive Treatise, P.K. Stumpf, ed. (New York: Academic Press), pp. 175-214.
20. Griffiths, G., Stobart, A.K., and Stymne, S. (1988). D6- and D12-Desaturase Activities and Phosphatidic Acid Formation in Microsomal Preparations from The Developing Cotyledons of Common Borage (*Borage offiicinalis*). Biochemical Journal. *252,* 641-647.

21. Griffiths, G., Jackson, F., Brechany, E.Y., Christie, W.W., Stymne, S., and Stobart, A.K. (1996). Octadecatetraenoic acid (C18:4; Stearidonic acid) Biosynthesis in Borage (*Borago officinalis*). Phytochemistry *43*, 381-386.
22. Harwood, J.L. (2010). Plant Fatty Acid Synthesis. Volume 2010. (Lipid Library).
23. Napier, J.A. (2007). The Production of Unusual Fatty Acids in Transgenic Plants. Annual Reviews in Plant Biology *58*, 295-319.
24. Robert, S. (2006). Production of Eicosapentaenoic and Docosahexaenoic Acid-Containing Oils in Transgenic Land Plants for Human and Aquaculture Nutrition. Journal of Marine Biotechnology *8*, 103-109.
25. Truksa, M., Vrinten, P., and Qiu, X. (2009). Metabolic Engineering of Plants for Polyunsaturated Fatty Acid Production. Journal of Molecular Breeding *23*, 1-11.
26. Tonon, T., Harvey, D., Larson, T.R., Li, Y., and Graham, I.A. (2005). Acyl-CoA Elongase activity and Gene from The Marine Microalga *Pavlova lutheri* (Haptophyceae). Journal of Applied Phycology *17*, 111-118.
27. R.Petrie, J., Shrestha, P., P.Mansour, M., D.Nichols, P., Liu, Q., and P.Singh, S. (2010). Metabolic Engineering of Omega-3 Long-Chain Polyunsaturated Fatty Acids in Plants Using an Acyl-CoA D6-Desaturase with Omega 3-Preference from The Marine Microalga *Micromonas pusilla*. Journal of Metabolic Engineering *12*, 233-240.
28. Grogan, D.W., and Cronan, J.E. (1997). Cyclopropane Ring Formation in Membrane Lipids of Bacteria. Journal of Microbiology and Molecular Biology Reviews *61*, 429-441.
29. Bao, X., Katz, S., Pollard, M., and Ohlrogge, J.B. (2002). Carbocyclic Fatty Acids in Plants: Biochemical and Molecular Genetic Characterization of Cyclopropane Fatty acid Synthesis of *Sterculia foetida*. Proceeding of the Natural Academy of Sciences *99*, 7172-7177.
30. Bao, X., Thelen, J.J., Bonaventure, G., and Ohlrogge, J.B. (2003). Characterization of Cyclopropane Fatty-acid Synthase from *Sterculia foetida*. The Journal of Biological Chemistry *278*, 12846–12853.
31. Bafor, M., Smith, M.A., Jonsson, L., Stobart, K., and Stymne, S. (1991). Ricinoleic Acid Biosynthesis and Triacylglycerol Assembly in Microsomal

Preparations from Developing Castor-Bean (*Ricinus communis*) Endosperm. Journal of Biochemistry. *280*, 507-514.

32. Broun, P., and Somerville, C. (1997). Accumulation of Ricinoleic, lesquerolic, and Densipolic Acids in Seeds of Transgenic Arabidopsis Plants That Express a Fatty Acyl Hydroxylase cDNA from Castor Bean. Journal of Plant Physiology *113*, 933-942.
33. Loo, F.J.V.D., Broun, P., Turner, S., and Somerville, C. (1995). An Oleate 12-Hydroxylase from *Ricinus communis* L. is a Fatty Acyl Desaturase Homolog. Proceeding of the Natural Academy of Sciences *92*, 6743-6747.
34. Moon, H., Smith, M.A., and Kunst, L. (2001). A Condensing Enzyme from the Seeds of *Lesquerella fendleri* That Specifically Elongates Hydroxy Fatty Acids. Journal of Plant Physiology *127*, 1635-1643.
35. Engeseth, N., and Stymne, S. (1996). Desaturation of Oxygenated Fatty Acids in *Lesquerella* and Other Oil Seeds. Planta *198*, 238-245.
36. Reed, D.W., Scha¨fer, U.A., and Covello, P.S. (2000). Characterization of The Brassica napus Extraplastidial Linoleate Desaturase by Expression in *Saccharomyces cerevisiae*. Journal of Plant Physiology *122*, 715-720.
37. Lee, M., Lenman, M., Banas, A., Bafor, M., Singh, S., Schweizer, M., Nilsson, R., Liljenberg, C., Dahlqvist, A., Gummeson, P., et al. (1998). Identification of Non–Heme Diiron Proteins That Catalyze Triple Bond and Epoxy Group Formation. science *280*, 915-918.
38. Cahoon, E.B., Ripp, K.G., Hall, S.E., and McGonigle, B. (2002). Transgenic Production of Epoxy Fatty Acids by Expression of a Cytochrome P450 Enzyme from *Euphorbia lagascae* Seed. Journal of Plant Physiology *128*, 615-624.
39. Feussner, I., and C.Wasternack (2002). The Lipoxygenase Pathway. Annual Reviews in Plant Biology *53*, 275-297.
40. Stenzel, I., Hause, B., Miersch, O., Kurzl, T., Maucher, H., Weichert, H., Ziegler, J., Feussner, I., and Wasternack, C. (2003). Jasmonate Biosynthesis and The Allene Oxide Cyclase Family of *Arabidopsis thaliana*. Journal of Plant Molecular Biology *51*, 895-911.

41. Farmer, E.E., Almeras, E., and Krishnamurthy, V. (2003). Jasmonates and Related Oxylipins in Plant Responses to Pathogenesis and Herbivory. Current Options In Plant Biology *6*, 372-378.
42. Vick, B.A., and Zimmerman, D.C. (1984). Biosynthesis of Jasmonic Acid by Several Plant Species. Journal of Plant Physiology *75*, 459-461.
43. Weber, H., Chételat, A., Caldelari, D., and Farmer, E.E. (1999). Divinyl Ether Fatty Acid Synthesis in Late Blight–Diseased Potato Leaves. The Plant Cell *11*, 485-493.
44. Gunstone, F.D., and Norris, F.A. (1983). Lipids in Foods. Chemistry, Biochemistry and Technology, (Pergamon Press Ltd).
45. Spiteller, G. (2005). Furan Fatty Acids: Occurrence, Synthesis, and Reactions. Are Furan Fatty Acids Responsible for the Cardioprotective Effects of a Fish Diet? Lipids *40*, 755-771.
46. Spitzer, V., Tomberg, W., and Pohlentz, G. (1997). Structure Analysis of an Allene-Containing Estolide Tetraester Triglyceride in the Seed Oil of *Sebastiana commersoniana* (Euphorbiaceae). Lipids *32*, 549-557.
47. Cahoon, E.B., Carlson, T.J., Ripp, K.G., Schweiger, B.J., Cook, G.A., Hall, S.E., and Kinney, A.J. (1999). Biosynthetic Origin of Conjugated Double Bonds: Production of Fatty Acid Components of High-value Drying Oils in Transgenic Soybean Embryos. Proceeding of the Natural Academy of Sciences *96*, 12935-12940.
48. Dyer, J.M., Chapital, D.C., Kuan, J.W., Mullen, R.T., Turner, C., McKeon, T.A., and Pepperman, A.B. (2002). Molecular Analysis of a Bifunctional Fatty Acid Conjugase/Desaturase from Tung. Implications for the Evolution of Plant Fatty Acid Diversity. Journal of Plant Physiology *130*, 2027-2038.
49. Ohlrogge, J., and Benning, C. (2000). Unraveling Plant Metabolism by EST Analysis. Plant Biology *3*, 224-228.
50. Hamberg, M. (1992). Metabolism of 6,9,12-Octadecatrienoic Acids in The Red Alga *Lithothamnion corallioides*: Mechanism of Formation of a Conjugated Tetraene Fatty Acid. Biochemical and Biophysical Research Communications *188*, 1220-1227.

51. Wise, M.L., Hamberg, M., and Gerwick, W.H. (1994). Biosynthesis of Conjugated Triene-Containing Fatty Acids by a Novel Isomerase from the Red Marine Alga *Ptilotu filicinui*. Journal of Biochemistry *33*, 15223-15232.

52. Zheng, W., Wise, M.L., Wyrick, A., Metz, J.G., Yuan, L., and Gerwick, W.H. (2002). Polyenoic Fatty Acid Isomerase from The Marine Alga *Ptilota filicina*: Protein Characterization and Functional Expression of The Cloned cDNA. Archives of Biochemistry and Biophysics *401*, 11-20.

53. Cahoon, E.B., Ripp, K.G., Hall, S.E., and Kinney, A.J. (2001). Formation of Conjugated D8,D10-Double Bonds by D12-Oleic-acid Desaturase-related Enzymes. The Journal Of Biological Chemistry *276*, 2637-2643.

54. Hornung, E., Pernstich, C., and Feussner, I. (2002). Formation of Conjugated D11 D13-Double Bonds by D12-Linoleic Acid (1,4)-Acyl-Lipid-desaturase in Pomegranate Seeds. European Journal of Biochemistry. *269*, 4852-4859.

55. Kaneda, T. (1991). Iso- and Anteiso-Fatty Acids in Bacteria: Biosynthesis, Function, and Taxonomic Significancet. Microbiological Reviews *55*, 288-302.

56. Griffiths, G., and Morse, N. (2006). Clinical Applications of C18 and C20 Chain Length Polyunsaturated Fatty Acids and Their Biotechnological Productions in Plants. Journal of American Oil Chemists Society *83*, 1-15.

57. Dahlqvis, A., Ståhl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., and Stymne, S. (2000). Phospholipid:Diacylglycerol Acyltransferase: An Enzyme That Catalyzes The Acyl-CoA-Independent Formation of Triacylglycerol in Yeast and Plants. Proceeding of the Natural Academy of Sciences *97*, 6487-6492.

58. Griffiths, G., Stobart, A.K., and Stymne, S. (1985). The Acylation of sn-glycerol 3-phosphate and The Metabolism of Phosphatidate in Microsomal Preparations from The Developing Cotyledons of Safflower (Carthamus tinctorius L.) Seed. Biochemical Journal. *230*, 379-388.

59. Ichihara, K. (1984). *sn*-Glycerol-3-phosphate Acyltransferase in a Particulate Fraction from Maturing Safflower Seeds. Archives of Biochemistry and Biophysics *232*, 685-698.

60. Stobart, K., Mancha, M., Lenman, M., Dahlqvist, A., and Stymne, S. (1997). Triacylglycerols are Synthesised and Utilized by Transacylation Reactions in

Microsomal Preparations of Developing Safflower (*Carthamus tinctorius L.*) seeds. Planta *203*, 58-66.

61. Stobart, A.K., and Stymne, S. (1985). The Interconversion of Diacyiglycerol and Phosphatidylcholine during Triacylglycerol Production in Microsomal Preparations of Developing Cotyledons of Safflower (Carthamus tinctorius L.). Journal of Biochemistry. *232*, 217-221.

62. Stymne, S., and Stobart, A.K. (1984). Evidence for The Reversibility of The Acyl-CoA:lysophosphatidylcholine Acyltransferase in Microsomal Preparations from Developing Safflower (*Carthamus tinctorius L.*) Cotyledons and Rat Liver. Journal of Biochemistry *223*, 305-314.

63. Napier, J.A. (2006). The Production of n-3 Long-Chain Polyunsaturated Fatty Acids in Transgenic Plants. European Journal of Lipid Science and Technology *108*, 965-972.

64. Nakanishi, H., Shindou, H., Hishikawa, D., Harayama, T., Ogasawara, R., Suwabe, A., Taguchi, R., and Shimizu, T. (2006). Cloning and Characterization of Mouse Lung-type Acyl-CoA:Lysophosphatidylcholine Acyltransferase 1 (LPCAT1)Expession in Alveolar Type II Cells and Possible Involvement in Surfactant Production. Journal of Biological Chemistry *281*, 20140-20147.

65. Chen, X., Hyatt, B.A., Mucenski, M.L., Mason, R.J., and Shannon, J.M. (2006). Identification and Characterization of a Lysophosphatidylcholine Acyltransferase in Alveolar Type II Cells. Proceeding of the Natural Academy of Sciences *103*, 11724-11729.

66. Slack, C.R., Bertaud, W.S., Shaw, B.D., Holland, R., Browse, J., and Wright, H. (1980). Some Studies on The Composition and Surface Properties of Oil Bodies from The Seed Cotyledons of Safflower (*Carthamus tinctorius*) and Linseed (*Linum ustatissimum*). Journal of Biochemistry *190*, 551-561.

67. Huang, A.H.C. (1992). Oil Bodies and Oleosins in Seeds. Annual Reveiws Plant Physiology *43*, 177-200.

68. Wanner, G., Formanet, H., and Theimer, R.R. (1981). The Ontogeny of Lipid Bodies. (spherosomes) in Plant Cells. Ultrastructural evidence. . Planta *151*, 109-123.

69. Millichip, M., Tatham, A.S., Jackson, F., Griffiths, G., Shewry, P.R., and Stobart, A.K. (1996). Purification and Characterization of Oil-bodies (Oleosomes) and Oil-body Boundary Proteins (Oleosins) from The Developing Cotyledons of Sunflower (*Helianthus annuus* L.). Biochemical Journal *314*, 333-337.

70. Dutta, P.C., Appelqvist, L., Gunnarssonb, S., and Hofsten, A.v. (1991). Lipid Bodies in Tissue Culture, Somatic and Zygotic Embryo of Daucus Carota L.: A Qualitative and Quantitative Study. Journal of Plant Science *78*, 259-267.

71. Tzen, J.T.C., Cao, Y., Laurent, P., Ratnayake, C., and Huang, A.H.C. (1993). Lipids, Proteins, and Structure of Seed Oil Bodies from Diverse Species. Journal of Plant Physiology *101*, 267-276.

72. Murphy, D.J., Keen, J.N., O'Sullivan, J.N., Au, D.M., Edwards, E.W., Jackson, P.J., Cummins, I., Gibbons, T., Shaw, C.H., and Ryan, A.J. (1991). A Class of Amphipathic Proteins Associated with Lipid Storage Bodies Plants. Possible Similarities with Animal Serum Apolipoproteins. Biochimica et Biophysica acta *1088*, 86-94.

73. Qu, R., and Huang, A.H.C. (1990). Oleosin KD 18 On The Surface of Oil Bodies in Maize: Genomic and cDNA Sequences, and The Deduced Protein Structure. Journal of Biological Chemistry *265*, 2238-2243.

74. Vance, V.B., and Huang, A.H.C. (1987). The Major Protein from Lipid Bodies of Maize. Characterization and Structure Based on cDNA Cloning. Journal of Biological Chemistry *262*, 275-279.

75. Hatzopoulos, P., Franz, G., Choy, L., and Sung, R.Z. (1990). Interaction of Nuclear Factors with Upstream Sequences of a Lipid Body Membrane Protein Gene from Carrot. Plant Cell *2*, 457-467.

76. Lee, K., and Huang, A.H.C. (1991). Genomic Nucleotide Sequence of a *Brassica napus* 20 kDa Oleosin Gene. Journal of Plant Physiology *96*, 1395-1397.

77. Kalinski, A., Loer, D.S., J. M. Weisenann, Matthews, B.J., and Herman, E.M. (1991). Isoforms .of Soybean Seed Oil Body Membrane Protein 24 kDa Oleosin are Encoded by Closely Related cDNAs. Journal of Plant Molecular Biology *17*, 1095-1098.

78. Andrikopoulos, N.K. (2002). Triglyceride Species Compositions of Common Edible Vegetable Oils and Methods Used for Their Identification and Quantification. Food Reviews International *18*, 71-102.
79. Library, L. (2009). Commidity Oils and Fats The Four Major Vegetable Oils. Volume 2010, *http://lipidlibrary.aocs.org/market/fourmain.htm*.
80. Library, L. (2008). Oils and Fats In The Market Place Non Food Uses. Volume 2010, *http://lipidlibrary.aocs.org/market/nonfood.htm*.
81. Library, L. (2009). Commodity Oils and Fats Palm Oil. Volume 2010, *http://lipidlibrary.aocs.org/market/palmoil.htm*.
82. Library, L. (2009). Commidity Oils and Fats Soybean Oil. Volume 2010, *http://lipidlibrary.aocs.org/market/soybean.htm*.
83. Library, L. (2009). Commodity Oils and Fats Rapeseed (Canola) Oil. Volume 2010, *http://lipidlibrary.aocs.org/market/rapeseed.htm*.
84. Gunstone, F.D. (2010). Oils and Fats In The Market Place: Biodiesel. (Lipid Library), pp. 1-2, *http://lipidlibrary.aocs.org/market/biodiesel.htm*.
85. Library, L. (2009). Commodity Oils and Fats Sunflower Seed Oil. Volume 2010, *http://lipidlibrary.aocs.org/market/sunflower.htm*.
86. Kodad, O., and Company, R.S.I. (2008). Variability of Oil Content and of Major Fatty Acid Composition in Almond (Prunus amygdalus Batsch) and Its Relationship with Kernel Quality. Journal of Agricultural and Food Chemistry *56*, 4096-4101.
87. Sabate, J., and Hook, D.G. (1996). Almonds, Walnuts, and Serum Lipids. In Lipids in Human Nutrition, G.A. Spiller, ed. (CRC Press: Boca Raton), pp. 137-444.
88. Fraser, G.E. (1999). Nut Composition, Lipids and Risk of a Coronary Event. Clynical Cardiology *22*, 1-5.
89. Etherton, P.M.K., Poth, S.Y., Sabate, J., Ratcliffe, H.E., Zhao, G., and Etherton, T.D. (2001). The Effects of Nuts on Coronary Heart Disease Risk. Nutrition Reviews *4*, 103-111.
90. Lopez-Ortiz, C.M., S.Prats-Moya, Ana Beltra´n Sanahuja, Maestre-Pe´rez, S.E., Grane´-Teruel, N., and Carratala, M.L. (2008). Comparative Study of Tocopherol

Homologue Content in Four Almond Oil Cultivars During Two Consecutive Years. Jounal of Food Composition and Analysis *21*, 144-151.

91. Sabate, J., Radak, T., and Brown, J. (2000). The Role of Nuts in Cardiovascular Disease Prevention. In Handbook of Nutraceuticals and Functional Foods, R.E.C. Wildman, ed. (CRC Press: Boca Raton), pp. 477-495.
92. Aparicio, R., and Mcintyre, P. (1998). Oils and Fats. In Food Authenticity: Issues and Methodologies, M. Lees, ed., pp. 214-273.
93. Antonio, L.L., Alfredo, M., Victoria, R.M.M., and Antonio, G.F. (2008). Sterols, Fatty Alcohols, and Triterpenic Alcohols. Commerical Table Olives *85*, 253-262.
94. Cherif, A., Belkacemi, K., Kallel, H., Angers, P., Arul, J., and Boukhchina, S. (2009). Phytosterols, Unsaturated Fatty Acid Composition and Accumulation in the Almond Kernel During Harvesting Period Importance for Development Regulation. Biochemistry *332*, 1069-1077.
95. Harwood, J., and Aparicio, R. (1999). Analysis and Properties. In Handbook of Olive Oil, J. Harwood, ed. (Aspen Publischers), p. 620.
96. Kaijser, A., Dutta, P., and Savage, G.R. (2000). Oxidative Stability and Lipid Composition of Macadamia Nuts Grown in New Zealand. Food Chemistry *71*, 67-70.
97. Alasalvar, C., Shahidi, F., Liyanapathirana, C.M., and Ohsima, T. (2003). Turkish Tombul Hazelnut (Corylus avellana L.). 1. Compositional Characteristics. Journal of Agricultural and Food Chemistry *51*, 3790-3796.
98. Alasalvar, C., Shahidi, F., Ohshima, T., Wanasundara, U., Yurttas, H.C., Liyanapathirana, C.M., and Rodrigues, F.B. (2003). Turkish Tombul Hazelnut (Corylus avellana L.). 2. Lipid Characteristics and Oxidative Stability. Journal of Agricultural and Food Chemistry *51*, 3793-3805.
99. Acukurt, F., Ozdemir, M., Biringen, G., and Locker, M. (1999). Effects of Geographical Origin and Variety on Vitamin and Mineral Composition of Hazelnut (*Corylus aVellana L.*) Varieties Cultivated in Turkey. Journal of Food Chemistry *65*, 309-313.

100. Ozdemir, M., and Devres, O. (1999). Turkish Hazelnuts: Properties and Effect of Microbiological and Chemical Changes on Quality. Food Reviews International *15*, 309-333.

101. Anderson, J.W., and Bridges, S.R. (1988). Dietary Fiber of Selected Foods. American Journal of Clinical Nutrition *47*, 440-447.

102. Brown, L., Rosner, B., Willett, W.W., and Sacks, F.M. (1999). Cholesterol Lowering Effects of Dietary Fiber: A Meta-Analysis. American Journal for Clinical Nutrition *69*, 30-42.

103. Yang, B., and Kallio, H. (2002). Composition and Physiological Effects of Sea Buckthorn (*Hippophae*) Lipids. Trends in Food & Technology *13*, 160-167.

104. Yang, B., and Kallio, H.P. (2001). Fatty Acid Composition of Lipids in Sea Buckthorn (Hippophae rhamnoides L.) Berries of Different Origins. Journal of Agricultural and Food Chemistry *49*, 1939-1947.

105. Yang, B. (2003). Natural Vitamin E: Activities and Sources. Lipid Technology *15*, 125-130.

106. Horrobin, D.F. (1993). Fatty acid Metabolism in Health and Disease: The Role of Delta-6-desaturase. American Journal for Clinical Nutrition *57*, 732-737.

107. Horrobin, D.F. (2000). Essential Fatty Acid Metabolism and its Modification in Atopic Eczema. American Journal for Clinical Nutrition *71*, 67-72..

108. Beveridge, T.H.J., Girard, B., Kopp, T., and Drover, J.C.G. (2005). Yield and Composition of Grape Seed Oils Extracted by Supercritical Carbon Dioxide and Pertroleum Ether: Varietal Effects. Journal of Agricultural and Food Chemistry *53*, 1799-1804.

109. Xu, C., Zhang, Y., Wang, J., and Lu, J. (2010). Extraction, Distribution and Characterisation of Phenolic Compounds and Oil in Grapeseeds. Journal of Food Chemistry *122*, 688-694.

110. Yamakoshi, J., Saito, M., Kataoka, S., and Tokutake, S. (2002). Procyanidin-Rich Extract from Grape Seeds Prevents Cataract Formation in Hereditary Cataractous (ICR/f) Rats. Journal of Agricultural and Food Chemistry *50*, 4983-4988.

111. Pinent, M., Blay, M., Blade, M.C., Salvado, M.J., Arola, L., and Aedevol, A. (2004). Grape Seed-Derived Procyanidins Have an Antihyperglycemic Effect in

Streptozotocin-Induced Diabetic Rats and Insulinomimetic Activity in Insulin-Sensitive Cell Lines. Journal of Endocrinology *145*, 4985-4990.

112. T Terra, X., Valls, J., Vitrac, X., Merillion, J.M., Arola, L., Ardeovol, A., Bladea, C., Ndez-larrea, J.F., Pujadas, G., Salvadoa, J., et al. (2007). Grape-Seed Procyanidins Act as Antiinflammatory Agents in Endotoxin-Stimulated RAW 264.7 Macrophages by Inhibiting NFkB Signaling Pathway. Journal of Agricultural and Food Chemistry *55*, 4357-4365.
113. Lafka, T., Sinanoglou, V., and Lazos, E.S. (2007). On The Extraction and Antioxidant Activity of Phenolic Compounds from Winery Wastes. Journal of Food Chemistry *104*, 1206-1214.
114. Turan, S., Topcu, A., Karabulut, I., Vural, H., and Hayaloglu, A.A. (2007). Fatty Acid, Triacylglycerol, Phytosterol, and Tocopherol Variations in Kernel Oil of Malatya Apricots from Turkey. Journal of Agricultural and Food Chemistry *55*, 10787-10794.
115. Ozdemir, F., and Topuz, A. (2004). Changes in Dry Matter, Oil Content and Fatty Acids Composition of Avocado During Harvesting Time and Post-Harvesting Ripening Period. Journal of Food Science *86*, 79-83.
116. Arao, K., Wang, Y.-M., Inoue, N., Hirata, J., Jae-Young Cha, Nagao, K., and Yanagita, T. (2004). Dietary Effect of Pomegranate Seed Oil Rich in 9cis, 11trans, 13cis Conjugated Linolenic Acid on Lipid Metabolism in Obese, Hyperlipidemic OLETF Rats. Lipids in Health and Disease *3*, 1-7.
117. Wang, R.-F., Xie, W.-D., Zhang, Z., Xing, D.-M., Yi Ding, W.W., Ma, C., and Du, L.-J. (2004). Bioactive Compounds from the Seeds of *Punica granatum* (Pomegranate). Journal of Natural Products *67*, 2096-2098.
118. Pande, G., and Akoh, C.C. (2009). Antioxidant Capacity and Lipid Characterization of Six Georgia-Grown Pomegranate Cultivars. Journal of Agricultural and Food Chemistry *57*, 9427-9436.
119. Anwar, F., Ashraf, M., and Bhanger, M.I. (2005). Interprovenance Variation in the Composition of *Moringa oleifera* Oilseeds from Pakistan. Journal of American Oil Chemists Society *82*, 45-51.

120. Anwar, F., and Bhanger, M.I. (2003). Analytical Characterization of *Moringa oleifera* Seed Oil Grown in Temperate Regions of Pakistan. Journal of Agricultural and Food Chemistry *51*, 6558-6563.

121. Anwar, F., Hussain, A.I., Iqbal, S., and Bhanger, M.I. (2007). Enhancement of the Oxidative Stability of Some Vegetable Oils by Blending with *Moringa oleifera* Oil. Journal of Food Chemistry *103*, 1181-1191.

122. Siddhuraju, P., and Becker, K. (2003). Antioxidant Properties of Various Solvent Extracts of Total Phenolic Constituents from Three Different Agroclimatic Origins of Drumstick Tree (*Moringa oleifera* Lam.) Leaves. Journal of Agricultural and Food Chemistry *51*, 2144-2155.

123. Research, C.o.S.a.I. (1962). The Wealth of India (A Dictionary of Indian Raw Materials and Industrial Products): Raw Materials, Volume 6.

124. Dahot, M.U. (1988). Vitamin Contents of Flowers and Seeds of Moringa *oleifera*, Pak. Journal of Biochemistry *21*, 21-24.

125. Frankel, E.N. (2005). Lipid Oxidation, 2nd Edition, (The Oily Press).

126. Connor, W.E. (2000). Importance of n-3 fatty acids in health and disease. American Journal for Clinical Nutrition *71*, 171-175.

127. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977). Analysis of Autoxidised Fats by Gas Chromatography-Mass Spectrometry: Methyl Oleate. Lipids *12*, 901-907.

128. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977). Analysis of Autoxidised Fats by Gas Chromatography-Mass Spectrometery: Methyl Linoleate. Lipids *12*, 908-913.

129. Frankel, E.N., Dufek, E.J., and Neff, W.E. (1980). Analysis of Autoxidised Fats by Gas Chromatography-Mass Spectrometry: Methyl 9, 15- and 12, 15-Octadecadienoate. Lipids *15*, 661-667.

130. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977). Analysis of Autoxidised Fats by Gas Chromatography-Mass Spectrometry: Methyl Linolenate. Lipids *12*, 1055-1061.

131. Frankel, E.N., Evans, C.D., McConnell, D.G., Selke, E., and Dutton, H.J. (1961). Autoxidation of Methyl Linolenate. Isolation and Characterisation of Hydroperoxides. Journal of Organic Chemistry *26*, 4663-4669.

132. Kamal-Eldin, A. ed. (2003). Lipid Oxidation Pathways (AOCS Press).

133. Shahidi, F., and Zhong, Y. eds. (2005). Lipid Oxidation: Measurment Methods, Volume 6, 6 Edition (John Wiley & Sons).

134. Delong, J.M., Prange, R.K., Hodges, D.M., Forney, C.F., Bishop, M.C., and Quilliam, M. (2002). Using a Modified Ferrous Oxidation-Xylenol Orange (FOX) Assay for Detection of Lipid Hydroperoxides in Plant Tissue. Journal of Agricultural and Food Chemistry *50*, 248-254

135. Griffiths, G., Leverentz, M., Silkowski, H., Gill, N., and Sanchez-Serrano, J.J. (2000). Lipid Hydroperoxide Levels in Plant Tissues. Journal of Experimental Botany *51*, 1363-1370.

136. Nourooz-Zadeh, J., Tajaddini-Sarmadi, J., Birlouez-Aragon, I., and Wolff, S.P. (1995). Measurment of Hydroperoxides in Edible Oils Using Ferrous Oxidation in Xylenol Orange Assay. Journal of Agricultural and Food Chemistry *43*, 17-21.

137. White, P.J. (1995). Conjugated Diene, Anisidine Value and Carbonyl Value Analyses. In Methods to Access Quality and Stability of Oils and Fat-Containing Foods, K. Warner and N.A.M. Eskin, eds. (AOCS), pp. 159-162.

138. Rice-Evans, C., and Miller, N.J. (1995). Antioxidants – The Case for Fruit and Vegetables in The Diet. British Food Journal *97*, 35-40.

139. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. Free Radical Biology & Medicine *26*, 1231-1237.

140. Liang, Y.C., May, C.Y., Foon, C.S., Ngan, M.A., Hock, C.C., and Basiron, Y. (2006). The Effect of Natural and Synthetic Antioxidants on The Oxidative stability of Palm Diesel. Journal of Fuel *85*, 867-870.

141. Cook, N.C., and Samman, S. (1996). Flavonoids Chemistry, Metabolism, Cardioprotective Effects, and Dietary Sources. Journal of Nutritional Biochemistry *7*, 66-76.

142. Heim, K.E., Tagliaferro, A.R., and Bobilya, D.J. (2002). Flavonoid Antioxidants: Chemistry, Metabolism and Structure-Activity Relationships. The Journal of Nutritional Biochemistry *13*, 572-584.

143. Acker, S.A.B.E.v., Balen, G.P.v., Berg, D.v.d., Bast, A., and Vijgh, W.J.F.v.d. (1998). Influence of Iron Chelation on the Antioxidant Activity of Flavonoids. Journal of Biochemical Pharmacology *56*, 935-943.

144. Frankel, E.N., Huang, S.-W., Aeschbach, R., and Prior, E. (1996). Antioxidant Activity of a Rosemary Extract and Its Constituents, Carnosic Acid, Carnosol, and Rosmarinic Acid, in Bulk Oil and Oil-in-Water Emulsion. Journal of Agricultural and Food Chemistry *44*, 131-135.

145. Sies, H., and Stahl, W. (1995). Vitamins E and C, a-Carotene, and Other Carotenoids as Antioxidants. American Journal of Nutrition *62*, 1315s-1321s.

146. Stahl, W., and Sies, H. (2003). Antioxidant Activity of Carotenoids. Molecular Aspects of Medicine *24*, 345-351.

147. KamaI-Eldin, A., and Appelqvist, L.-A. (1996). The Chemistry and Antioxidant Properties of Tocopherols and Tocotrienols. Lipids *31*, 671-701.

148. Chan, H., W.S. (1987). The Mechanism of Autoxidation, in Autoxidation of Unsaturated Lipids, 1 Edition, (London: Academic Press).

149. Maillard, B., Ingold, K.U., and Scaniano, J.C. (1983). Rate Constants for the Reactions of Free Radicals with Oxygen in Solution. Journal of American Chemical Society *105*, 5095-5099.

150. Nagaoka, S., Sawada, K., Fukumoto, Y., Nagashima, U., Katasumata, S., and Mukai, K. (1992). Mechanism of Antioxidant Reaction of Vitamin E: Kinetic, Spectroscopic and ab initio Study of Proton-Transfer Reactions. Journal of Physical Chemistry *96*, 6663-6668.

151. Burton, G.W., and Ingold, K.U. (1981). Autooxidation of Biological Molecules. 1. The Antioxidant Activity of Vitamin E and Related Chain-Breaking Phenolic Antioxidants in vitro. Journal of American Chemical Society *103*, 6472-6477.

152. Schaich, K.M. (1992). Metals and Lipid Peroxidation: Contemporary Issues. Journal of Lipids *27*, 209-218.

153. Campbell, T.W., and Coppinger, G.M. (1952). The Reaction of tert-Butyl Hydroperoxide with Some Phenols. Journal of American Chemical Society *74*, 1467-1469.

154. Patterson, L.K., Rodgers, M.A.J., and Powers, E.L. eds. (1981). Studies of Radiation-lnduced Peroxidation in Fatty Acid Micelles, in Oxygen and Oxy-Radicals in Chemistry and Biology (New York: Academic Press).

155. Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L., and Ingold, K.U. (1985). Autoxidation of Biological Molecules. 4. Maximizing the Antioxidant Activity of Phenols. Journal of The American Chemical Society *107*, 7053-7065.

156. Lea, C.H. (1960). On the Antioxidant Activities of the Tocopherols. II. Influence of Substrate, Temperature and Level of Oxidation. Journal of Science. Food and Agriculture *11*, 212-218.

157. Lea, C.H. (1960). Antioxidants in Dry Fat Systems. I. Influence of the Fatty Acid Composition of the Substrate. Journal of Science. Food and Agriculture *11*, 143-150.

158. Chan, H.W.S., and Pokorny, J. (1987). Major Factors Affecting the Autoxidation of Lipids, in Autoxidation of Unsaturated Lipids, (London: Academic Press).

159. Cort, W.M., Mergens, W., and Greene, A. (1987). Stability of alpha - and gamma -Tocopherol: Fe 3+ and Cu 2+ Interactions. Journal of Food Science *43*, 797-798.

160. Koskas, J.P., Cillard, J., and Cillard, P. (1984). Autooxidation of Linoleic Acid and Behavior of Its Hydroperoxides with and without Tocopherols. Journal of American Oil Chemists Society *61*, 1466-1469.

161. Cillard, J., Cillard, P., Cormier, M., and Girre, L. (1980). Alpha-Tocopherol Prooxidant Effect in Aqueous Media: Increased Autooxidation Rate of Linoleic Acid. Journal of American Oil Chemists Society *57*, 252-255.

162. Cillard, J., Cillard, P., and Cormier, M. (1980). Effect of Experimental Factors on the Prooxidant Behavior of alpha-Tocopherol. Journal of American Oil Chemists Society *57*, 255-261.

163. Peers, K.E., and Coxon, D.T. (1983). Controlled Synthesis of Monohydroperoxides by alpha-Tocopherol Inhibited Autoxidation of Polyunsaturated Lipids. Journal of Chemistry and Physics of Lipids *32*, 49-56.

164. Peers, K.E., Coxon, D.T., and Chan, H.W.S. (1981). Autoxidation of Methyl Linolenate and Methyl Linoleate: The Effect of alpha-Tocopherol. Journal of Science. Food and Agriculture *32*, 898-904.

165. Lea, C.H., and Ward, R.J. (1959). Relative Antioxidant Activity of the Seven Tocopherols. Journal of Science. Food and Agriculture *10*, 537-548.

166. Marinova, E.M., and Yanishlieva, N.V. (1992). Effect of Ternperature on the Antioxidative Action of Inhibitors in Lipid Autoxidation. Journal of Science. Food and Agriculture *60*, 313-318.

167. Saucy, F., Ducret, F., Lambelet, P., and Loliger, J. (1990). The Fate of Antioxidant Radicals During Lipid Autoxidation. II. The Influence of Oxygen Supply on Lipid Autooxidation. Journal of Chemistry and Physics of Lipids *55*, 215-221.

168. Yamauchi, R., and Matsushita, S. (1977). Quenching Effect of Tocopherols on the Methyl Linoleate Photooxidation and Their Oxidation Products. Journal of Agricultural Biology and Chemistry *41*, 1425-1430.

169. Warner, K., INFORM 4,, and 529. (1993). Effects of Adding Various Tocopherol Ratios on the Stability of Purified Vegetable Oils. Inform *4*.

170. Cao, Y., Gao, H.L., Chen, J.N., Chen, Z.Y., and Yang, L. (2006). Identification and Characterization of Conjugated Linolenic Acid Isomers by Ag+-HPLC and NMR. Journal of Agricultural and Food Chemistry *54*, 9004-9009.

171. Rowbottom, S. (2006). Determination of Peroxide Value in Commerical Oils and Fats. Mylnefield Lipid Analysis *4*, 1-6.

172. AOCS (2003). AOCS Official Method Cd 3d-63 Acid Value. In Official Methods and Recommended Practices of the AOCS, 6th Edition., pp. 1-2.

173. AOCS (2003). AOCS Official Method Cd 18-90. In Official Methods and Recommended Practices of the AOCS p-Anisidine, 6th Edition., pp. 1-4.

174. AOCS (2003). AOCS Official Method Cd 3-25 saponification Value. In Official Methods and Recommended Practices of the AOCS, 6th Edition.

175. Christie, W.W. (1982). Lipid Analysis, 2nd Edition, (Pergamon Press).
176. Bligh, E.G., and Dyer, W.J. (1959). A Rapid Method of Total Lipid Extraction and Purification. Canadian Journal of Biochemistry and Physiology *37*, 911-917.
177. Oomah, B.D., Ladet, S., Godfrey, D.V., Liang, J., and Girard, B. (2000). Characteristics of raspberry (Rubus idaeus L.) seed oil. Food Chemistry *69*, 187-193.
178. Cao, Y., Yang, L., Gao, H.-L., Chen, J.-N., Chen, Z.-Y., and Ren, Q.-S. (2007). Re-characterization of Three Conjugated Linolenic Acid Isomers by GC–MS and NMR. Chemistry and Physics of Lipids *145*, 128-133.
179. Yoshida, H., Kondo, I., and Kajimoto, G. (1992). Participation of Free Fatty Acids in the Oxidation of Purified Soybean Oil During Microwave Heating. Journal of American Oil Chemists Society *69*, 1136-1140.
180. Frankel, E.N., Cooney, P.M., Moser, H.A., Cowan, J.C., and Evans, C.D. (1959). Effect of Antioxidants and Metal lnactivators in Tocopherol-Free Soybean Oil. Sonderd ruck aus Fette· Seifen . Anstrichmiltel . Die Erniihrungsindustrie *61*, 1036-1039.
181. Library, L. (2010). Methyl Palmitic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_sat/M0016.htm*.
182. Library, L. (2010). Methyl Palmitoleic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_to2db/M0228.htm*.
183. Library, L. (2010). Methyl Stearic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_sat/M0018.htm*.
184. Library, L. (2010). Methyl Oleic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_to2db/M0258.htm*.
185. Library, L. (2010). Methyl Linoleic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_to2db/M0478.htm*.
186. Library, L. (2010). Methyl *alpha*-Linolenic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_pufa/M0672.htm*.
187. Library, L. (2010). Methyl *gamma*-Linolenic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_pufa/M0673.htm*.

188. Library, L. (2010). Methyl Stearidonic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_pufa/M0772.htm*.

189. Library, L. (2010). Methyl Eicosanoic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_sat/M0020.htm*.

190. Library, L. (2010). Methyl Eicosenoic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_to2db/M0285.htm*.

191. Library, L. (2010). Methyl Ricinoleic acid. Volume 2010. (AOCS), *http://lipidlibrary.aocs.org/ms/arch_me/me_misc/M1118.htm*.

192. Prasad, N.B.L., and Azeemoddin, G. (1994). Characteristics and Composition of Guava (*Psidium guajava L*.) Seed and Oil Journal. of American Oil Chemists Society *71*, 457-458.

193. Fadavi, A., Barzegar, M., and Azizi, M.H. (2006). Determination of Fatty Acids and Total Lipid Content in Oilseed of 25 Pomegranates Varieties Grown in Iran. Journal of Food Composition and Analysis *19*, 676-680.

194. Zlatanov, M.D. (1999). Lipid Composition of Bulgarian Chokeberry, Black Currant and Rosehip Seed oils. Journal of The Science of Food and Agriculture *79*, 1620-1624.

195. Christie, W.W. (1990). Gas Chromatography and Lipids : A Practical Guide. W.W. Christie, ed. (The Oily Press).

196. AN, M.A.O. (2004). Chemical and Nutrient Analysis of Baobab (Adansonia digitata) Fruit and Seed Protein Solubility. Plant Foods for Human Nutrition *59*, 29-33.

197. Kleiman, R., Ashley, D.A., and Brown, J.H. (2008). Comparison of Two Seed Oils used in Cosmetics, Moringa and Marula. Industrial Crops and Products *28*, 361-364.

198. Lampi, A., Kataja, L., Kamal-Eldin, A., and Vieno, P. (1999). Antioxidant Activities of Alpha and Gamma-Tocopherols in the Oxidation of Rapeseed Oil Triacylglycerols. Journal of American Oil Chemists Society *76*, 749-755.

199. Piombo, G., Barouh, N., Barea, B., Boulanger, R., Brat, P., Pina, M., and Villeneuve, P. (2006). Characterization of The Seed Oils from Kiwi (*Actinidia*

chinensis), Passion Fruit (*Passiflora edulis*) and Guava (*Psidium guajava*). Fondamental *13*, 195-199.

200. Assuncao, F.P., Bentes, M.H.S., and Serruya, H. (1984). A Comparison of The Stability of Oils from Brazil Nut, Para Rubber and Passion Fruit Seeds. Journal of American Oil Chemists Society *61*, 1031-1036.
201. Christie, W.W. (1999). The Analysis of Evening Primrose Oil. Industrial Crops and Products *10*, 73-83.
202. Senyanayake, S.P.J.N., and Shahidi, F. (2002). Chemical and Stability Characteristics of Structured Lipids from Borage (Borago officinalis L.) and Evening Primrose (Oenothera biennis L.) Oils. Journal of Food Science *67*, 2038-2045.
203. Traitler, H., Wille, H.J., and Studer, A. (1988). Fractionation of Blackcurrant Seed Oil. Journal of American Oil Chemists Society *65*, 755-760.
204. Goffman, F.D., and Galletti, S. (2001). Gamma-Linolenic Acid and Tocopherol Contents in the Seed Oil of 47 Accessions from Several *Ribes* Species. Journal of Agricultural and Food Chemistry *49*, 349-354.
205. Khan, M.A., and Shahidi, F. (2001). Effects of Natural and Synthetic antioxidants on The Oxidative Stability of Borage and Evening Primrose Triacylglycerols. Food Chemistry *75*, 431-437.
206. Ramadan, M.F., and Morsel, J.-T. (2002). Characterization of Phospholipid Composition of Black Cumin (*Nigella sativa L.*) seed oil. Food *4*, 240-244.
207. Ramadan, M.F., and Morsel, J.-T. (2003). Analysis of Glycolipids from Black Cumin (*Nigella sativa L.*), Coriander (*Coriandrum sativum L.*) and Niger (*Guizotia abyssinica Cass.*) Oil Seeds. Food Chemistry *80*, 197-204.
208. Chen, J., Cao, Y., Gao, H., Yang, L., and Chenc, Z.-Y. (2007). Isomerization of Conjugated Linolenic Acids During Methylation. Chemistry and Physics of Lipids *150*, 136-142.
209. Shaker, E.S. (2006). Antioxidative Effect of Extracts from Red Grape Seed and Peel on Lipid Oxidation in Oils of Sunflower. LWT *39*, 883-892.
210. Mitei, Y.C., Ngila, J.C., Yeboah, S.O., Wessjohann, L., and Schmidt, J. (2009). Profiling of Phytosterols, Tocopherols and Tocotrienols in Selected Seed Oils

from Botswana by GC–MS and HPLC. Journal of American Oil Chemists Society *86*, 617-625.

211. AOCS (1997). *p*-Anisidine Value Cd 18-90. AOCS Offical Methods, 1-2.

212. Przybylski, R. ed. (2005). Flax Oil and High Linolenic Oils, Volume 6, 6 Edition (John Wiley & Sons Inc).

213. Kornsteiner, M., Wagner, K., and Elmadfa, I. (2006). Tocopherols and Total Phenolics in 10 Different Nut Types. Journal of Food Chemistry *98*, 381-387.

214. Khan, M.A., and Shahidi, F. (2000). Tocopherols and Phospholipd Enhance the Oxidative Stability of Borgae and Evening Primrose Triacylglycerols. Journal of Food Lipids *7*, 143-150.

215. Malecka, M. (2002). Antioxidant Properties of The Unsaponifiable Matter Isolated from Tomato Seeds, Oat Grains and Wheat Germ Oil. Journal of Food Chemistry *79*, 327-330.

216. Krings, U., El-Saharty, Y.S., El-Zeany, B.A., Pabel, B., and Berger, R.G. (2000). Antioxidant Activity of Extracts from Roasted Wheat Germ. Journal of Food Chemistry *71*, 91-95.

217. Abbasi, H., Rezaei, K., Emamdjomeh, Z., and Mousavi, S.M.E. (2008). Effect of Various Extraction Conditions on The Phenolic Contents of Pomegranate Seed Oil. European Journal of Lipid Science and Technology *110*, 435-440.

218. Hämäläinen, T.I., Sundberg, S., Hase, T., and Hopia, A. (2002). Stereochemistry of The Hydroperoxides Formed During Autoxidation of CLA Methyl Ester in the Presence of Alpha-Tocopherol. Journal of Lipids *37*, 533-540.

219. Pajunen, T.I., Johansson, M.P., Hase, T., and Hopia, A. (2008). Autoxidation of Conjugated Linoleic Acid Methyl Ester in the Presence of *a*-Tocopherol: The Hydroperoxide Pathway. Journal of Lipids *43*, 599-610.

220. Luna, P., Fuente, M.A.d.l., Salvador, D., and Marquez-Ruiz, G. (2007). Differences in Oxidation Kinetics Between Conjugated and Non-Conjugated Methyl Linoleate. Journal of Lipids *42*, 1085-1092.

221. Yang, L., Cao, Y., Chen, J. N., and Chen, Z. Y. (2009). Oxidative Stability of Conjugated Linolenic Acids. Journal of Agricultural and Food Chemistry *57*, 4212-4217.

222. Sensidoni, A., Bortolussi, G., Orlando, C., Lognay, G., Fantozzi, P., and Paquot, M. (1995). Composition and Oxidative Stability of Borage (*Borago officinalis L*.) and Borage-Virgin Olive Oil Blends. *28*, 343-346.

223. Wahle, K.W.J., Heys, S.D., and Rotondo, D. (2004). Conjugated Linoleic Acids: Are They Beneficial or Detrimental to Health? Progress In Lipid Research *43*, 553-587.

224. Toomey, S., McMonagle, J., and Roche, H.M. (2006). Conjugated Linoleic Acid: A Functional Nutrient in The Different Pathophysiological Components of The Metabolic Syndrome? Current Opinion in Clinical Nutrition & Metabolic Care *9*, 740-747.

225. Chen, Z.Y., Chan, P.T., Kwan, K.Y., and Zhang, A. (1997). Reassessment of The Antioxidant Activity of Conjugated Linoleic Acids. Journal of American Oil Chemists Society *73*, 749-753.

226. Berg, J.J.M.v.d., Cook, N.E., and Tribble, D.L. (1995). Re-investigation of The Antioxidant Properties of Conjugated Linoleic Acid. Journal of Lipids *30*, 599-605.

227. Lee, S., and Min, D.B. (1990). Effects, Quenching Mechanisms, and Kinetics of Carotenoids in Chlorophyll-Sensitized Photooxidation of Soybean Oil. Journal of Agricultural and Food Chemistry *38*, 1630-1634.

228. Choe, E., and Min, D.B. (2006). Mechanisms and Factors for Edible Oil Oxidation. Comprehensive Food Science and Food Safety *5*, 169-186.

229. Lee, J.H., Ozcelik, B., and Min, D.B. (2003). Electron Donation Mechanisms of β-carotene as a Free Radical Scavenger. Journal of Food Science *68*, 861-865.

230. Heber, D., and Lu, Q. Y. (2002). Overview of Mechanisms of Action of Lycopene. Exeperimental Biology and Medicine, 920-923.

231. Tsaknis, J., Lalas, S., and Lazos, E.S. (1997). Characterization of Crude and Purified Pumpkin Seed Oil. Grasas y Aceites *48*, 267-272.

232. List, G.R., Friedrich, J.P., and Pominski, J. (1984). Characterization and Processing of Cottonseed Oil Obtained by Extraction with Supercritical Carbon Dioxide. Journal of American Oil Chemists Society *61*, 1847-1849.

233. Lazos, E.S., Tsaknis, J., and Lalas, S. (1998). Characteristics and Composition of Tomato Seed Oil. Grasas y Aceites *49*, 440-445.

234. F Falaras, P., Kovanis, I., Lezou, F., and Seiragakis, G. (1999). Cottonseed Oil Bleaching by Acid Activated Montmorillonite. Journal of Fine Particle Science: Clay Minerals *34*, 221-232.

235. Berbesi, R. (2006). Achieving Optimal Bleaching Performance. In Oil-Mill Gazatteer. (Oil-Dri Corporation), pp. 1-4.

Appendix

Data for Graph 1. Oxidative stability of plant seed oils

Oil	Time						
	0	**1**	**4**	**6**	**24**	**48**	**120**
Grape	20.2	24.9	27.3	36.6	47.6	89.0	170.1
Kakui	1.1	2.1	4.3	5.1	9.6	18.1	47.4
Lime	28.7	33.2	29.9	43.5	72.1	76.2	94.5
Manketti	3.5	3.8	4.5	6.0	6.7	8.8	9.1
Marula	2.1	3.9	3.0	5.1	7.7	15.5	31.6
Moringa	2.7	nd	Nd	nd	3.2	15.8	32.9
Papaya	1.2	1.6	2.0	2.7	4.8	8.4	21.9
Passion	4.9	5.1	6.2	6.0	8.9	11.9	20.8
Grapefruit	4.0	6.4	12.2	nd	21.5	39.1	85.1
Pomegranate	1.1	1.2	2.8	2.4	3.2	7.8	11.8
Sesame	1.0	3.6	4.2	4.5	6.9	8.8	13.5

nd = not determined

Data for Graph 2. Peroxide accumulation in Moringa oil

Oil	Time					
	0hr	**1hr**	**4hr**	**24hr**	**48hr**	**120hr**
Moringa	0.4	3.0	6.6	40.7	82.8	182.8
Moringa 1% PO	3.0	6.1	12.1	28.4	43.2	82.5
Moringa 5% PO	1.4	4.0	12.1	19.9	42.3	68.0
Moringa 13% PO	3.3	5.9	11.6	17.7	28.5	71.7