SCHOOL OF PHARMACY

A STUDY OF THE BIOCHEMICAL DEVELOPMENT AND TOXICOLOGY OF THE SEED OF *SANTALUM SPICATUM*

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This thesis is dedicated to the memory of my father.

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ABBREVIATIONS

AMP	2-amino-2-methyl-1-propanol
amu	atomic mass unit
AOAC	Association of Official Analytical Chemists
ASAT	serum aspartate aminotransferase
DMOX	4,4-dimethyloxazoline
ECN	equivalent carbon number
FAME	fatty acid methyl ester
FID	flame ionisation detection
GC	gas chromatography
HPLC	high performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
IR	infrared spectrometry
J	coupling constant
MS	mass spectrometry
m/z	mass-to-charge ratio
NAD	nicotinamide-adenine-dinucleotide
NADH	nicotinamide-adenine-dinucleotide reduced
NMR	nuclear magnetic resonance
NSAID	nonsteroidal anti-inflammatory drugs
RI	refractive index
TIC	total ion chromatogram
TLC	thin layer chromatography
UV	ultraviolet
XMYA	ximenynic acid

ABSTRACT

The seed of *Santalum spicatum* is rich in a fixed oil (59% by weight), which is characterised by a high percentage of acetylenic, ethylenic ximenynic acid (35% of total fatty acids). A number of important aspects of the seed fixed oil, its composition in developing seeds, its triacylglycerols molecular species in the oil, the nutrition and toxicity of the oil feeding, and the possible bioactivity of ximenynic acid in mice were investigated.

The identification of *cis* and *trans* isomers of ximenynic acid in the seed oil, and the metabolite of ximenynic acid in mouse liver lipid fractions were achieved using 2-amino-2-methyl-1-propanol to form 2-substituted 4,4-dimethyloxazoline derivatives, which were analysed by gas chromatography with mass spectrometric detection.

Changes in proximate and fatty acid composition were investigated in developing seed collected weekly from about seven days after flowering to maturity. It was determined that moisture and carbohydrate contents decreased significantly during the development sequence, while fixed oil content increased from 0.3% to 50% (by weight) with seed development. A corresponding increase in the proportions of both oleic and ximenynic acids occurred suggesting a precursor/product relationship. Mature seed collected from different locations in Western Australia showed minor differences in characteristics and lipid composition, which may have been influenced by geographical origin and harvesting year of samples.

The lipid components from the seed oil were separated using thin-layer chromatography and the individual triglyceride bands were characterised by high performance liquid chromatography and gas chromatography using flame ionisation and mass spectrometric detection after removal from the plate. The triximenynin (trisantalbin) band showed no other contaminating fatty acids and was obtained in a relatively pure state.

A nutrition and toxicity study was performed by feeding a semi-synthetic diet containing sandalwood seed oil to a level of 15% of total energy content to a group of mice for one month and another group for two months. The most significant effect of sandalwood seed oil ingestion when compared with a standard lab diet (5% fat, by weight) and a canola oil-enriched diet (15% fat, by weight) was an apparent reduction in body weight gain, which may be the effect of ximenynic acid as a growth retardant. Serum aspartate aminotransferase levels were determined in the mice as an indicator of hepatotoxicity. These levels were higher in mice fed the sandalwood seed oil diet than those fed the standard lab diet, suggesting that ximenynic acid may affect liver-specific enzyme activity. Analysis of the total lipid fatty acids of various tissues and organs of mice showed only a low incorporation of ximenynic acid into the general tissues (0.3-3% by weight), and its absence in the brain.

This study suggests a few health benefits from consumption of large quantities of sandalwood seed oil in the diet. These include a low lipid content in blood, heart and muscle, increase in the 16:1/16:0 and 18:1/18:0 ratios, production of increased levels of 18:1(n-9) and docosahexaenoic acid, and decreased levels of arachidonic acid in certain tissues. There were no specific pathological, morphological or mortality changes observed in the mice.

Sandalwood seed may be both a food and a medicine.

CHAPTER 1: INTRODUCTION

1.1 SANDALWOOD

1.1.1 General information

Sandalwood, *Santalum spicatum* (R.Br.) A.DC., a member of the Santalaceae family, is a small tree or shrub three to eight metres tall, having a ten to thirty centimetre trunk diameter, with dull grey-green fleshy leaves (Figure 1.1) (1, 2). The tree is an obligate root hemi-parasitic species, requiring a range of hosts for growth, and often taking fifty to ninety years to reach harvestable size in the naturally arid habitat. Its fruit is a drupe yielding a mostly brown endocarp with seed inside, and which ripens over a number of months mostly between September and November (1, 3). The seed has an average weight of 2.2 g (range 1.0-5.0 g), but may vary considerably in size (3); the seed kernel nestles in a strong endocarp shell, and forms an average 28% of the whole nut with a moisture content of 3% (4).

Santalum spicatum is a local species of Western Australia and South Australia. It occurs over a wide range of climatic conditions, and is naturally distributed throughout much of Western Australia arid region (Figure 1.2) (2). For this reason, it becomes essential that crops having good commercial values and the ability to thrive in poor soil conditions be identified and characterised.

1.1.2 The commercial value of sandalwood

Santalum spicatum is an integral part of the history and ecology of Western Australia. It is valued commercially for its scented wood which has been exported since the 1840s (5). The scented wood and the associated volatile oil was mainly used in perfumes, incense and soap, and also in the treatment of gonorrhoea and other illnesses by the aborigines (6, 7). Recently, it was reported that the volatile oil of sandalwood showed a high inhibitory activity against viral (HIV-1) growth (8).

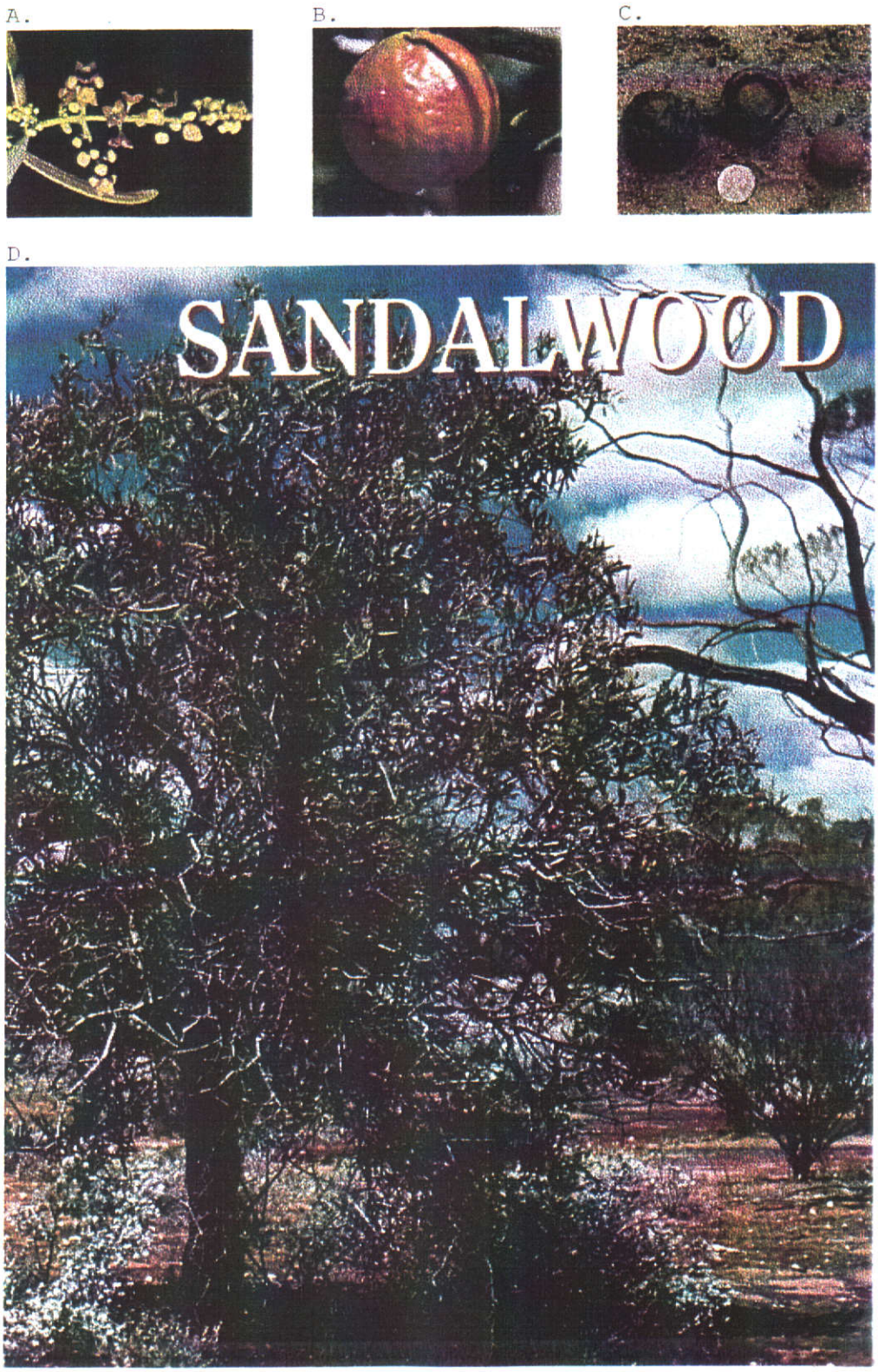


Figure 1.1 Sandalwood, *Santalum spicatum*: A) flowers, B) fruit, C) entire drupe fruit with five cents coin for scale and D) tree. (Reproduced by kind permission of the Department of Conservation and Land Management) (2).

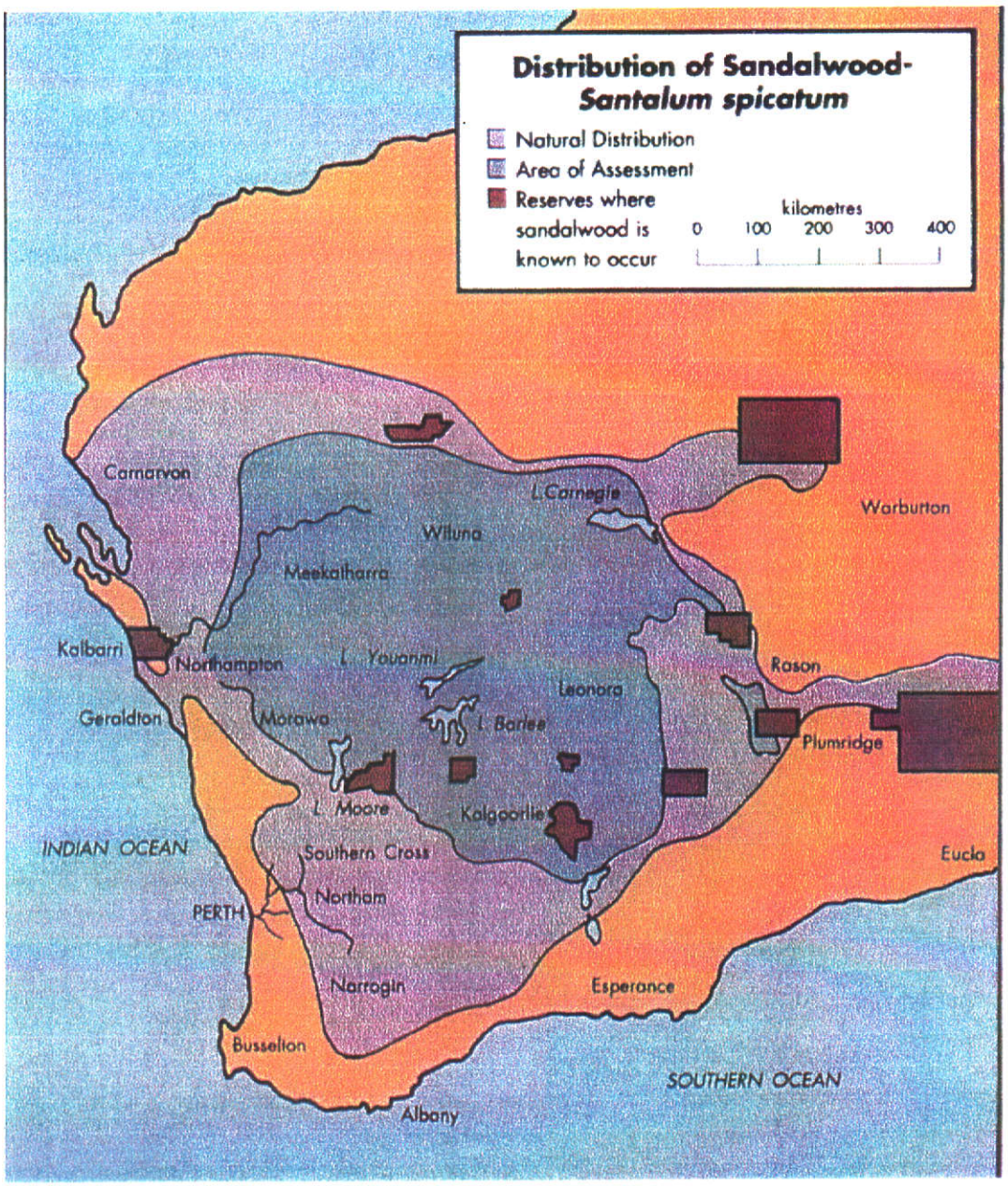
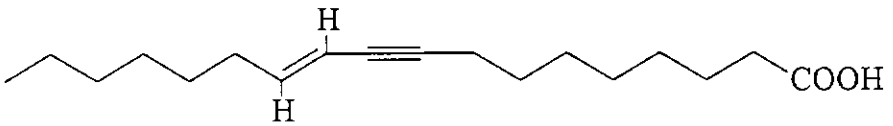


Figure 1.2. Distribution of *Santalum spicatum* in Western Australia. (Reproduced by kind permission of the Department of Conservation and Land Management) (2).

The tree bears significant numbers of large fruits, which are drupes with large seeds contained within a hard endocarp. These seeds are typically very rich in a drying fixed oil (50-60%) (4). The seed kernels have formed a valuable part of the traditional Australian Aboriginal diet (6), and have also been used by them as a topical application for skin lesions and taken by mouth as a treatment for rheumatoid arthritis (Dr. Bob Longmore, personal communication from Marr Mooditj, Western Australia). It has also been reported that the kernel was used as a 'rubbing medicine' for cold and stiffness (9).

The seed oil is characterised by containing a high percentage of unusual acetylenic fatty acids such as *trans*-ximenynic acid [XMYA, (34%), *trans*-11-octadecen-9-ynoic acid, 18:2(9a, 11t) where 'a' denotes the presence of the triple bond; 't' (or 'c') indicates the double bond in *trans* (or *cis*)-configuration], and stearolic acid [octadecen-9-ynoic, 18:1(9a), (1%)] both occurring as glycerides (4). XMYA is sometimes called santalbic acid; the term XMYA is used throughout this thesis.



Common names:	ximenynic acid, santalbic acid
Chemical name:	*trans*-11-octadecen-9-ynoic acid
Shorthand notation:	18:2(9a,11t)
Molecular weight:	278.4
Empirical formula:	$C_{18}H_{30}O_2$

XMYA has been reported to form, with diethanolamine or alkali-hydrolysate of gelatin, a surface activity product with good foaming capacity and foam stability (10). XMYA was also found to have skin moisturising and trophic properties (11). More recently, Jones and his coworkers have demonstrated that XMYA has antimicrobial and antifungal properties, and its sodium and potassium salts have been found to be

effective inhibitors of some Gram-positive bacteria and a number of pathogenic fungi (12). XMYA may be easily separated from the other fatty acids in the oil, using steps of alkaline hydrolysis, then acidification, solvent extraction, and multiple re-crystallisation from hexane to constant melting point (13). According to previously published data by Gunstone and Russell (14), the triple bond of XMYA can be selectively hydrogenated using a Lindlar catalyst to produce a conjugated linoleic acid [*cis*-9, *trans*-11-octadedienoic acid, 18:2(9c, 11t)], which has been newly recognised as an effective antioxidant and anticarcinogenic fatty acid (15-17).

1.2 THE PRESENT STUDY

1.2.1 Background

Due to large scale commercial harvesting of the scented wood, *Santalum spicatum* as a resource is diminishing (2, 3). In order to replenish the stocks, research has been undertaken on propagation and cultivation in recent years. The tree has been planted by individuals as a garden specimen and in trial plantations by research groups.

A number of studies on diverse topics such as germination techniques, suitable soil and fertiliser regimes, water and host requirements have been carried out in the School of Environmental Biology, Curtin University of Technology since 1981 (18). In the last decade, attention has been drawn to the use of the seed as an edible quality nut (19, 20). The tree may produce a profusion of fruits, with the seeds value being similar to other commercially available nuts (21). It has been suggested that if the nut could be developed as a food crop, this would provide continuing cash benefits for growers down the years until the trees become ready for harvesting for their aromatic wood (22). The seed contains high levels of a potentially valuable fixed oil (4, 6, 21). Thus, economic and efficient utilisation of the seed and the seed oil will stimulate the development of this economically important species. Hence the seed and seed oil of *Santalum spicatum* have potential commercial significance both as a possible food

stuff and as a natural source materials for making soaps, detergents, personal-care products and pharmacological agents.

1.2.2 Chemical composition of sandalwood seed and seed oil

1.2.2.1 Proximate composition

The so-called oilseeds are important for at least two reasons. Firstly, they provide a 'storage energy' to fuel the germination of the seed; secondly, they are a food source for humans and animals (23). The world production of oils and fats has increased in recent years and is expected to go on increasing to meet demand. Over the 40 year period 1962 to 2002 the world production of oils and fats is expected to increase from 29 to 105 million tones (24). The commercial importance of an oilseed in general is based on the quantity and composition of the oil present (23).

Chemical composition studies usually determine level of lipids, moisture, crude protein, ash, fibre and carbohydrate (by difference). There have been few reports on the chemical composition of *Santalum spicatum* seeds and these demonstrate an oil content of *ca.* 60% and protein of 16% (4, 25-26). More detailed findings of the proximate analysis of the seeds are lacking and provide a reason for this study.

1.2.2.2 Fatty acid composition of sandalwood seed oil

The reported fatty acid composition of *Santalum spicatum* seed oil is shown in Table 1.1 (25-26). Fatty acid analyses in these studies (25, 26) have demonstrated that oleic acid, 18:1(n-9) (52%) is the predominant fatty acid, followed by XMYA (31%), which is a significant fatty acid in the seed oil. Other fatty acids occurred only as minor components. Differences of fatty acid profiles have been observed, however the variation demonstrated by different authors may generally be considered to be insignificant.

Table 1.1 Fatty Acid Percentage Composition of *Santalum spicatum* Seed Oil.

Fatty acid	Notation	Relative % (25)	Relative % (26)
Palmitic	16:0	3.5	3.3
Palmitoleic	16:1(n-7)*	0.7	0.7
Stearic	18:0	1.9	3.5
Oleic	18:1(n-9)	54.4	50.9
Linoleic	18:2(n-6)	0.6	1.7
α-linolenic	18:3(n-3)	nd	2.5
Stearolic	18:1(9a)	nd	1.9
XMYA	18:2(9a, 11t)	33.4	28.4
Others		5.5	7.1

*: where the term 'n' indicates the position of double bond counted from terminal methyl group as carbon number 1.

nd = not detected

XMYA is the simplest of the conjugated acetylenic acids, and was first discovered by South African workers in the seed oil of three species of Ximenia (27-28). It was then found in the seed oil of Australian Santalum species in the 1950s (4, 13, 29). An early report noted that some unusual fatty acids may be restricted to certain plant species or families (30). For example, XMYA and its homologues were found to occur in plant species of the families Olacaceae and Santalaceae (4, 31-33). A selection of acetylenic acids found in sweet quandong (*Santalum acuminatum*) seed oil is shown below.

$CH_3 (CH_2)_5 CH{=}CH\ C{\equiv}C\ (CH_2)_7 COOH$	XMYA
$CH_3 (CH_2)_7 C{\equiv}C\ (CH_2)_7 COOH$	Stearolic acid
$CH_3 (CH_2)_3 CH{=}CH\ (C{\equiv}C)_2\ (CH_2)_7 COOH$	Exocarpic acid
$CH_3 CH_2 (CH{=}CH)_2\ (C{\equiv}C)_2\ (CH_2)_7 COOH$	13,15-Octadecadiene-9,11-diynoic acid
$CH_3 CH_2 CH{=}CH\ (C{\equiv}C)_3\ (CH_2)_7 COOH$	15-Octadecene-9,11,13-triynoic acid

Clearly, the conjugated acetylenic fatty acids shown above have certain common features. For example, they all have eighteen carbon atoms with an acetylenic bond at the carbon 9-position, and this may be accompanied by acetylenic or ethylenic bonds (which may be *cis* or *trans*) further away from the number carboxyl group (34).

1.2.3 Fatty acids and lipids

1.2.3.1 Fatty acid structure and nomenclature

Fatty acids are of widespread occurrence in the natural fats, oils, and allied substances, particularly in the vegetable kingdom (35). According to the abundance and importance of the various fatty acid in nature, these compounds have been distinguished as 'major' (>10%), 'minor' (<10%) and 'unusual' (such as conjugated ethylenic, acetylenic, substituted and branched-chain) fatty acids (30).

The fatty acid nomenclature system has varying degrees of complexity. Generally a fatty acid may be referred to by a common name, such as oleic acid; a systematic name established by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUPAC-IUB Commission on Biochemistry Nomenclature, 1967) (36), such as *cis*-9-octadecenoic (where 9 indicates the position of monounsaturation counted from the carboxyl group as carbon number one); and finally a shorthand nomenclature, such as 18:1(n-9), the term 'n' standing for the fact that the terminal methyl group of the fatty acid chain is taken as carbon number 1. These have been used together as shown in Table 1.2 (37). Shorthand nomenclature will generally be used in this thesis. Unsaturated fatty acids are further qualified by the terms *cis* (or Z) or *trans* (or E) used to express double bond configuration according to the nomenclature agreed by IUPAC-IUB (36). Most natural sources of unsaturated fatty acids are of the *cis* configuration, however *trans* fatty acids also occur naturally in some plants (30, 38).

The commonly occurring unsaturated fatty acids include 18:1(n-9), 18:2(n-6) and 18:3(n-3). These three fatty acids are also important as the precursors for biosynthesis of polyunsaturated fatty acids of the (n-3), (n-6) and (n-9) families. The biosynthetic

Table 1.2 Fatty Acid Nomenclature (of fatty acids referred to in this study)

No	Common name	Systematic name	Shorthand notation	Registry number*
1	myristic	tetradecanoic	14:0	544-63-8
2	palmitic	hexadecanoic	16:0	57-10-3
3	palmitoleic	*cis*-9-hexadecanoic	16:1(n-7)	2091-29-4
4	margaric	hepatadecanoic	17:0	506-12-7
5	stearic	octadecanoic	18:0	57-11-4
6	oleic	*cis*-9-octadecenoic	18:1(n-9)	112-80-1
7	vaccenic	*trans*-11-octadecenoic	18:1(11t)	143-25-9
8	linoleic	*cis,cis*-9,12-octadecadienoic	18:2(n-6)	60-33-3
9	conjugated linoleic	*cis*-9-*trans*-11-octadecadienoic	18:2(9c,11t)	872-23-1
10	α-linolenic	all *cis*-9,12,15-octadetrienoic	18:3(n-3)	463-40-1
11	stearolic	9-octadecynoic	18:1(9a)	506-24-1
12	ximenynic	*trans*-11-octadecen-9-ynoic	18:2(9a, 11t)	557-58-4
13	*cis*-ximenynic	*cis*-11-octadecen-9-ynoic	18:2(9a,11c)	136145-04-5
14	arachidic	eicosanoic	20:0	506-30-9
15	gadoleic	*cis*-11-eicosaenoic	20:1(n-9)	29204-02-2
16	arachidonic	all *cis*-5,8,11,14-eicosatetraenoic	20:4(n-6)	506-32-1
17	eicosapentae-noic	all *cis*-5,8,11,14,17-eicosapentaenoic	20:5(n-3)	1553-41-9
18	adernic	all *cis*-7,10,13,16-docosatetraenoic	22:4(n-6)	28874-58-0
19	docosahexae-noic	all *cis*-4,7,10,13,16,19-docosahexaenoic	22:6(n-3)	6217-54-5
20	nervonic	*cis*-15-tetracosaenoic	24:1(n-9)	506-37-6
21	furanoid fatty acid	8,11-epoxy-8,10-octadecadienoic	$F_{8,11}$	N/A

*: Chemical abstracts service registry number.

processes are known to be controlled by microsomal chain elongation and desaturation enzyme systems (39-41). In addition, 18:2(n-6) and 18:3(n-3), known as essential fatty acids, are synthesised by plants and must be obtained in the diet by animals including humans (37). The (n-3) and (n-6) polyunsaturated fatty acids play an important role in the modulation of inflammation and the possible cellular and biochemical mechanisms responsible (37, 39).

Although *trans* fatty acids are chemically unsaturated, the *trans* double bond is less biochemically active than a *cis* double bond (42, 43). Recent data indicate that *trans* fatty acids are subject to the same metabolic control mechanisms which regulate the metabolism of saturated and *cis* fatty acids (43). The health aspects of *trans* fatty acid will be further discussed in section 1.2.7.

A separate group of unusual polyunsaturated fatty acids has been characterised by the presence of conjugated double bonds (-C=C-C=C-) as distinct from the usual methylene-interrupted (-C=C-C-C=C-) bond arrangement (30). These fatty acids are generally found above trace levels only in rare oilseeds. However, conjugated linoleic acid, a mixture of *cis* and *trans* isomers of an 18:2 fatty acid, was initially isolated from grilled ground beef and then shown to be present in a variety of dairy products resulting from rumen and bacteria biohydrogenation (15-17, 39).

Most natural acetylenic fatty acids are conjugated C18 compounds, and occur in some higher plants mostly in the families of Santalaceae and Olacaceae, or in some fungi and bryophytes (34, 44-45). The present study was designed to obtain data on the biosynthesis of XMYA in *Santalum spicatum*, and the distribution and metabolism of XMYA in laboratory animal.

In addition to saturated and unsaturated fatty acids, a number of oxygenated fatty acids such as ricinoleic acid (12-hydroxy-9-octadecanoic acid) and furanoid fatty acids (e.g., 9,12-epoxy-9,11-octadecadienoic acid), are also found in nature (30).

Less common fatty acid such as those with a branched chain or a carbocyclic entity have also been found in bacteria, some oils from marine and animal fats (40).

One unusual family of fatty acids comprises the prostaglandins, C-20 carboxylic acids containing a cyclopentane ring. The naturally occurring prostaglandins are normally biosynthesised via the arachidonic [all-*cis*-5,8,11,14-eicosatetraenoic, 20:4(n-6)] cascade initially catalysed by the cyclo-oxygenase regulatory enzymes, (illustrated further in Figure 1.6) which can be affected by dietary fatty acid composition (39, 46).

The presence of unsaturated ethylenic or acetylenic bonds in fatty acid means that they are active in a number of chemical reactions involving hydrogenation and oxidation (37). Another important reaction is the autoxidation of the unsaturated fatty acids, involving free radical mechanisms (47), leading to fat and oil rancidity which may have considerable biological significance in the stability of biological membranes and in the acceptability of foods (37). This will be discussed later in section 1.2.7.

1.2.3.2 Lipid structural chemistry and nomenclature

Lipid is a term that has been used to describe the group of natural compounds of carbon, hydrogen, and oxygen which are seen as glyceryl esters of fatty acids in fats, oils, waxes and phosphoglycerides (48). The kinds of fatty acids present in a triacylglycerol have marked effects upon its physical and chemical behaviours (49). The phosphoglycerides have two ester bonds from glycerol to fatty acids (conferring the lipophilic character) plus one ester bond to phosphoric acid (conferring the hydrophilic character), producing a more polar lipid, which forms an integral part of the structure and function in biological membranes (50, 51). Phosphoglycerides are constructed from two combinations of apolar and 'backbone' moieties: a glycerol (or other polyol) moiety substituted with one or two acyl or alkyl chains; or an *N*-acylated sphingoid base (i.e., a ceramine) (50).

The abbreviated system of triacylglycerol nomenclature uses the initial letter of the fatty acid common name, e.g., 'O' for oleic acid, to construct a code for the triacylglycerol (49). Thus a simple triacylglycerol such as triolein is 'OOO', while a mixed triacylglycerol such as *sn*-2-palmito-1,3-diolein is 'OPO'.

1.2.4 The analysis of fatty acids and lipids

1.2.4.1 Extraction of lipid from natural samples

The analysis of natural fats and oils is difficult for two main reasons: firstly, because of the extremely large number of possible molecular species; and secondly, the very similar chemical and physical properties of most of these molecules (52).

Lipids may be physically entrained within the structure of the sample matrix, or physically and chemically bound to proteins, polysaccharides and other cellular components by covalent ionic, hydrogen bonding and Van der Waals forces (30). Various extraction and purification procedures are used to obtain lipids in a relatively pure state before analysis. The common method for extraction of lipids is that of Bligh and Dyer (53). It has been shown to be effective for the extraction of lipid from animal tissues. The advantage of this method is that it is simple, reproducible and quantitative.

A low-toxicity solvent system developed by Hara and Radin (54), called the 'cold solvent method', uses isopropanol/hexane (2:3, by volume) for the determination of the oil content in oilseeds. This method has been extensively used over the years, and has been shown to be comparable to or better than the Association of Official Analytical Chemists (AOAC) standard method (55).

1.2.4.2 Chromatographic techniques for separating lipids

The lipid composition of seed oils or animal tissues is an important parameter measured regularly in many laboratories (51). Chromatographic methods such as column chromatography, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) have mainly been employed for lipid analysis, providing highly specific information on lipid and fatty acid compositions. Generally chromatographic methods depend on adsorption or partition processes for separating complex lipids into simple lipid classes by solvent flow.

The classical technique of TLC is the most important method for the purification, fractionation and tentative identification of neutral and complex lipid classes (56-58). The fatty acid composition of each isolated lipid fraction can then be determined by complementary techniques, such as gas chromatography with mass spectrometric detection (GC/MS) of their methyl esters. Although TLC offers relative cheapness, simplicity and speed for lipid analysis, it is of limited value for large capacity and accurate quantification (56-58).

HPLC usually involves the use of a column of silica-based packed stationary phase, a mobile phase and a ultra/violet (UV)-detector. This method has been employed for lipid analysis only in recent years (60-62). The advantages of HPLC over TLC for lipid analysis include easy quantification, high selectivity and sensitivity (63).
Because many lipids lack chromophoric groups and may contain both saturated and unsaturated fatty acid residues (64), detection may be difficult. Refractive index detectors can provide acceptable detection of non-UV absorbing compounds providing strict instrumental control is maintained (63). The mobile phase generally employed consists of a mixture of acetone and acetonitrile, and occasionally tetrahydrofuran or hexane may be added to produce better separation characteristics (61-63).

In addition to the HPLC method, the separation of triacylglycerols by GC has also been reported (65-67). Although this technique requires column temperatures of approximately 300°C to 350°C (68-69), excellent resolution of the different types of triacylglycerols without the need for derivatisation techniques can be achieved (64, 70). Flame ionisation detectors are very sensitive and are the most widely used of the GC detector types.

Due to industrial interest, agricultural production of *Santalum spicatum* seeds is increasing. Information on the lipid classes present in the seeds is virtually nonexistent. The present study uses a combination of rapid TLC, HPLC and GC methods allowing the resolution of *Santalum spicatum* seeds oil into various molecular species present in each lipid class.

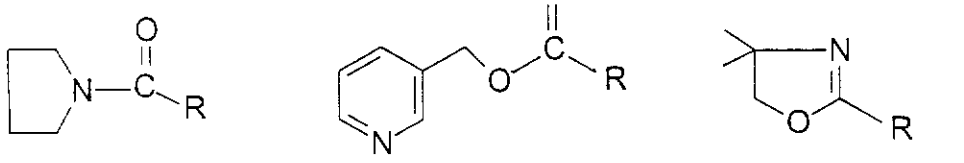
1.2.4.3 The preparation of fatty acid derivatives

The determination of fatty acids is an essential part of lipid analysis. It is usual to convert lipids into volatile derivatives such as fatty acid methyl esters (FAME) (70). A rapid methylation/transmethylation reaction may be used for this preparation. FAME are formed from interactions between fatty acid or triacylglycerol and methanol with catalytic reagents such as acids (71-73). The reaction is termed 'methylation' or 'transmethylation (esterification)' (74).

FAMEs are ideally suited for separation and general identification by GC/MS for quantitative analytical purposes. The analysis of saturated fatty acid by GC/MS is easy and accurate, however problems may arise in the analysis of unsaturated fatty acid as their methyl esters. This is because the mass spectra of FAME generally exhibit molecular ions, but may lack ions indicative of structural features such as the position of unsaturated centres (75). There are no diagnostic ions suitable to determine the position of the double bond because the double bond is ionised, resulting in double bond migration.

The use of methyl esters may be unsuitable for the unequivocal identification of fatty acids and their configuration, and some other means of specific identification is required. In addition, complex fatty acids which contain branched and poly-unsaturated chains or additional functional groups may give unsatisfactory fragmentation patterns when using GC/MS analysis of FAME derivatives.

Between 1978 and 1988, a number of different powerful analytical techniques, such as transformations to pyrrolidides (76), picolinyl esters (77) and 4,4-dimethyloxazolinyl (DMOX) derivatives (78) (as shown below, where 'R' denotes the alkyl chain), were proposed for GC/MS identification of various fatty acid with different functional groups. These methods require hydrolysis of lipids and acidification to yield free fatty acid which are then converted into the respective ester derivatives.



pyrolidide ester picolinyl ester DMOX derivatives

It has been found that double bonds and functional groups may be 'fixed' by these derivatives to a better extent than with the corresponding FAME. The mass spectra of the heterocyclic derivatives contain strong diagnostic ions as a result of specific cleavage processes, thus locating the position of double bond and/or the functional groups (77-82).

Of these techniques, the most useful is that involving DMOX derivatives (78, 79). The DMOX derivatives of fatty acid shows several advantages over other derivatives: 1) they are readily prepared and purified; 2) they yield abundant diagnostic ions due to directed fragmentation; and 3) they demonstrate good chromatographic properties (79). Saturated, unsaturated (79), methyl branched (80), hydroxy and epoxy groups (81) have all been analysed and identified by the low resolution electron impact mass spectra of their DMOX derivatives. Specific details of the characteristic fragmentation of fatty acid DMOX derivatives are discussed in Chapter 3 (page 53). DMOX derivative preparation and fragmentation were evaluated and applied to the analysis of XMYA isomers in *Santalum spicatum* seed oil.

1.2.5 The biosynthesis of fatty acids in plants

The general field of fatty acid biosynthesis has been investigated over several decades. Early studies reported that the synthesis of fatty acid could arise from various precursors, such as carbohydrates, non-carbohydrate and even proteins (84). The

biosynthesis of fatty acid may follow two distinct pathways. One is the repetitive condensation of C_2 acetate precursor units to construct a long-chain fatty acid, the other is by C_2 unit elongation of a pre-formed fatty acid (38). The latter sequence may occur with both saturated and unsaturated precursor molecules. More recent reports indicate that even though CO_2 is the ultimate source of carbon for fatty acid synthesis, acetyl-CoA or malonyl-CoA should be regarded as more direct precursors rather than CO_2 for fatty acid synthesis (51, 85-86).

The acetate-malonate pathway is of primary importance, and the major pathways of fatty acid synthesis in plant cells are summarised in Figure 1.3.

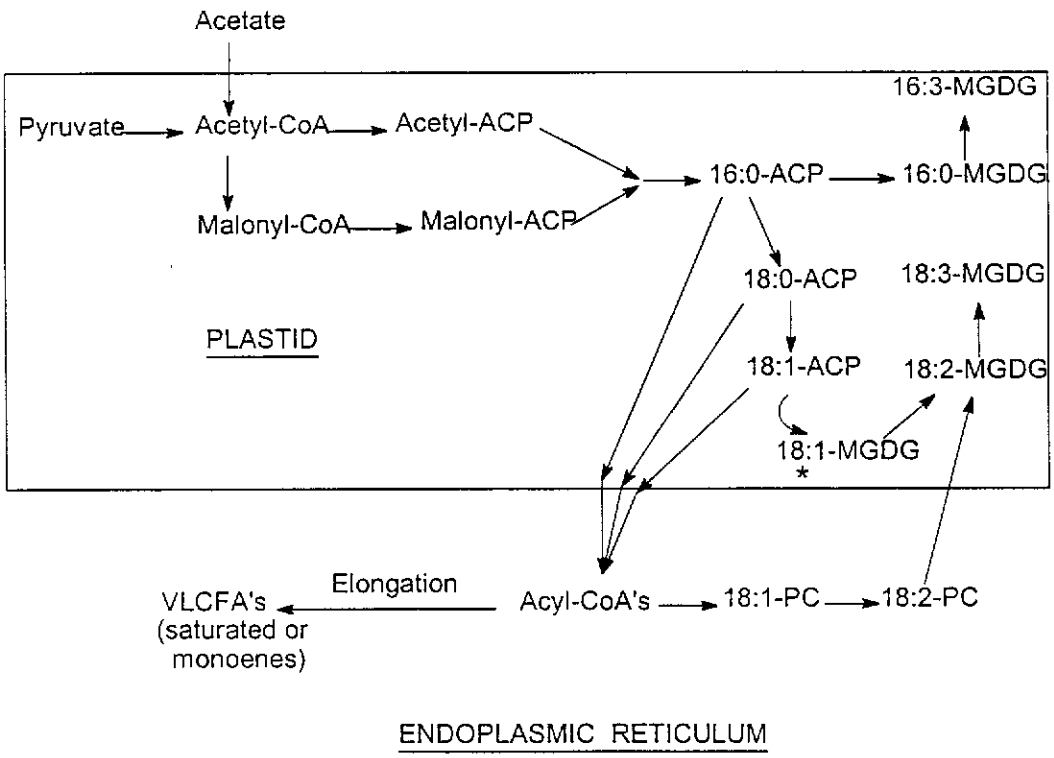


Figure 1.3 Major pathways of fatty acid synthesis in plant cells. * Indicates other lipids are probably involved in "16:3" plants. ACP = acyl carrier protein
PC = Phosphatidylcholine; MGDG = monogalactosyldiacylglycerol.
VLCFA = very long chain (>C18) fatty acid. Redrawn from reference (85).

Thus, the mechanisms of fatty acid synthesis occurring in plant systems are considered to be: 1) the *de novo* synthesis of saturated acids from acetate; 2) chain elongation; 3) Δ9-desaturation to produce monoenes; and 4) further desaturation (51).

Oleic acid, 18:1(n-9) was the first unsaturated fatty acid to be demonstrated to arise by oxidative desaturation of a saturated precursor, 18:0 in plant tissue (87). Furthermore, oleoyl-CoA itself may be used as a substrate to form linoleate and α-linolenate by aerobic desaturation (88). In contrast, although animals can desaturate 18:0 to 18:1(n-9), they cannot further desaturate the latter to 18:2(n-6) or 18:3(n-3), which are the precursors of polyene acids of the n-6 and n-3 families (37, 39). Thus, 18:2(n-6) and 18:3(n-3) are the essential fatty acids, important for good health, and can only be obtained from dietary vegetable sources.

The biosynthesis of acetylenic fatty acid has been suggested to involve the formation of triple and double bonds either 1) by elimination reaction from enols and alcohols respectively, or 2) by dehydrogenation of saturated compounds (89, 90). In a study of quandong (*Santalum acuminatum*) seed germination, a series of acetylenic acids was demonstrated (page 7) (33). It was suggested that the conversion of oleic acid into acetylenic fatty acids replaces the usual conversion into linoleic acid, following a regular pattern (90): **oleic acid → stearolic acid → ximenynic acid.**

The biosynthesis of crepenynic acid [*cis*-9-octadecen-12-ynoic, 18:2(9c, 12a)], a non-conjugated isomer of XMYA, has been studied in developing seeds of *Crepis ruba* (91, 92). It was demonstrated that the introduction of the acetylenic bond at the carbon 12-13 position arose after formation of an 18 carbon chain (92). Investigations of the synthesis of XMYA and crepenynic acid have shown the probable common precursor as 18:1(n-9) emphasising its importance in further fatty acid biosynthesis (33, 91). Many aspects of the biosynthesis of XMYA are still unknown. The objective of this study was to provide additional information on the biosynthesis of XMYA in developing seed of *Santalum spicatum*.

1.2.6 Fatty acid metabolism

1.2.6.1 Digestion and absorption of fatty acid

Fats provide 37 KJ/g as a energy source (93, 94). Most of the fatty acids from seed oils in the human diet belong to the C18 series with lesser amounts of C16, and approximately 99% of them are in the form of triacylglycerol (30). When fat is consumed, triacylglycerols must be partly hydrolysed to a mixture of free fatty acids and monoacylglyceride before efficient absorption by the body (37, 39, 95).

The major site of digestion of triacylglycerol is the small intestine where the action of pancreatic lipase, a mixture of hydrolytic enzymes, release fatty acid from triacylglycerol (37, 95-97). After absorption, free fatty acids released from both dietary and endogenous triacylglycerol are transported in plasma bound to albumin and undergo utilisation in liver, cardiac and skeletal muscle or are stored as triacylglycerol in adipose tissue (39, 95). This complex process of triacylglycerol metabolism involves hydrolysis, emulsification, chemical combinations and various other biochemical reactions (39).

1.2.6.2 Degradation of fatty acids

Fatty acid molecules provide a source of cellular energy through a series of reactions such as α- β-, and ω-oxidation, which are named depending on which carbon of the acyl chain is attacked (95). Most dietary fatty acids are oxidised for energy via tissue β-oxidation in mitochondria (95). During β-oxidation, fatty acids are degraded when two carbon atoms are removed at each reaction cycle (95).

There have been few studies of the metabolism of acetylenic fatty acids (98-100). For example, crepenynic acid was found to be incorporated into rat depot fat but only to a minor extent; and 4-decynedioic acid, a metabolite of crepenynic acid by ω-oxidation, has been isolated from the urine of rats fed on *Crepis rubra* seeds (98). The stearolic

metabolism of acid in rats was found to proceed by the main metabolic steps of ω-, β-, and presumably α-oxidation, being finally oxidised and expired as CO_2, with a low level being incorporated into body lipids (99). More recently, studies of rat fed quandong (*Santalum acuminatum*) seed oil have indicated that XMYA was readily absorbed, and widely distributed in tissue in the lipid fractions (100). However, little was determined about the effects of XMYA on tissue fatty acid composition and the activities of the biochemical pathways of XMYA metabolism.

Dehydrogenation is a common reaction during fatty acid metabolism. By contrast, the reverse process, 'biohydrogenation', is less common in nature (39, 101-102). An early study has indicated that elaidic acid [*trans*-9-octadecanoic acid, 18:1(9t)] was mainly biohydrogenated to 18:0 when methyl elaidate-1-^{14}C was fed to fat-deficient guinea pigs (101). Recently, a few workers have reported that biohydrogenation of double bonds in nature is only found in ruminant animals, such as the cow and the sheep, and a few bacteria (39, 102). This reaction may also involve double bond migration together with partial or complete hydrogenation. For example, 18:2(n-6) can be biohydrogenated to produce either stearate or an octadecenoate [18:1(9c) or 18:1(11t)], the mechanism following reaction pathways shown in Figure 1.4 for *Butyrivibrio fibrosolvens* (102):

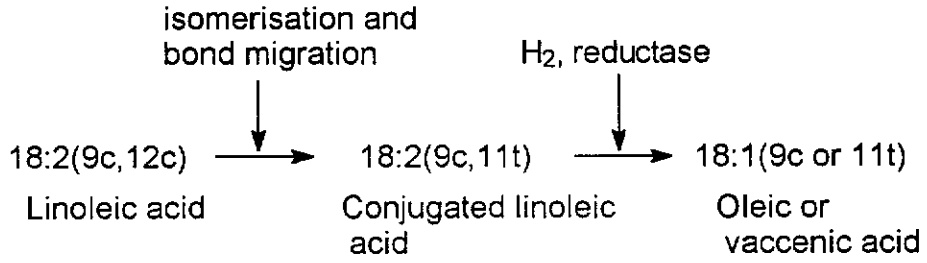


Figure 1.4 Biohydrogenation reactions of linoleic acid.
Adapted from reference (39).

The effects of dietary fatty acids on lipid metabolism have been concluded to involve: 1) *de novo* fatty acid synthesis and fatty acid oxidation, 2) incorporation into circulating lipoproteins, blood lipids, and cholesterol, and 3) conversion to eicosanoids (103).

1.2.6.3 Fatty acids and eicosanoids

Dietary fat is a diverse collection of biological materials, which plays an important and essential role in human and animal nutrition, e.g., supplying essential fatty acids. It is known that essential fatty acids are necessary for the integrity of all living animal tissues, and the body cannot synthesise them in sufficient quantity to meet its needs (104-106). Essential fatty acids were first discovered by Burr and Burr in 1928 (104), who found that rats fed on a totally fat-free diet exhibited a wide range of acute symptoms such as reduced growth rate and a reproductive deficiency, and that these deficiencies could be cured by the addition of 18:2(n-6) and 18:3(n-3) to the diet. Although 18:2(n-6) is a major constituent in some seed oils, for example, in sunflower seed (67%) and soybean (54%) oils (23), the human requirement for this acid is known to be only 1-2% of the total daily caloric intake (107).

It has been demonstrated that 18:2(n-6) and 18:3(n-3) can act as substrates for conversion to the corresponding C20 and C22 derivatives such as arachidonic [20:4(n-6)], eicosapentaenoic [all-*cis*-5,8,11,14,17-eicosapentaenoic, 20:5(n-3)] and docosahexaenoic [all-*cis*-4,7,10,13,16,19-docosahexaenoic, 22:6(n-3)] acids by successive desaturation (i.e., oxidation with double bond formation) and elongation (i.e., lengthening of the chain by two methylene groups) reactions, primarily in the liver of most mammals (95, 106). Accordingly, 18:1(n-9) can also act as precursors to form corresponding long-chain (n-9) polyunsaturated fatty acid series. As a result of such modification, polyunsaturated fatty acid of various chain lengths are readily synthesised without altering the methyl end of the molecule, as shown in Figure 1.5.

One of the biological function of polyunsaturated fatty acids is their incorporation into cell membrane phospholipids. It was found that long-chain polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids are incorporated mainly into the 2-position of phospholipids via *de novo* synthesis and acylation-deacylation mechanism, and help to maintain a high degree of membrane fluidity (108). Animal studies have found that the developing rat brain has a high capacity to synthesise arachidonic and docosahexaenoic acids from their precursor molecules by desaturation and elongation (109).

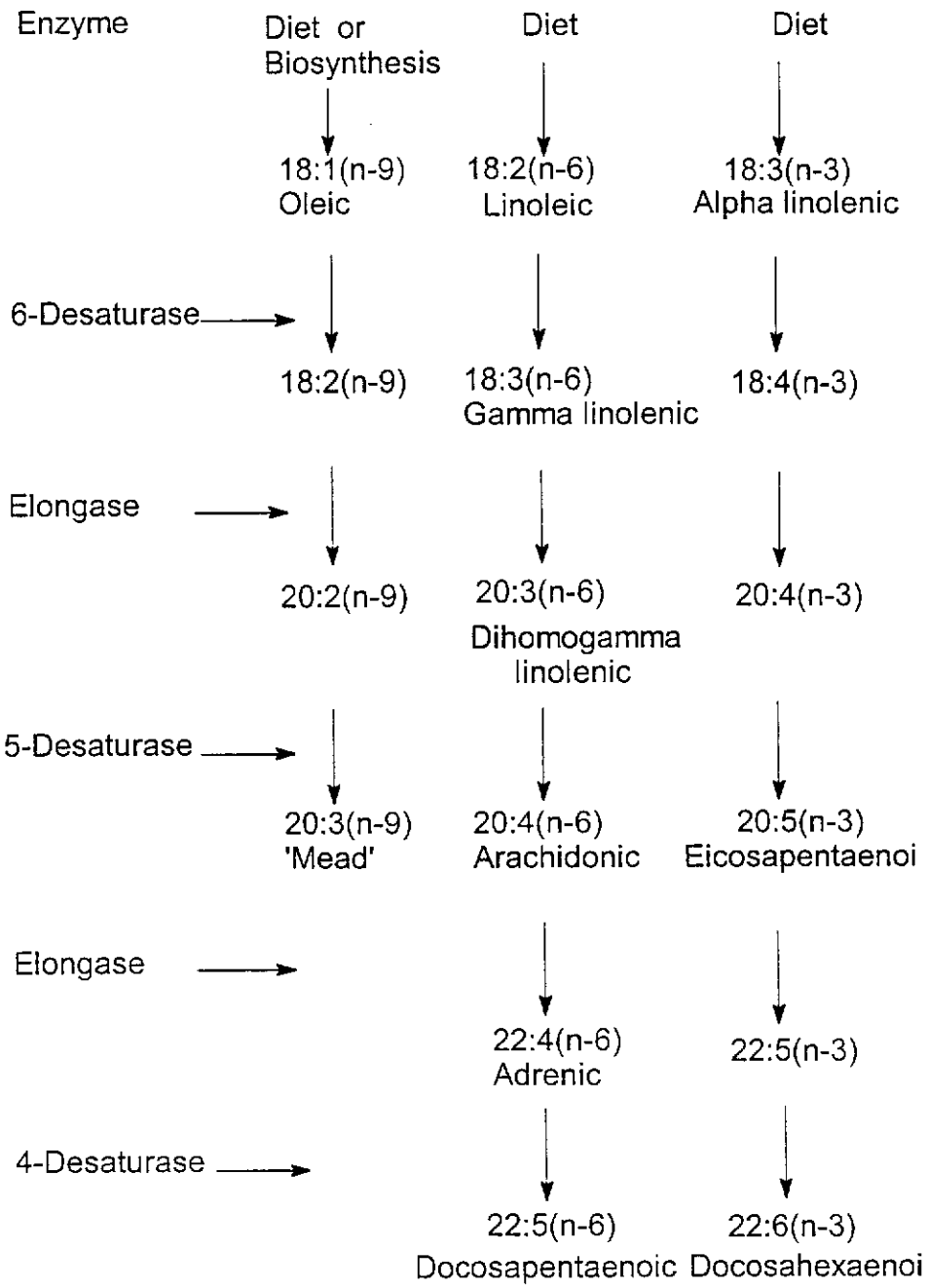


Figure 1.5 Metabolic transformation of oleic, linoleic and linolenic acids by desaturation and elongation. Redrawn from ref. (37).

Although the desaturase and elongase enzymatic systems are the same for different polyunsaturated fatty acid series, there is no crossover between fatty acid metabolites from the 18:2(n-6) and 18:3(n-3) sequences. In addition to acting as energy substrates, some polyunsaturated fatty acids have critical physiological functions. They may be metabolised by oxygenation into a variety of oxygenated fatty acid such as prostaglandins, leukotrienes and thromboxanes, which are collectively referred to as 'eicosanoids' (110). Eicosanoids play a prominent physiological roles in different organs, including muscle contraction/relaxation, platelet aggregation/inhibition, blood vessel contraction/dilation, inflammation responses, immune responses and regulation of hormone secretion and other processes (39). Prostaglandins are not normally stored in tissues but are biosynthesised from essential fatty acids when required. The biosynthesis consists of many steps that involve many different enzymes and cofactors (37, 46, 106). The pathway of the biosynthetic origins of prostaglandins from essential fatty acid is shown in Figure 1.6.

Since the biologically active prostaglandins are synthesised from unsaturated fatty acids, inhibitors of their biosynthesis have attracted a great deal of attention as potential therapeutic agents (37, 39, 46, 106). The first oxygenation step regulated by cyclo-oxygenase enzyme in prostaglandins biosynthesis can be inhibited by certain non-steroidal anti-inflammatory drugs (e.g., aspirin or indomethacin) (46, 106). Early studies have also reported the effects of (n-3) polyunsaturated fatty acids on arachidonic acid incorporation into membrane lipids and changes in its metabolism by cyclo-oxygenase and lipoxygenase (111-115). In fact, eicosanoids derived from (n-3) polyunsaturated fatty acids have different biological potencies to these derived from (n-6) polyunsaturated fatty acids (106). A number of acetylenic fatty acids are known to inhibit cyclo-oxygenase and lipoxygenase enzymes in a variety of tissues (116-117). Crepenynic acid and XMYA were demonstrated to be competitive inhibitors and alternative substrates for the essential cyclo-oxygenase and lipoxygenase enzymes which produce inflammatory intermediates *in vitro* leukocyte assay (118). Although sandalwood seeds have been used for a therapeutic effect to treat arthritis by oral ingestion, the mechanisms responsible for the effect on eicosanoids are on clear. This study investigates the metabolism of XMYA as an unusual fatty acid and its effect on the incorporation of other fatty acids into various mouse tissues.

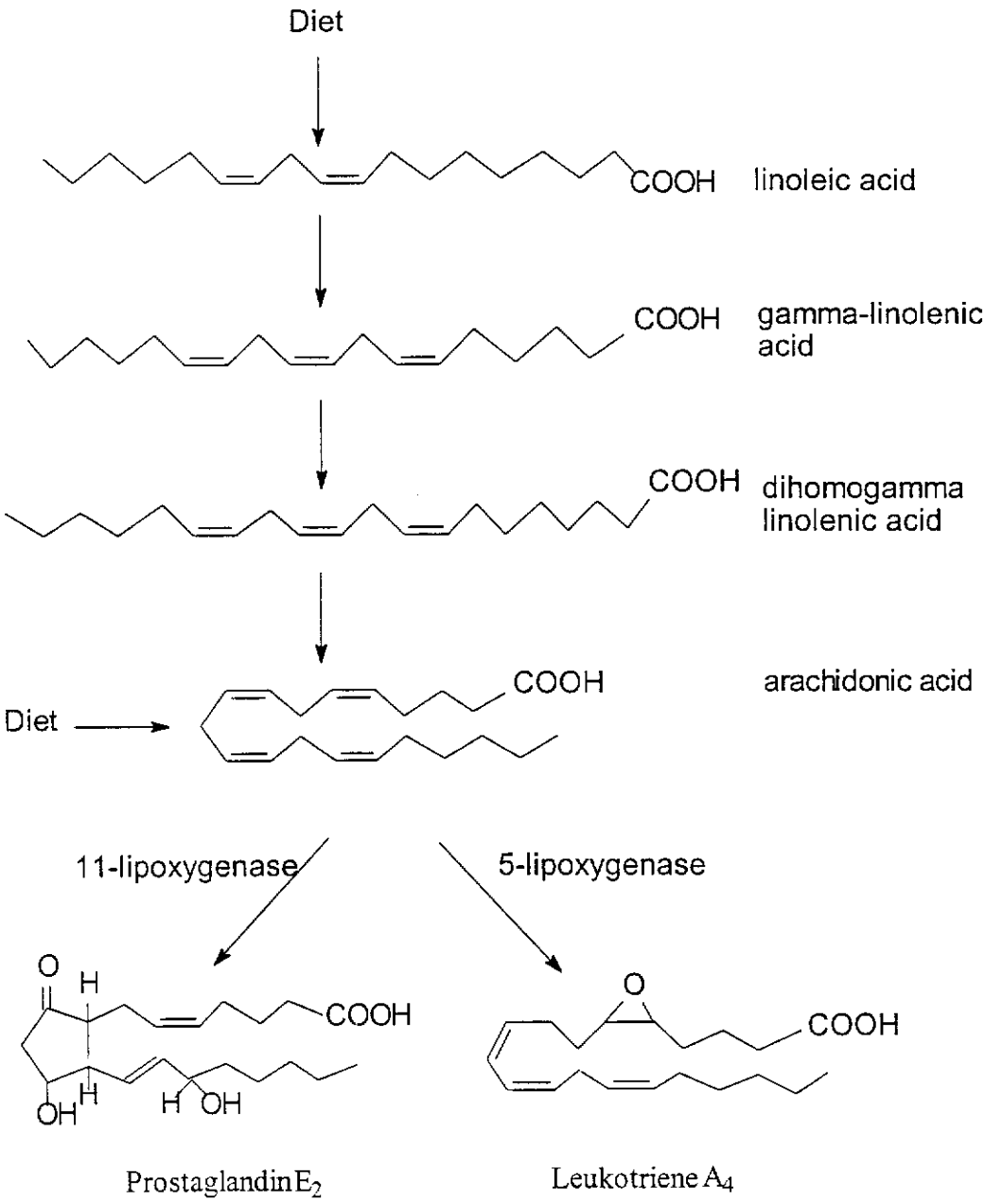


Figure 1.6 The biosynthesis of prostaglandin and leukotriene from fatty acid. Adapted from reference (37, 39).

1.2.7 Health aspects of fatty acids

In contrast to the traditional view of lipids mainly supplying energy, recent researches have shown that dietary fatty acids can effect numerous biochemical and physiological reactions (111). In general, the intake of high dietary fat, especially saturated fat intake, is strongly associated with elevated serum cholesterol and low density lipoprotein cholesterol increasing the risk of coronary heart disease. Replacement of saturated fatty acids by the (n-3) and (n-6) polyunsaturated fatty acids may reduce heart disease risk. Considerable attention has been given to the relationship of dietary (n-3) polyunsaturated fatty acid to human disease such as thrombosis, atherosclerosis, inflammation, cancer, autoimmune disorders and aging (107). This relationship has been extensively studied in humans and in different animal species. On this basis, plant oils rich in γ-linolenic acid [all *cis*-6,9,12-octadecatrienoic acid, 18:3(n-6)] and fish oils rich in eicosapentaenoic and docosahexaenoic acids are the most interesting in their potential for use as therapeutic agents. These oils are in growing demand for nutritional, clinical and pharmaceutical applications.

1.2.7.1 Fatty acid autoxidation

Lipid autoxidation is an important issue in the food industry, where antioxidants were used to prevent food oil deterioration (23). The major pathway for oxidative spoilage of foods is attack by oxygen at the allylic methylene position in a fatty acid. This reaction is catalysed by metals such as iron or copper or enzymes such as lipoxygenase to form a 'free radical' peroxide ($ROO^{\cdot}$) (39, 119). Lipid peroxidation has been suggested to occur in three separate steps: initiation ($RH \rightarrow R^{\cdot} + H^{\cdot}$), propagation ($R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$) and termination ($R^{\cdot} + R^{\cdot} \rightarrow$ non-radical products). The cycle of lipid peroxidation is shown in Figure 1.7.

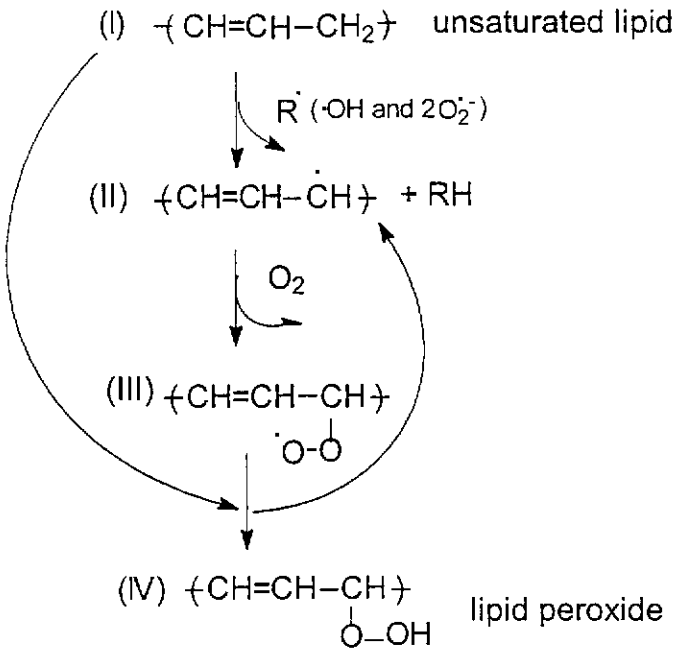


Figure 1.7 Cycle of lipid hydroperoxidation
Adapted from (121).

It is known that a number of external factors such as various types of radiation, including sunlight, and environmental pollutants can initiate autoxidation *in vitro*. Internal factors such as a number of enzymes involved in various oxidation processes could potentially lead to lipid peroxide formation *in vivo* (120).

In living systems, the autoxidation of polyunsaturated fatty acid may have important physiological and/or pathological consequences. Clinical investigations have found that continued lipid peroxidation destroys membranes, inhibiting the function of cell organelles and producing degenerative changes in cell and tissue structure (121). Thus antioxidants such as vitamin E (α-tocopherol), are of current interest as prophylactic agents for the treatment of cancer, atherosclerosis, rheumatoid arthritis, AIDS, Parkinson's disease and Alzheimer's disease (122). There is considerable interest in the role of some fatty acids such as conjugated linoleic acid as antioxidants.

1.2.7.2 Safety and health effects of trans fatty acids

As *trans* fatty acids are naturally present in foodstuffs, their role in nutrition, their possible effects on essential fatty acids metabolism and the consequences for human health have been extensively investigated (123). Most of the reports in the literature on *trans* fatty acid are conflicting. In recent years, concern has been expressed regarding the possible deleterious effects of *trans* fatty acid on elongation and desaturation of essential fatty acids, especially in relation to coronary heart disease (124). For instance, the U.S. Food and Drug Administration does not permit use of trans fatty acid content on labels of food products (125). In contrast to the above report, other investigations have yielded different results. In 1994, Thomson *et al.* found that there was no difference in the uptake of varying concentration of D-glucose, and no diet-associated changes in the uptake of fatty acid or cholesterol between rats fed *trans*-18:1, *cis*-18:1 or no 18:1 (126).

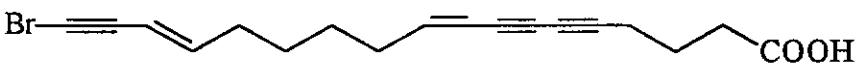
Overall consensus seems to indicate that there are no adverse effects to be expected from ingestion of *trans* fatty acid. In 1985, a special Federation of American Societies for Experimental Biology Committee and the 1987 British Nutrition Foundation report on the health aspects of dietary *trans* fatty acid concluded that there is little reason for concern with the safety of dietary *trans* fatty acid both at their present and expected levels of consumption (127). As the double bond of XMYA is in the *trans* configuration, part of the present study is to obtain additional information on these aspects.

1.2.7.3 Biological effects of less common fatty acids

Understanding the physiological significance of fatty acid, particular the unusual fatty acids is important. Evidence from animal studies has demonstrated that synthetic conjugated linoleic acid [18:2(9c,11t)] inhibits the initiation of mouse forestomach tumorigenesis by benzo[*a*]pyrene, and has also suggested that conjugated linoleic acid

is incorporated from the diet into the phospholipid of cell membranes, providing a defence mechanism against membrane attack by free radicals (15).

Mid-chain (C10-C14) acetylenic fatty acids have been suggested to be physiologically innocuous (128). In contrast, a number of long-chain acetylenic fatty acids (C18 or C20) have been reported to have more physiological and biochemical activities on the biosynthesis of eicosanoids (117-118, 128-130). Furthermore, brominated polyacetylenic acids, e.g. 18-bromo-*cis*, *cis*-9,15-octadecadiene-5,7,17-triynoic acid, extracted from the sponge *Xestospongia muta*, have been shown to inhibit human immunodeficiency virus (HIV) protease, a critical enzyme in the replication of HIV (131). Thus, some acetylenic acids may be considered to have potential application as therapeutic agents.



Finally an interesting ethnobotanic observation is that crushed sandalwood seed kernels have been used by Aboriginal Australians as a topical application for skin lesions and they were eaten to treat arthritis, providing circumstantial evidence of the general activity of the study material (6).

1.3 THESIS OBJECTIVES

The preceding review has shown that the biosynthesis of fatty acid in plants, fatty acid and lipid analysis and the effect of dietary fatty acid on numerous biochemical and physiological reactions are broadly understood. With respect to sandalwood seed oil much of these aspects are still unknown. The aim of this thesis is to provide:

- An understanding of the metabolic changes of selected biochemical constituents of *Santalum spicatum* seed during its development.
- A determination of the proximate composition and lipid component of such seeds collected from different locations of Western Australia.
- Lipid class analysis of this seed oil.
- Nutrition and toxicity studies of this seed oil in mice.
- A consideration of the metabolic fate of ingested XMYA in mice.

1.4 THESIS OUTLINE

This thesis consists of seven chapters.

Chapter 1 is a general introduction.

Chapter 2 describes the methodologies used in this study.

Chapter 3 presents information on the analysis of fatty acid with different derivatives. The isomers of XMYA in the seed oil, and the metabolite of XMYA in animal tissue are elucidated using mass spectrometry of their dimethyloxazoline derivatives.

Chapter 4 reports the metabolic changes of biochemical constituents of sandalwood seed during its development, and gives information on XMYA biosynthesis. This chapter also shows the influence of geographical origin on proximate and fatty acid compositions of the seeds from different locations of Western Australia and provides further information on the biosynthesis of XMYA.

Chapter 5 describes the analysis of triacylglycerol molecular species in sandalwood seed oil by various chromatographic methods. Triximenynin is separated and identified from the seed oil.

Chapter 6 assesses the nutrition and toxicity of sandalwood seed oil feeding. The presence of XMYA regulates the incorporation of alternative fatty acids into various tissues, which may suggest a biochemical basis for its potential anti-inflammatory activity.

Chapter 7 provides a general conclusion using results from each of the studies.

CHAPTER 2: METHODOLOGY

2.1 STANDARDS AND REAGENTS

All the chemicals were either analytical reagent grade or HPLC quality. Silica gel G60 and precoated F_{254} plates were supplied by Merck Company (Germany). Aspartate aminotransferase kits were supplied by Boehringer Mannheim GmbH (Germany). The standard heptadecanoic acid (17:0), and triacylglycerols (triolein, tripalmitin) were supplied by Sigma Chemical Company (U.S.A.).

2.2 MATERIALS

2.2.1 Sample sources

Samples of the fruit and seed of *Santalum spicatum* (R.Br.) A.DC. were obtained during different stages of growth and development from 10-12 year old trees, grown under irrigation conditions in the Field Trial Area, School of Environmental Biology, Curtin University of Technology, Western Australia. Each tree was given a specific code for identification purposes, and the mature seeds were harvested late October 1993 (Table 2.1). Mature seeds collected from wild trees from other growing areas of Western Australia were provided from another study (3) (Table 2.2). These seeds were stored at about 20°C before analysis.

2.2.2 Animal sources

Female mice, strain ARC(S) of six weeks of age and weighing 20 to 30g were obtained from Animal Resources Centre, Western Australia, and were fed standard laboratory diet for four weeks before use. The experimental protocol was reviewed and proved

Table 2.1 Codes of analysed *Santalum spicatum* trees in Field Trial Area (Curtin University, Perth, Western Australia)

Code	Bed ID*	Tree ID*	Date planted	Seed source
93Y02	6	52	08.81	Kalgoorlie
93Y03	6	53	08.81	Kalgoorlie
93Y08	5	100	08.81	Kalgoorlie
93Y09	7	13	04.84	Dryandra
93Y10	7	16	04.84	Dryandra
93Y11	2	106	08.81	Ex Bullock Holes
93Y12	1	5	08.81	Kalgoorlie

*ID = identification

Table 2.2 Location of *Santalum spicatum* Seed Collection Sites

Location	Collection Date	Latitude	Longitude
Dryandra	1989	32°46'	117°06'
Everade	1988	25°10'	120°00'
Kalgoorlie	1988	31°42'	121°30'
Koorda	1987	30°50'	117°29'
Marvel Loch	1989	31°30'	119°26'
Mt. Wilkinson	1988	26°48'	120°08'
Nanga	1990	26°15'	113°40'
Norseman	1984	32°28'	121°24'
Shell Beach	1990	32°12'	113°40'

by the Animal Experimentation Ethics Committee of Curtin University of Technology (approval number: N44/94).

2.2.3 Facilities and resources

Equipment and resources necessary for this study were available in the School of Pharmacy, infrared spectrometry was used at the School of Applied Chemistry and scanning electron microscopy was used at the School of Applied Physics, Curtin University of Technology. Nuclear magnetic resonance spectroscopic analysis was provided by the Department of Chemistry, University of Western Australia.

2.2.3.1 Gas chromatography

A Hewlett Packard (HP; Palo Alto, CA) 5890 series II gas chromatograph with a HP 5971 mass selective detector (GC/MS), and a DB 23 capillary column (30 m × 0.25 mm id., 0.15 μm) (J & W Scientific, Folsom, CA) were used for the analysis of fatty acid derivatives.

A HP 5790 capillary gas chromatograph with flame ionisation detection (GC/FID), and HT-5 column (SGE; 6 m × 0.53 mm id., 0.1 μm film thickness) were also used for the analysis of triacylglycerols.

2.2.3.2 High performance liquid chromatography

The instrument used consisted of a Model 501 pump (Waters Assoc., U.S.A.), a Rheodyne loop (20 μL) injector (Model 7125, U.S.A.), a Waters R401 refractive index detector (Waters Assoc., Milford, CT), a Model 3396A integrator (HP, U.S.A.), and a μBondapak^{TM} C18 column (3.9 × 300 mm, Waters, U.S.A.).

2.2.3.3 Ultraviolet/visible spectrometry

A HP Model 8452A PC ultraviolet/visible scanning spectrophotometer equipped with a HP Vectra computer Model 486/33 N was used.

2.2.3.4 Infrared spectrometry

A Bruker IFS 66 (Karlsruhre, Germany) spectrophotometer was used for infrared spectra over the range 4000-600 cm^{-1}. *Trans*-ximenynic acid methyl ester was measured as a thin film and ximenynic acid as a melted solid between NaCl plates.

2.2.3.5 Nuclear magnetic resonance spectroscopy

^{1}H and ^{13}C nuclear magnetic resonance spectra (NMR) of *trans*-ximenynic acid methyl ester in C_6D_6 solution were obtained using a Bruker (Rheinstetten, Germany) AM300 NMR spectrometer. The ^{13}C signals were obtained as composite pulse decoupled spectra.

2.2.3.6 Scanning electron microscopy

A JEOL JSM-35C (Japan) scanning electron microscope with United Scientific X-Ray detector (energy dispersive spectrometry, U.S.A.) was used for sandalwood seed mineral constituents analysis.

2.3 METHODS

2.3.1 Proximate analysis

The fruits were gathered at weekly intervals commencing at 7 days (1994) and 14 days (1993) after observation of flower opening and immediately subjected to proximate analysis. Samples of the fruit were also dissected to examine kernel development.

The fruit and seed composition studies, moisture, ash, oil, protein (Kjeldahl) and carbohydrate content (by difference), were determined following standard procedures (55, 132). Moisture was determined as "loss on drying" by drying 0.5-1.0 g samples of crushed kernel to constant weight at 105°C. Ash content was determined using the previously dried kernel samples by complete incineration in a silica crucible at a temperature not exceeding 450°C in a muffle furnace until completely free from carbon. The samples were cooled and weighed to constant weight in the crucible to provide an ash value.

Lipid content was determined using a cold solvent method as described later in section 2.3.2. The protein content of the de-oiled seed meal was determined by the standard Kjeldahl procedure, using a Kjeltec Auto 1030 Analyzer; a conversion factor of N to protein content of 6.25 was used (132). All results are based on product dry weight.

2.3.2 Oil extraction

2.3.2.1 Soxhlet extraction

Using the method of AOAC (55), seed samples of *Santalum spicatum* (approximately 2 g) were ground with a mortar and pestle and extracted with 200 mL hexane in a Soxhlet apparatus for 2 hours. The solution was dried with anhydrous sodium sulphate, and the solvent was removed in a Büchi Rotatory Evaporator at reduced pressure at 70°C to yield a viscous, pale yellow oil.

2.3.2.2 Cold solvent method

Using the method of Hara and Radin (54), seed samples of *Santalum spicatum* (approximately 0.5 g) were ground with a mortar and pestle and extracted with 5 mL isopropanol/hexane (2:3, by volume) in 10-mm × 100-mm screw-cap glass tubes rotated in a vertical plane at room temperature overnight. After extraction, the tubes were then centrifuged at 2,500 × g for phase separation. The organic solvent phase was transferred to a clean tube through an anhydrous sodium sulphate column. The residue was extracted with 5 mL of the above mixed solvent by rotation for 4 hours, followed by centrifugation. The extracts were then combined, dried over anhydrous sodium sulphate, and the solvent was evaporated under nitrogen at 70°C in a Büchi Rotatory Evaporator at reduced pressure to yield a viscous, pale yellow oil.

2.3.3 Preparation of fatty acid derivatives

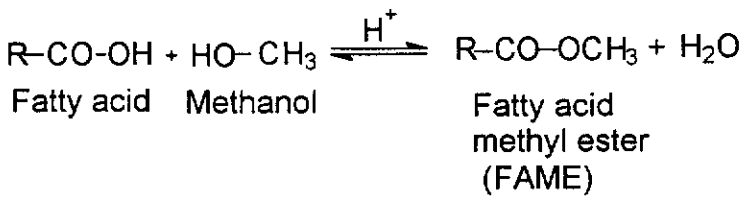
2.3.3.1 Fatty acid methyl esters

Fatty acid constituents in *Santalum spicatum* seed oil were determined as fatty acid methyl esters (FAMEs). A rapid methylation/transmethylation method was used to convert the fatty acids of the oil to their methyl esters. Following the addition of heptadecanoic acid (1 mg in 1 mL of hexane) as internal standard, the FAMEs were prepared in a mixture of toluene (2 mL) and 1% sulphuric acid in methanol (4 mL) at 50°C overnight (70). The reaction mixture was dissolved in 10 mL of hexane, and then washed twice with 10 mL of distilled water. The hexane layer was dried over anhydrous sodium sulphate then filtered to yield a hexane solution of the FAMEs. The reaction is reversible as shown in Scheme 1 (74)

Attempts were made on several occasions without real success to use the urea adduct concentration technique to enrich the polyunsaturated FAMEs of the total lipid preparation (70). This method requires the addition of urea to a hot methanol solution

of FAMEs, allowing crystals of the saturated fatty acid-urea adduct to separate out, and leaving a liquid supernatant enriched in polyunsaturated fatty acids (70).

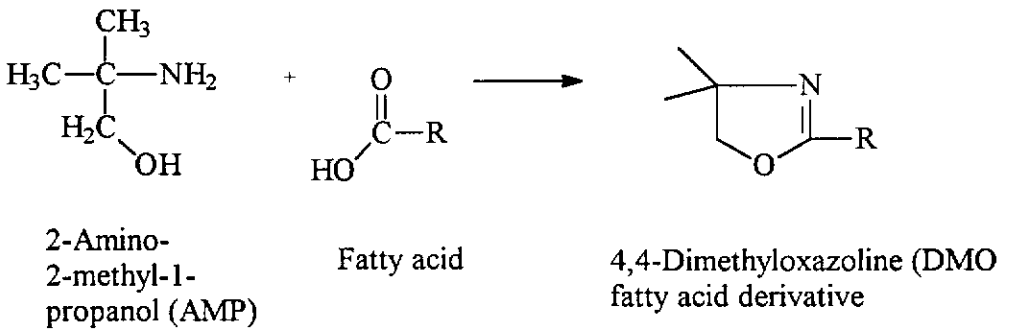
Scheme 1



2.3.3.2 4,4-Dimethyloxazoline derivative

Free fatty acids from *Santalum spicatum* seed oil preparations were then derivatised with 2-amino-2-methyl-1-propanol (AMP) to form 2-substituted 4,4-dimethyloxazoline (DMOX) according to the reaction shown in Scheme 2 (78).

Scheme 2



Two methods for the preparation of the DMOX derivatives were used. The first method required isolation of free fatty acid after saponification of the seed oil, followed by derivatisation. The seed oil (10 mg) was saponified with 1 M solution of potassium hydroxide in 95% ethanol (2 mL) at 95°C for 1 hour. After the mixture was cooled, hexane was added, and the solution was acidified with 6 M HCl to pH 1. Free fatty acids were extracted with hexane, the solution was dried over anhydrous sodium

sulphate, and evaporated at reduced pressure in a Buchi Rotatory Evaporator at 60°C to yield the free fatty acids (70).

The DMOX derivatives of the total fatty acids were prepared by heating 0.1 mL of free fatty acid solution (1 mg fatty acid dissolved in 1 mL toluene) with 0.05 mL of AMP for 2 hours at 210°C in a muffle furnace in a sealed, capped tube (78, 82).

The second method, a direct conversion of fatty acids in oil into their DMOX derivatives, was carried out by heating 4 mg of seed oil with 0.5 mL of AMP under a nitrogen atmosphere in a capped tube, for 18 hours at 180°C in a muffle furnace (133). The reaction mixture was cooled and dissolved in 5 mL of dichloromethane then washed twice with 2 mL of distilled water. The organic layer was dried over anhydrous sodium sulphate, and filtered to provide a solution suitable for direct injection into the GC.

2.3.4 Isolation and confirmation of identity of ximenynic acid

XMYA can be obtained by crystallisation from a mixture of free fatty acids in hexane at -25°C following the hydrolysis of sandalwood seed oil (13). Re-crystallisation in hexane gave relatively pure (>95%) *trans* XMYA as white flakes, melting point 38.9-39.3°C (27, 28). Melting points were determined in capillary tubes using an Electrothermal IA 900 digital melting point apparatus. We have concluded (178), on the basis of NMR (^{1}H and ^{13}C), IR and UV spectroscopy and GC/MS, that the isolated XMYA is *trans* XMYA.

2.3.5 Catalytic hydrogenation of ximenynic acid

XMYA was a recrystallised and characterised sample previously extracted from *Santalum spicatum* seed oils. Using the method of Gunstone and Russell (14), a solution of XMYA (2 g) in ethanol (30 mL) containing quinoline (0.08 g) and Lindlar's

catalyst (Pd-$CaCO_3$) (0.2 g) was shaken under hydrogen (200 mL) at room temperature until the required amount of hydrogen had been absorbed (14). The solution was evaporated to dryness, and the residue was prepared as FAMEs or DMOX derivatives for GC/MS analysis.

2.3.6 Chromatographic methods for separation and identification of lipids

2.3.6.1 Thin-layer chromatography

Dry precoated silica gel G 60 F_{254} plates (20 × 20 cm, 0.2 mm layer) were used.
A 5 mg (0.1 mL) amount of the sample of a 5% solution [in chloroform/methanol (1:1, by volume)] of *Santalum spicatum* seed oil was applied with a 10 µL disposable pipette as a line of spots 1.5 cm from the lower edge of the silica gel layer. The plate was developed using a mixture of *n*-hexane/diethyl ether/acetic acid (70:30:1, by volume) until the solvent front was 18 cm above the origin (58). After development, the silica gel G60 F_{254} plate was air-dried and the separated acetylenic fixed oil bands viewed under UV-254 light. Each band was scraped off from the areas of the chromatogram and was eluted from the silica gel using 10 mL chloroform/methanol (1:1, by volume). The extracts were evaporated under a stream of nitrogen at 50°C before further analysis. Early experiments using an iodine spray to view a shielded edge of the TLC failed to yield satisfactory results.

2.3.6.2. High performance liquid chromatography

A sample (20 µL) of a 20% solution of *Santalum spicatum* seed oil in tetrahydrofuran was injected onto a µBondapak™ C18 column. The sample components were eluted at a flow rate of 1.0 mL/min using a mixture of acetone/acetonitrile (7:3, by volume) as the mobile phase, and peaks were detected by a refractive index detector (62); this part work has been reported recently (228).

2.3.6.3 Gas chromatography with flame ionisation detection

Santalum spicatum seed oil sample solutions (2 μL) of approximately 1% (w/v) in hexane were analysed by GC/FID, using an HT-5 column. Helium was used as the carrier gas at a head pressure of 3×10^5 Pa. The injector and detector temperatures were set at 300°C and 420°C respectively, and the oven temperature was programmed from 200 to 370°C, at 10°C per min. and then maintained at 370°C for 5 minutes (70).

2.3.6.4 Gas chromatography with mass selective detector

FAMEs and DMOX derivatives were quantified by a GC/MS in a HP 5890 Series 11 GC with an HP 5971 mass selective detector. The separation was carried out on a DB 23 capillary column using the conditions described in section 2.2.3. The injection volume was 1 μL and the sample solutions were approximately 0.05% w/v in hexane, or occasionally dichloromethane. The instrument was set in scan mode with a gradient temperature program of 50°C for 5 min., then 5°C 1 min. increase to 240°C, which was maintained for a further 5 min. Helium was used as carrier gas with a head pressure of 25 Pa, and a linear velocity controlled to 47.5 cm sec^{-1} (1.40 mL min^{-1}) by an electronic pressure program with vacuum compensation.

2.3.7 Statistical methods

Statistical analysis of results were determined using analysis of variance (ANOVA) where appropriate. When the *P*-value was significant at the 0.05 level, Tukey's *t*-test was applied pairwise to detect group differences. All statistical analysis were performed using SPSS for Windows, standard version (SPSS Inc., Chicago, Illinois, U.S.A.).

2.4 ANIMAL EXPERIMENTS

2.4.1 Animals and maintenance

Young female mice were selected as experimental animals for this study based on the following criteria: 1) the mouse is the most widely used animal in biomedical research; 2) female mice can be easily maintained in groups (male mice tend to fight frequently when in groups); and 3) the use of young mice (6 weeks old) permitted analysis of the effects of dietary fat composition on tissue lipids in rapidly growing subjects (134). A high kinetic exchange of dietary fatty acids could be expected and hence analysed in different tissues.

The animals were randomly divided into four groups, three (A, B and C) each of 15 mice, and one group (D) of eight mice. In each group, mice were marked using an ear punch coding system allowing individual identification. The mice were housed per group per cage (standard laboratory cage) in a relative humidity 50% ± 5, temperature-regulated (21 ± 1°C), ventilated and light-controlled room (illuminated at 05.00 to 17.00 hours). The animals were adapted to their environment for four weeks, feed and water were given with free access. All animals were monitored on a daily basis, available water and feed quantities checked and the general health assessed. No mortality were seen during the test period. The total experimental feeding period was eight weeks.

2.4.2 Dietary treatments and tissue collection

The three required diets were modified from the American Institute Nutrition (AIN) 93 formulation (135) containing 5% (w/w) mixed feed oil (standard lab diet, group A); 15% (w/w) canola oil (group B) and 15% (w/w) sandalwood seed oil (group C), and were made up commercially (Glen Forrest Stockfeeders, Perth, Western Australia). The diets maintained nutritional densities generally similar to AIN-93 (229). The composition of diets is shown in Table 2.3. In Table 2.4 the fatty acid composition (of total fatty acids) of each dietary fats is summarized. The diets were stored in closed containers at room temperature. All of the diets were well accepted by the test animals,

however it was noted that initial feeding of sandalwood seed oil diet in group C did it cause loss of whiskers without any other apparent effects. This phenomenon is discussed late in section 6.2.2. At the end of the feeding period, the mice were fasted overnight and killed by decapitation under ether anesthesia. After decapitation, liver, heart, adipose tissue, kidney, brain, and muscles were quickly removed by dissection, washed with physiological saline, and stored in capped tubes at -20°C until the lipids were extracted. The protocol for mice dietary regime and blood sample collection is shown in Figure 2.1.

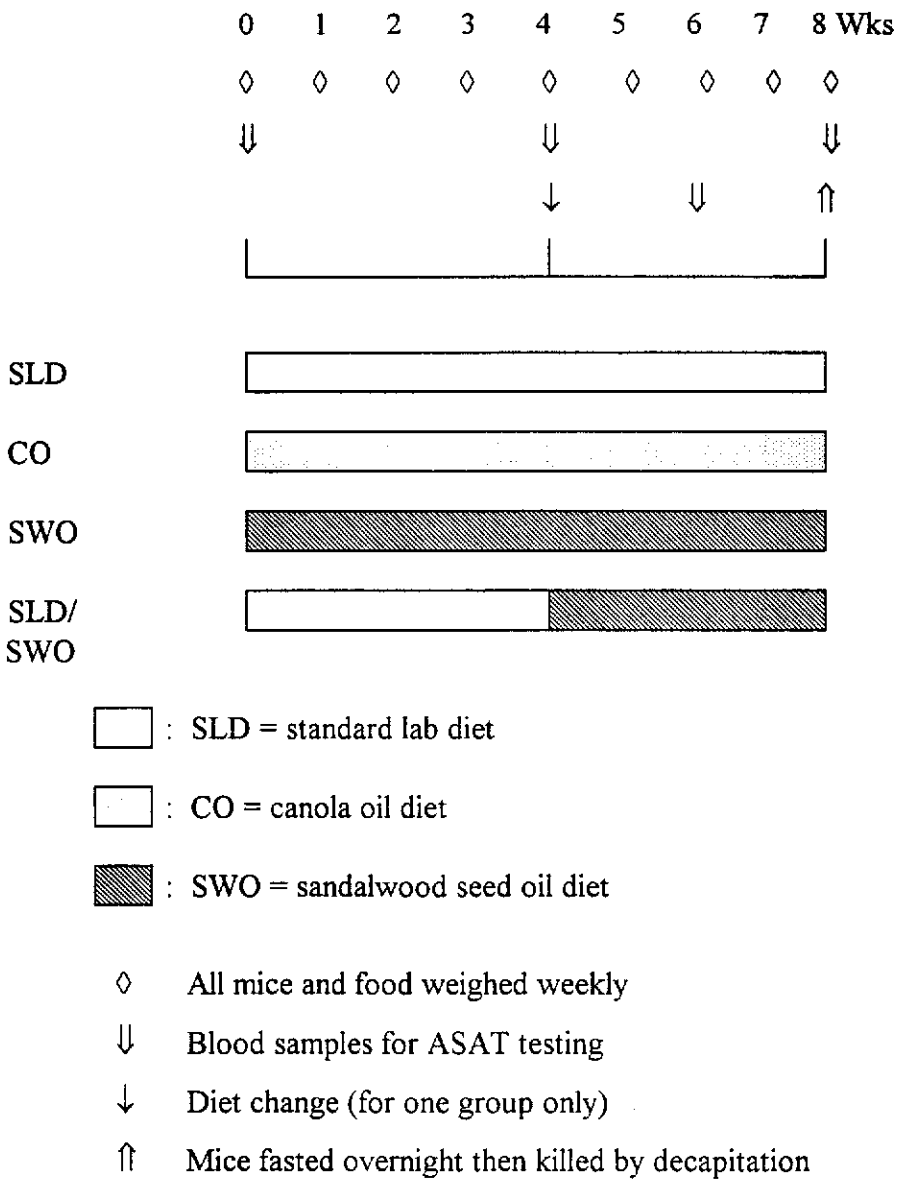


Figure 2.1 Experimental protocol for the mouse feeding study.

Table 2.3 Diet Percentage Composition (by weight of diet)

Nutrient	SLD*	CO*	SWO*
Protein	19.00	18.35	18.35
Fat	5.00	15.00	15.00
Crude Fibre	5.00	5.00	5.00
Calcium	0.78	0.50	0.50
Phosphorus	0.57	0.27	0.27
Salt [a]	0.41	0.26	0.26
Lysine	0.86	1.29	1.29
Methionine	0.31	0.68	0.68
Methionine cystine	0.56	0.73	0.73
Threonine	0.65	0.71	0.71
Tryptophane	0.21	0.24	0.24
Leucine	1.26	1.60	1.60
Isoleucine	0.75	1.04	1.04
Arginine	1.37	0.66	0.66
Histidine	1.37	0.66	0.66
Tyrosine	0.50	1.00	1.00
Phenylalanine	1.30	0.91	0.91
Valine	0.71	1.16	1.16
Digestible Energy MJ/Kg	14.47	17.85	17.85
Essential fatty acids	1.45	3.36	0.81
Vitamin mix [b]	1.00	1.00	1.00

[a]Salt	mg/kg	[b]Vitamin mix	Per kg
Copper	16.00	Vitamin A	10,000 IU
Magnesium	100.0	Vitamin B12	30.00 μg
Manganese	70.00	Vitamin D	2,000 IU
Molybdenum	0.50	Vitamin E	100.0 mg
Iodine	0.50	Vitamin K	2.00 mg
Iron	70.00	Calcium pantothenate	20.00 mg
Selenium	0.10	Folic acid	2.00 mg
Zinc	60.00	Nicotinic acid	25.00 mg
		Pyridoxine	6.00 mg
		Riboflavin	6.00 mg
		Thiamine	6.00 mg
		Viotin	100.0 μg

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil

Table 2.4 Relative Percentage of Fatty Acid in the Dietary Lipids

Fatty acid	SLD*	CO*	SWO*
14:0	1.09	nd	nd
16:0	33.73	4.16	3.53
16:1(n-7)	0.69	tr	0.15
18:0	8.09	2.38	1.92
18:1(n-9)	30.24	71.05	52.83
18:2(n-6)	24.88	18.89	1.86
18:3(n-3)	1.29	3.51	3.52
18:1(9a)	nd	nd	1.51
XMYA	nd	nd	34.68
SFA*	42.91	6.54	5.45
MUFA*	30.93	71.05	52.98
PUFA*	26.17	22.40	5.34
Acetylenic fatty acid	nd	nd	36.19

nd = not detectable; tr = trace (<0.01%)

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid;

PUFA* = polyunsaturated fatty acid

2.4.3 Aspartate aminotransferase assay

Blood (max 0.3 mL) was collected from the retro orbital venous plexus (136), as initial (week 0), middle (week 4) and final (week 8) blood samples over the 8 week period for groups A, B and C. In group D, blood was collected during weeks 4, 6 and 8 respectively as initial, middle and final samples, using a capillary pipette. After collection, the blood samples were kept at room temperature for 0.5 hour, cooled to 5°C for 1 hour and then centrifuged to separate the serum.

Aspartate aminotransferase (ASAT) activities were determined at an absorbency of 340 nm on a HP UV/visible spectrophotometer. A sensitive, high speed kinetic photometric method with aspartate aminotransferase (Boehringer Mannheim GmbH) as substrate employing only 0.2 mL diluted serum (1→10, in 0.9% NaCl) was used for the rapid determination of a large number of samples. The procedure is to pipette 0.2 mL diluted serum sample into ASAT solution (25°C), mix, then pour the mixture solution into a cuvette. After *ca.* 1 min, read initial absorbance and start stopwatch at the same time. The reading was repeated after exactly 1, 2 and 3 min. The mean absorbance change per minute (ΔA/min) is thus determined, and was used for the calculation. Each blood sample was analysed in triplicate. The UV absorption spectrum of a typical reaction mixture is shown in Figure 2.2, and a typical plot of changing UV absorption is shown in Figure 2.3.

Test principle:

The test principle is shown in the following reactions (page 47):

In this reaction, the oxidised (NAD) and reduced (NADH) forms have different spectrophotometric properties, NADH exhibits a broad absorption band at 340 nm, which is entirely absent from NAD^+. Thus the reduction or oxidation of NAD is relatively easy to monitor by the change in light absorption at 340 nm (137).

λ max NADH (pH 10.0) = 340 nm (E = 6200, ie. 1 mole in 1 litre corresponds to absorbency of 6200, when the light path is 1 cm).

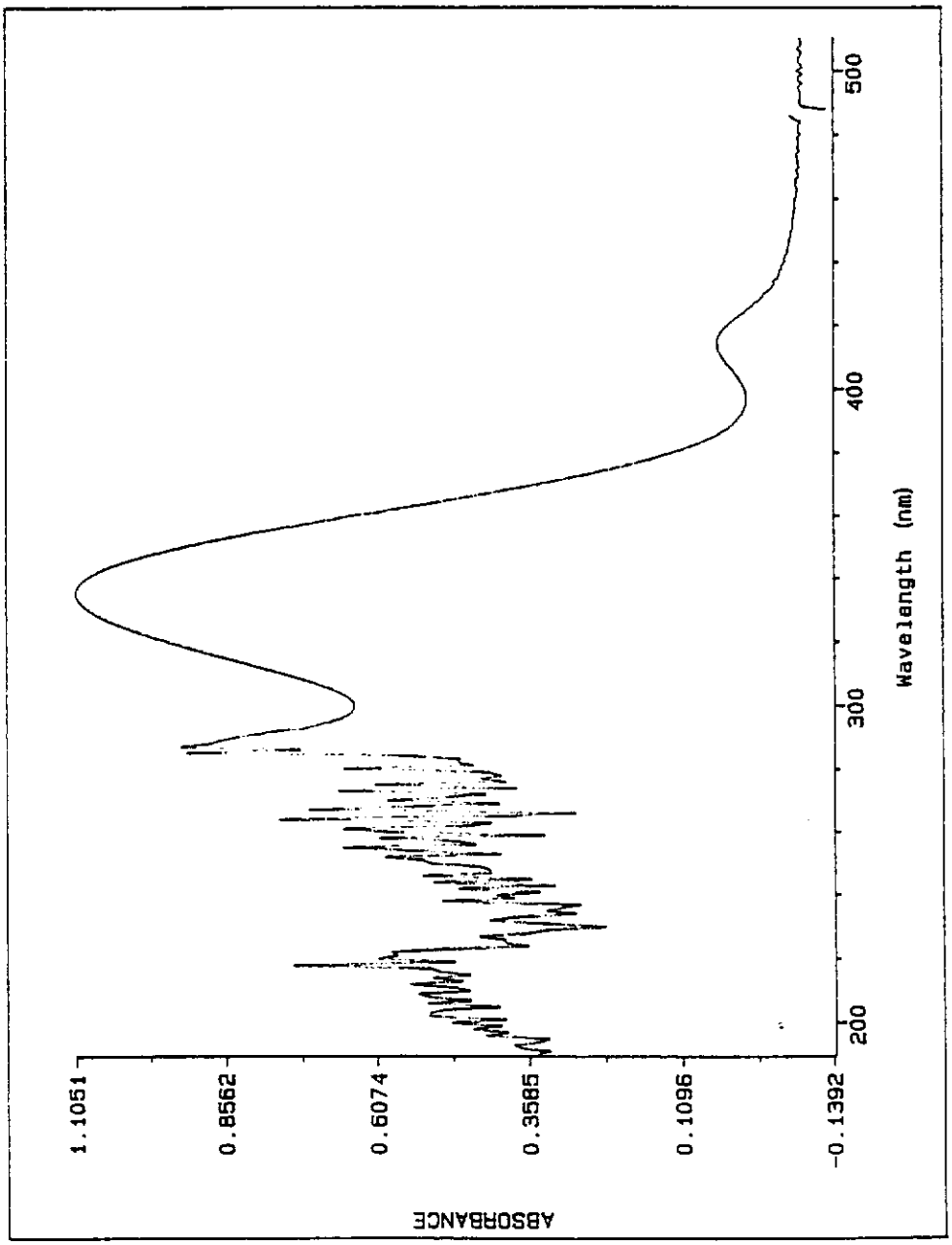


Figure 2.2 The UV absorption spectrum of mouse aspartate aminotransferase reaction mixture.

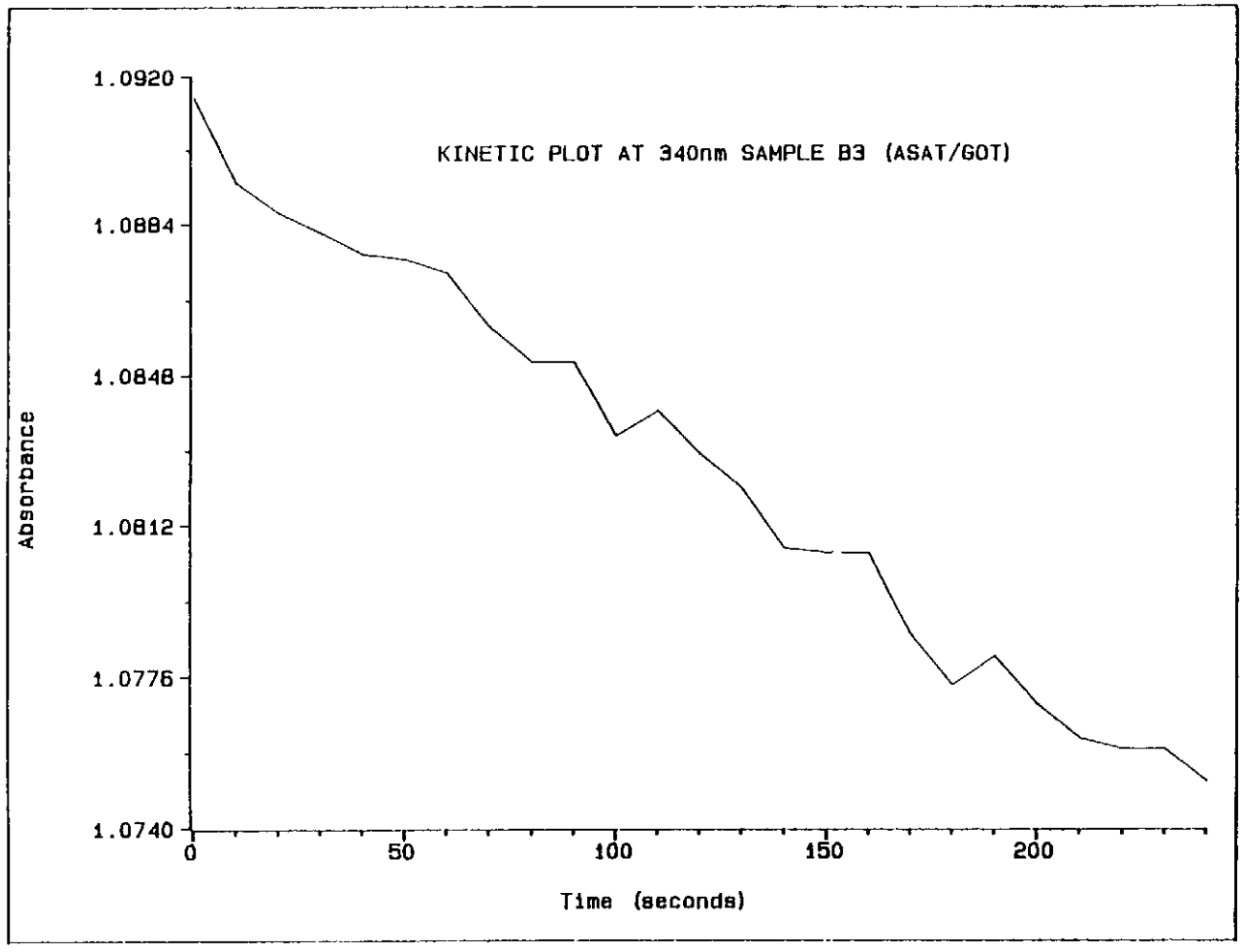
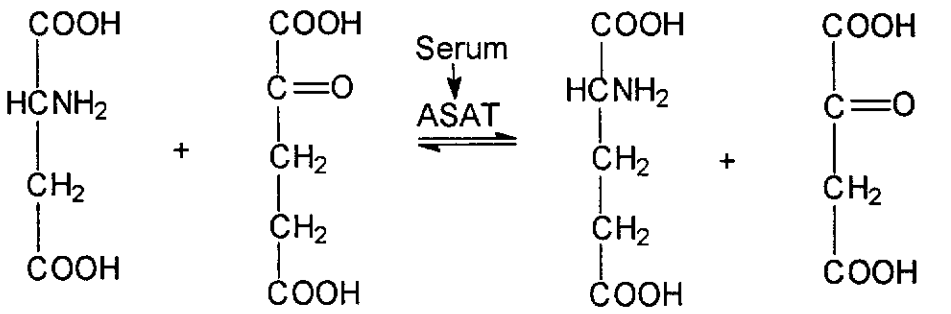
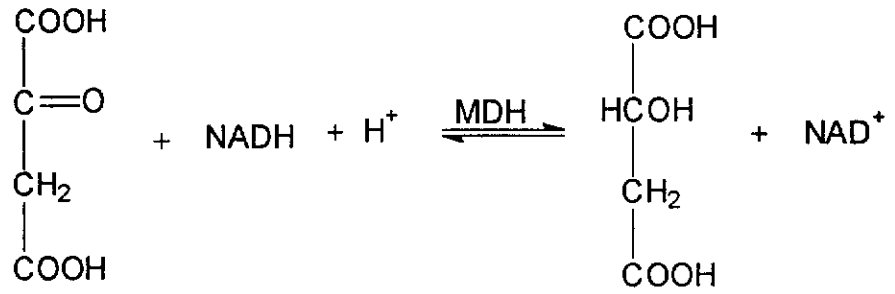


Figure 2.3 A kinetic plot of mouse aspartate aminotransferase reaction mixture at 340 nm.



L-aspartic acid | α-ketoglutaric acid | L-glutamic acid | oxalacetic acid



oxalacetic acid | malic acid

Calculation:

The activity of ASAT at 25°C in the sample may be calculated as follows:

$$U/l = \frac{T.V. \times 1000 \times \Delta\ A/min}{mMabsorptivity \times S.V. \times}$$

mM absorptivity = 6.2 at 340 nm

P = light path (1 cm)

S.V. = Sample volume (0.17 mL dilute); T.V. = Total assay volume (1.17 mL)

Therefore, the calculation for ASAT activity becomes:

$$U/l = \frac{1.17 \times 1000 \times \Delta\ A/min}{6.2 \times 0.17 \times 1}$$

$$= 1110 \times \Delta A/min \text{ (for diluted sample, results} \times 10)$$

2.4.4 Lipid analysis

Total lipid was extracted from the sample of the homogenised organ using the method of Bligh *et al.* (53). The tissues of the sample (weight approximately 0.2-0.5 g) were homogenised and extracted for 4h with 4.0 mL of dichloromethane/methanol/water (1:2:0.8, by volume) in 10-mm × 100-mm screw-cap glass tubes. After extraction, 1mL of dichloromethane, 0.9 mL of water, and 0.5 mL of a mixture containing 2 M KCl and 0.1 M HCl were added and the tubes were rotated in a vertical plane for 30 min., and then centrifuged at 2,500 × g to facilitate phase separation. The upper, aqueous phase was aspirated and discarded, and the lower dichloromethane phase was transferred to a clean tube through an anhydrous sodium sulphate column.
The tissue residue was extracted twice with dichloromethane (2 mL) by brief rotation followed by centrifugation. The dichloromethane extracts were combined and then evaporated under a stream of nitrogen at 50°C.

For lipid analysis, the dietary oils and the total lipids of various tissues were converted to FAMEs (70), and also the DMOX derivatives using the one step conversion method (133), as previously described in section 2.3.3. The FAMEs and DMOX derivatives were quantified by GC/MS as described in section 2.3.6.4.

2.4.5 Histopathological examination by visible light microscopy

After fixation in buffered neutral formalin, samples were routinely processed (138):

Cryostat sections: Small pieces of fresh tissues were quenched in dry ice at -70°C. Cryostat sections of 10 μm were cut on a AO Spencer Cryostat and received on gelatinised slides. The slides were placed in a Coplin jar containing formalin vapour for 5 min. to fix the sections permanently to the slide.

Haematoxylin and Eosin (H&E) staining: This is the routine method to demonstrate biological structure. The sections were stained in Harris's Haematoxylin, differentiated in acid alcohol and blued in Scott's Water. They were then counterstained in alcoholic Eosin, dehydrated, cleared and mounted in a resinous media (DPX).

Presence of lipid: Lipid is soluble in fat solvents such as xylols which are used after H&E staining. Any lipids (neutral and ester lipids) are removed from the sections leaving clear circular spaces in the tissues, which can be defined under light microscopy.

CHAPTER 3: FATTY ACID ANALYSIS

3.1 INTRODUCTION

Oleic acid and XMYA were found to be major fatty acids of mature *Santalum spicatum* seed. This chapter describes the GC/MS of DMOX derivatives to the structure determination of fatty acids from the seeds oil and the seed oil-fed mice tissues.

3.1.1 Derivatives of fatty acids

The analysis of fatty acid present in various lipids such as vegetable oils, fish oils, dairy and meat fats traditionally require conversion into fatty acid methyl esters (FAMEs) or alkyl 4,4-dimethyloxazolines (DMOX) derivatives for gas chromatographic (GC) analysis (70, 78). The use of GC to characterise fatty acid profiles of lipids has been the most commonly accepted analysis in the laboratory (70). In addition, GC/MS and associated electronic library system, make this method extremely powerful in providing analysis and identification of fatty acids (139).

The procedure of fatty acid analysis involves two steps: first is the preparation of the fatty acid derivatives, and second is the analysis of the derivatives by GC/MS. Since most lipids are conventional and can be directly transesterified to FAME by either base-catalysed methods ($NaOCH_3$, KOH and NaOH) or acid-catalysed methods (HCl, H_2SO_4 and BF_3) (71-73), there is no need to obtain the free fatty acid before preparing FAME. Both methods are acceptable as esterification procedures.

DMOX derivatisation is a relatively new method in fatty acid analysis (78, 133). It is also a powerful technique for structural elucidation (78-81).

3.1.2 Electron ionisation of fatty acid derivatives

Electron ionisation of saturated straight chain FAME gives similar ion yields for all FAME from medium- to long-chain acids (139). The mass spectra of methyl esters of those fatty acids produce a prominent molecular ion together with the most abundant ion at *m/z* 74, the diagnostic ion of FAME which is formed from the McLafferty rearrangement as shown in Figure 3.1.

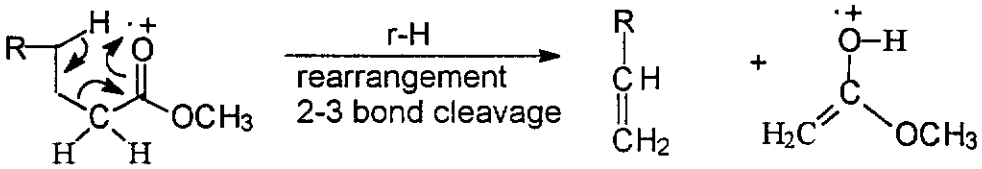
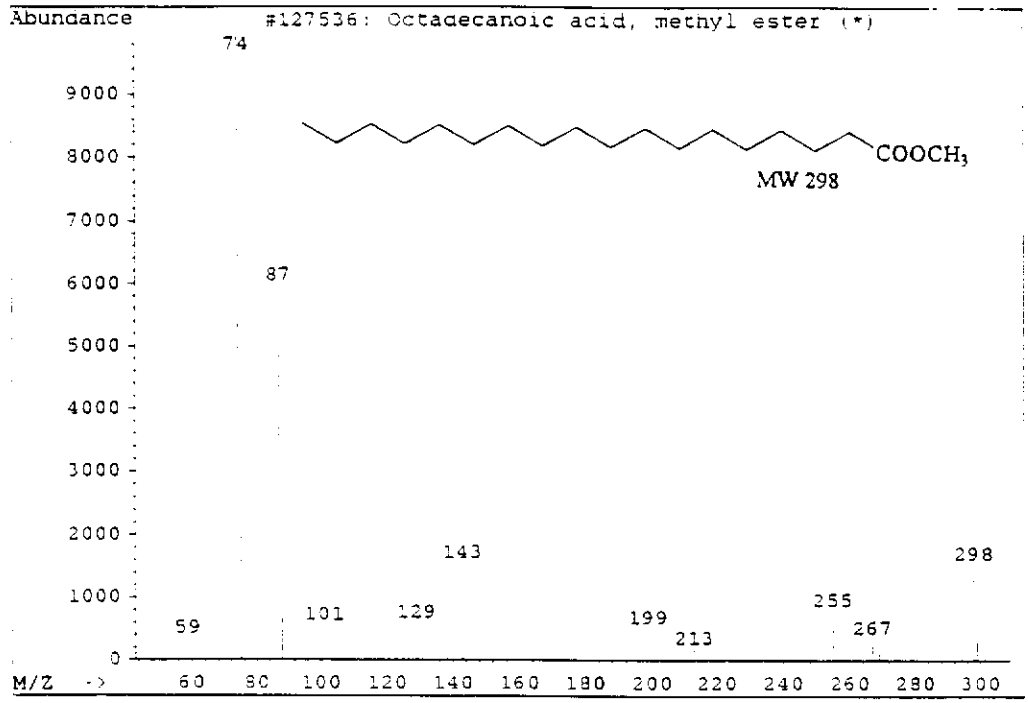


Figure 3.1 McLafferty rearrangement of fatty acid methyl ester. Adapted from reference (75).

The molecular ion is a valuable feature of the electron ionisation mass spectra of methyl esters, and other abundant ions may also provide useful information. However, if there are any additional functional groups such as double bonds, or hydroxyl groups in the fatty acyl chain, they will influence the fragmentation of FAME (139). The presence of unsaturation may be inferred under electron ionisation conditions on the basis of the mass of the molecular ion, and migration of double (or triple) bond along alkyl chain which occurs (139). However, these spectra provide little information to indicate the position of double or triple bonds (Figure 3.2).

To overcome this problem of structure assignment, a number of derivatives of fatty acid have been proposed for use in locating double bonds by GC/MS. Harvey (1982) investigated the picolinyl esters of fatty acid (77). In 1988, Zhang and his group first published information on the DMOX derivatives of fatty acid (78). Generally, picolinyl esters and DMOX derivatives are now recognised as the most useful derivatives (140).



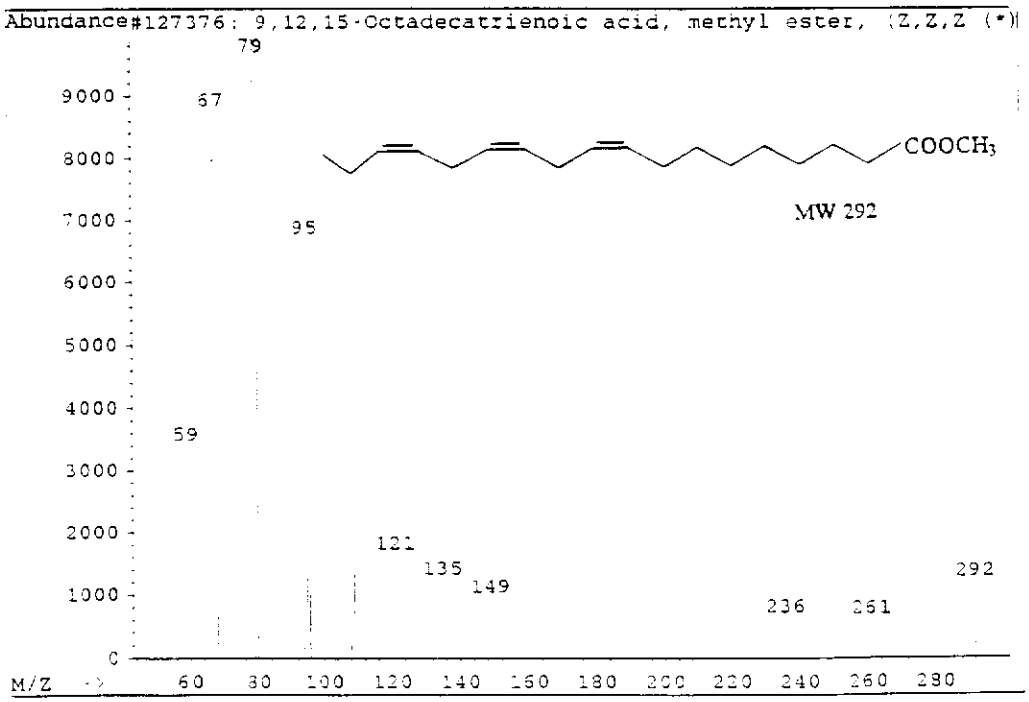
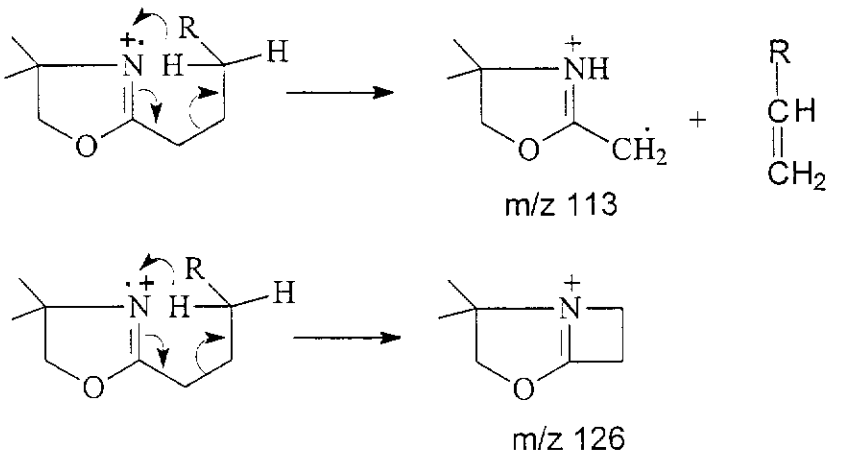


Figure 3.2. Electron ionization (70 ev) mass spectra of: A) methyl stearate, B) methyl linolenate.

Electron ionisation mass spectra of these derivatives are characterised by a molecular ion and a series of cluster ions, which display a fragmentation pattern produced by sequential radical-induced cleavage of each carbon-carbon bond from the terminal part of the fatty acid chain (70, 77-78, 139). Importantly, migration of double bonds does not occur during ionization of these derivatives. Murphy indicated that the proposed mechanism involves the initial ionisation of the molecule at the oxazole nitrogen. This then directs interaction with each carbon atom in the alkyl chain, resulting in a radical site-initiated cleavage (139).

The mass spectra of DMOX derivatives include characteristic fragments at *m/z* 113 and 126 (base peak), which are formed by a McLafferty rearrangement (*m/z* at 113) and cyclization-displacement reactions (*m/z* at 126) together with even-mass homologous series of ions at *m/z* 126 + (14)n as shown in Scheme 3.1 (78).

Scheme 3.1.



The mass spectra of picolinyl esters are similar to those of DMOX derivatives. There are intense ions at *m/z* 92 and 108 (base peak), and an ion from a McLafferty rearrangement at *m/z* 151 together with an even-mass homologous series of ions at *m/z* 164 + (14)n (77). Both picolinyl esters and DMOX derivatives give more abundant high mass diagnostic ions, however the DMOX derivatives are preferred for certain conjugated fatty acid, acetylenic fatty acid, and fatty acid with three or more double bonds, when picolinyl esters failed to give satisfactory mass spectra (140). The electron impact mass spectra of the DMOX derivatives of 18:0 and 18:1(n-9) from the present study are shown in Figure 3.3.

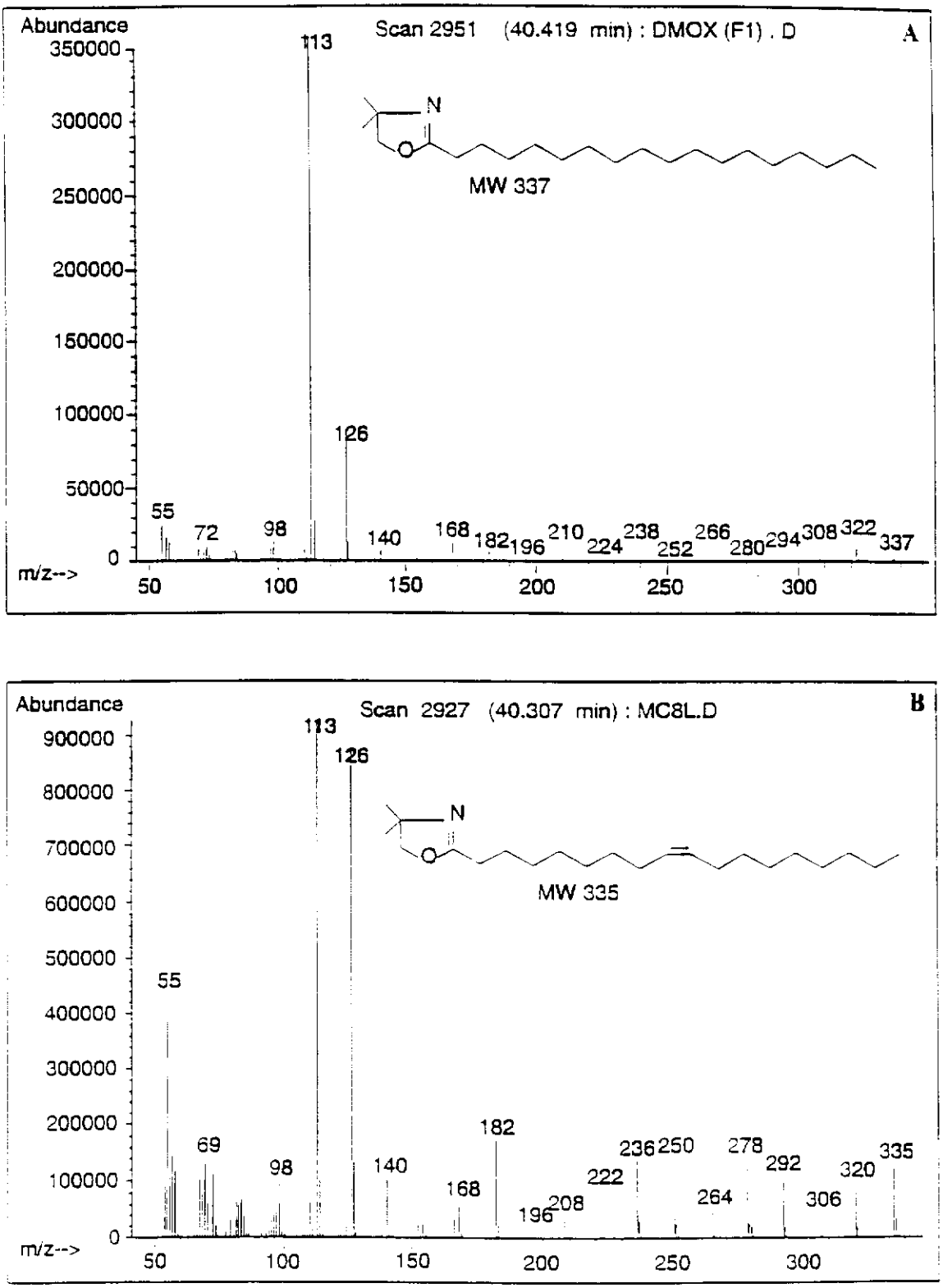


Figure 3.3 Mass spectrum of 2-substituted -4,4-dimethyloxazoline derivatives from A) stearic acid and B) oleic acid.

On electron impact, it appears that DMOX derivatives typically fragment to produce a series of ions as a result of successive cleavage of carbon-carbon bonds (79). The most pronounced ion is the even-mass homologous ion series at m/z 126 + (14)n regularised with peaks derived from cleavage at each bond (78), as described in Scheme 3.1 (only shows one of them at *m/z* 126). In contrast to the mass spectrum of saturated fatty acid, the unsaturated fatty acid such as 18:1(n-9) exhibited a mass interval of 12 amu instead of the usual 14 amu for a saturated chain between those at m/z 208 (C9) and 196 (C8), indicating a double bond at C9 (78) (Figure 3.3). The position of the double bond (s) are usually easy to interpret by recognising gaps of 12 amu.

For the spectra of acetylenic fatty acids, a rule has been formulated that a triple bond is indicated at carbon n by a mass interval of 10 amu between fragments containing *n-2* and *n-1* carbons of the acyl chain, and is further confirmed by intense peaks at carbon *n-2* and *n+2* (64). For example, five acetylenic acids with isolated or conjugated double bonds, have been thus identified in the seed kernel oil of *Pyrularia edulis* (79). More recently, both *cis-* and *trans-* isomers of XMYA were identified in the seed oil of *Curupira tefeensis* by their DMOX derivatives (45).

3.2 RESULTS AND DISCUSSION

3.2.1 Identification of ximenynic acid

This section describes the separation and identification of FAME and DMOX derivatives of fatty acids in the seed oil of *Santalum spicatum*. A typical gas chromatography total ion chromatogram (TIC) of FAME is shown in Figure 3.4. During the FAME analysis of the *Santalum spicatum* seed oil, some samples displayed an unknown peak which had the same mass spectrum as ximenynic (XMYA) (*trans-*) methyl ester. It was determined that the peak represented the less common *cis*-isomer of ximenynic acid which had been detected in other plant species such as in the seed oils of *Ongokea gore* Engler (isano) (141). (141). The identity of this fatty acid was

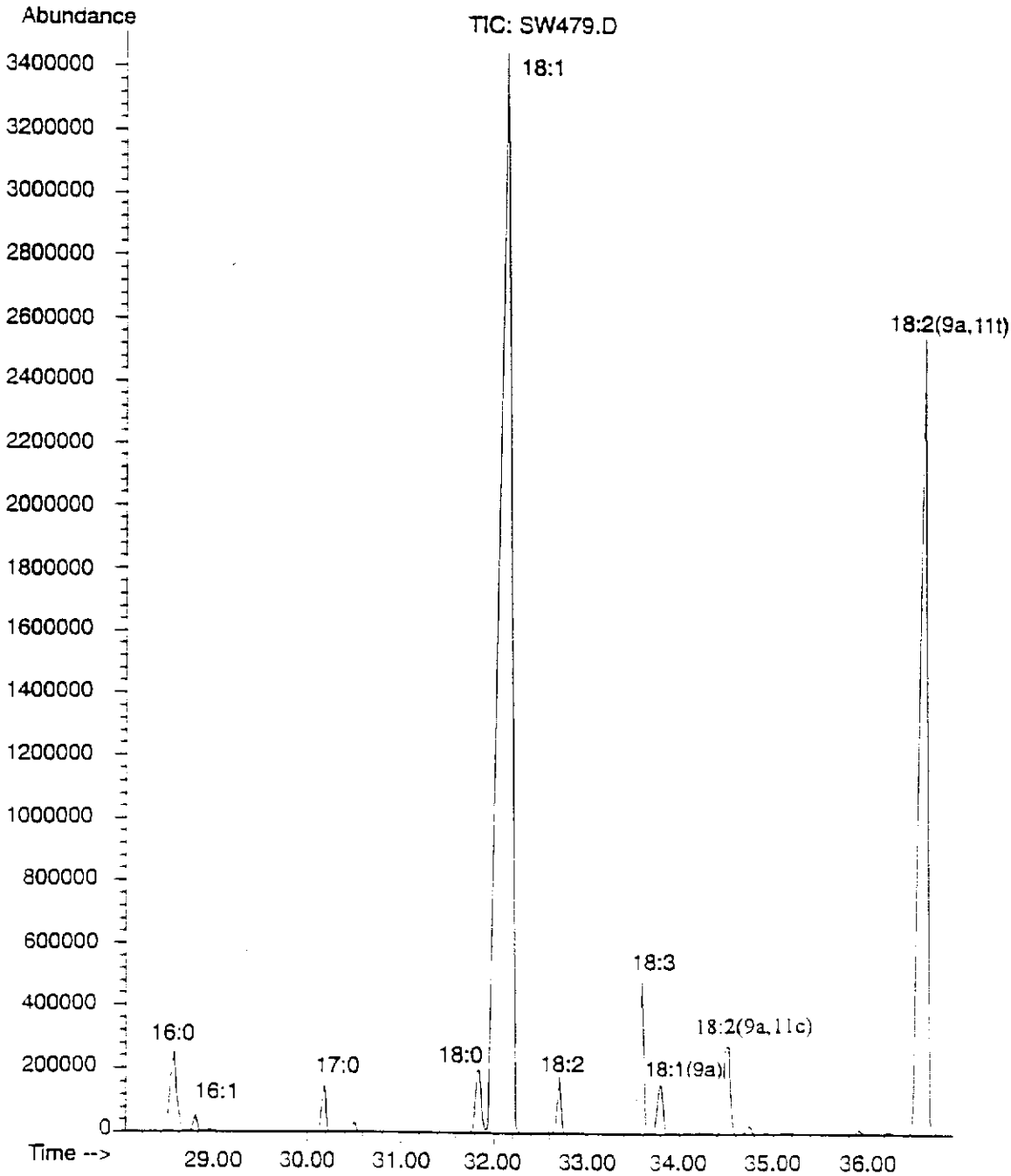


Figure 3.4 Gas chromatography (GC) of the fatty acid methyl esters of *Santalum spicatum* seed oil on a DB 23 column. GC conditions as described on page 39.

made using the DMOX derivatives to enable a precise location of double and triple bonds (78).

A previous study by Vickery *et al.* on a sandalwood seed oil derived from *Santalum obtusifolium* had identified *trans*-XMYA (71.5%) methyl ester according to the diagnostic ion at *m/z* 79 (94%) in the MS together with the UV spectrum, IR spectrum and ^{1}H- and ^{13}C-NMR spectra (142). In the present study, the question also arose about the configuration of the major XMYA present in sandalwood (*Santalum spicatum*) seed oil. In order to solve this problem, XMYA was obtained by crystallisation from hexane after the steps of alkaline hydrolysis of the seed oil (13). Re-crystallisation from hexane gave apparently XMYA as white flakes, melting point 38.9-39.3°C [38.5-39.5°C, (14)]. The UV spectral analysis of XMYA in hexane showed a λ_{max} at 229 nm (Figure 3.5), which is indicative of a conjugated system and is in good agreement with the value obtained by previous researchers (14, 142). The IR spectrum indicated an intense band for large CH_2 stretch at 2928 cm^{-1} (alkyl chain), and bands for an unsaturated =CH- at 3019 cm^{-1}, a central acetylenic group at 2216 cm^{-1}, C=O at 1742 cm^{-1} (ester) and O-Me at 2856 cm^{-1} (142). The IR absorption at 955 cm^{-1} of XMYA methyl ester and 956 cm^{-1} of the free acid provided confirmation of the *trans* double bond present in a conjugated system (142-143) (Figure 3.6). The structure of XMYA methyl ester was elucidated from high field ^{1}H-NMR and ^{13}C-NMR data. The ^{1}H-NMR spectrum of XMYA methyl ester in C_6D_6 (Figure 3.7) showed a high degree of agreement to that previously reported (142). In particular the spectrum showed a complex doublet centred at 5.56 ppm, J 15.7 Hz and a doublet of triplet centred at 6.14 ppm, J_1 15.7 H, J_2 7.1 Hz, which can be assigned to the *trans*-alkenic protons of a conjugated enyne system C11 (142, 144). The ^{13}C-NMR spectrum also showed a high degree of agreement to details previously reported and exhibited singlets at 80.06 and 88.89 ppm which were assigned to the acetylenic carbon atoms, C9 and C10, and at 110.91 and 143.11 ppm which were assigned to the olefinic carbon atoms, C11 and C12 (142, 145) (Figure 5.8).

It was thus concluded that the major XMYA isolated from the sample of *Santalum spicatum* seed oil was of the *trans* configuration (42).

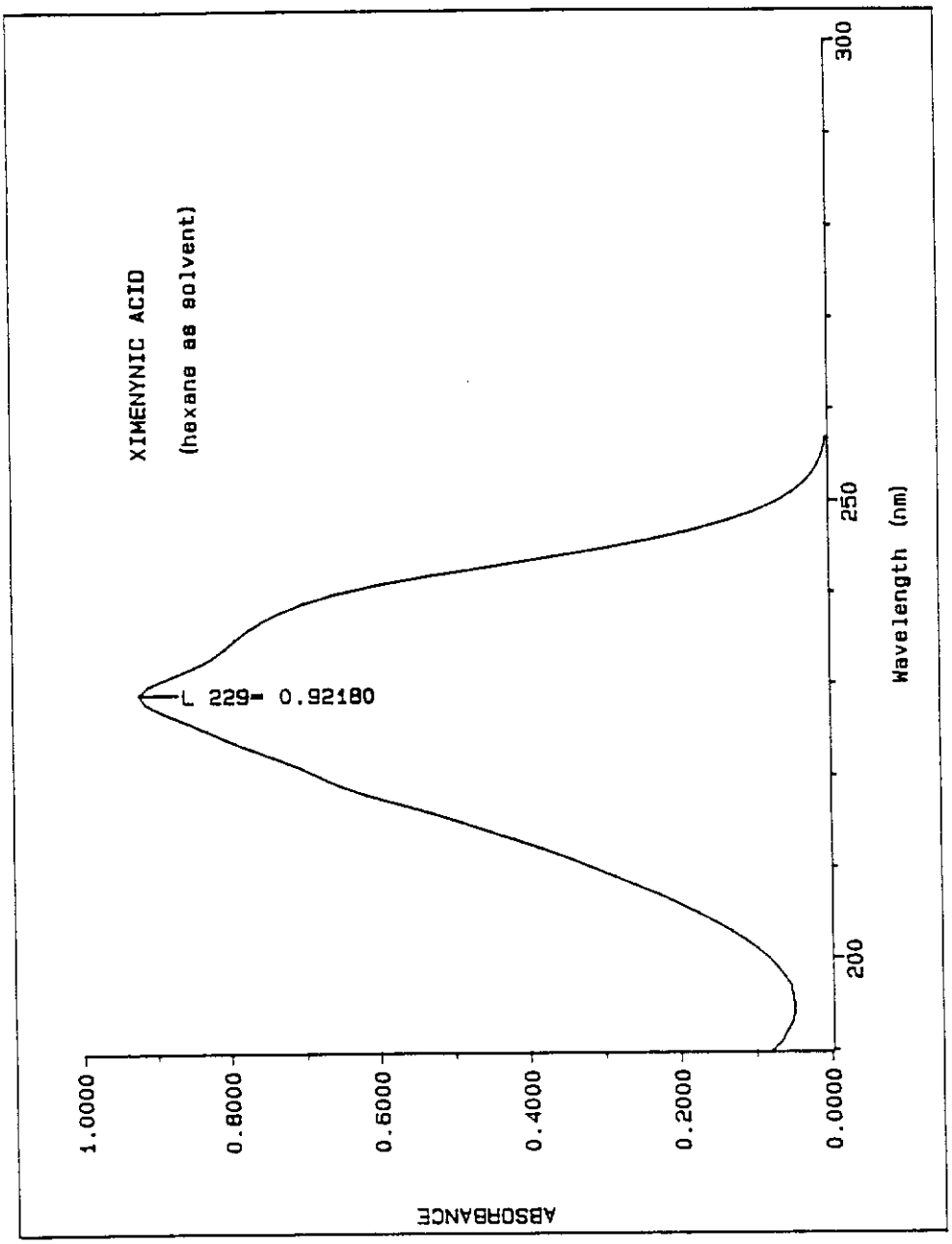


Figure 3.5 UV absorbance of ximenynic acid.

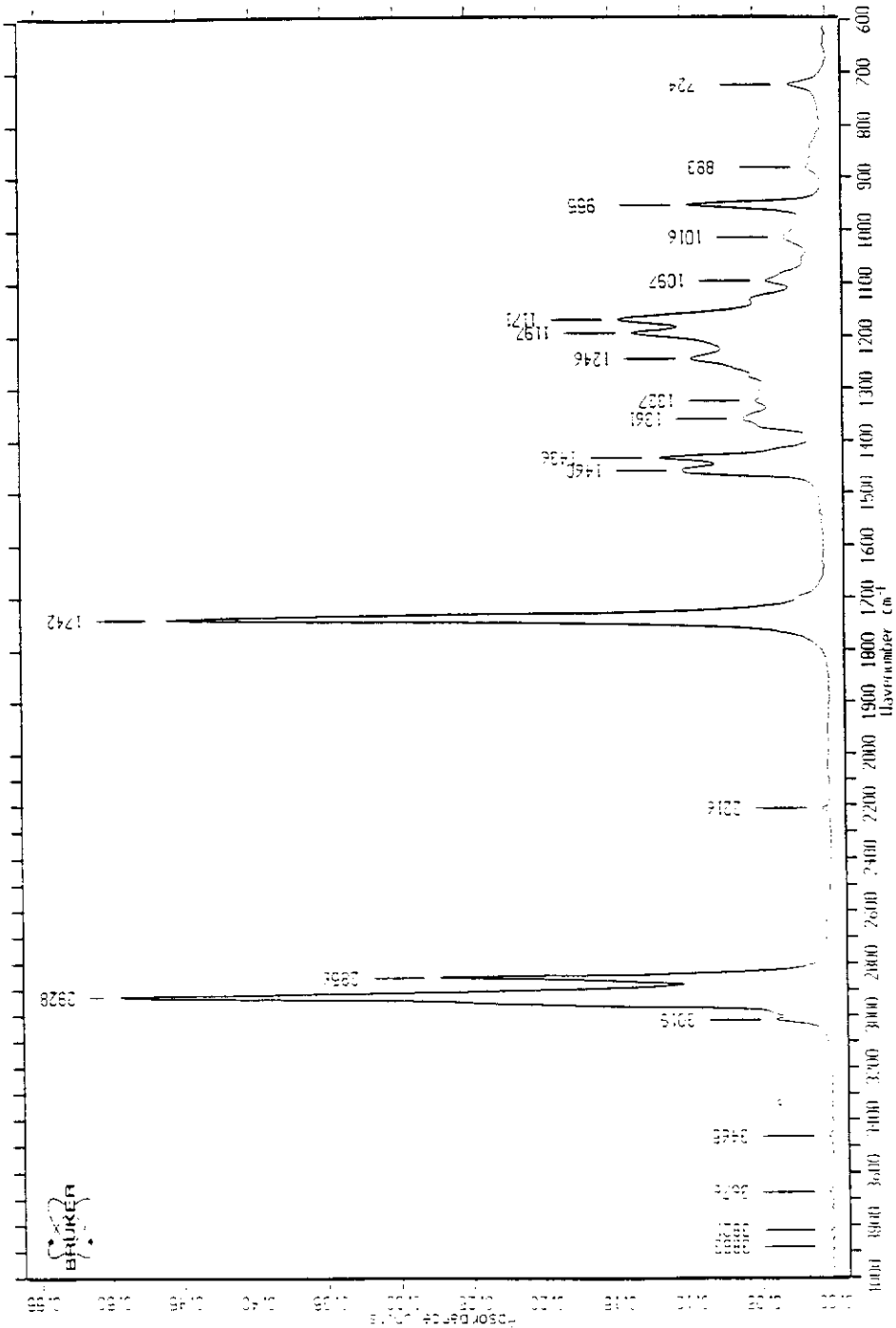


Figure 3.6 IR spectrum of ximenynic acid methyl ester (thin film).

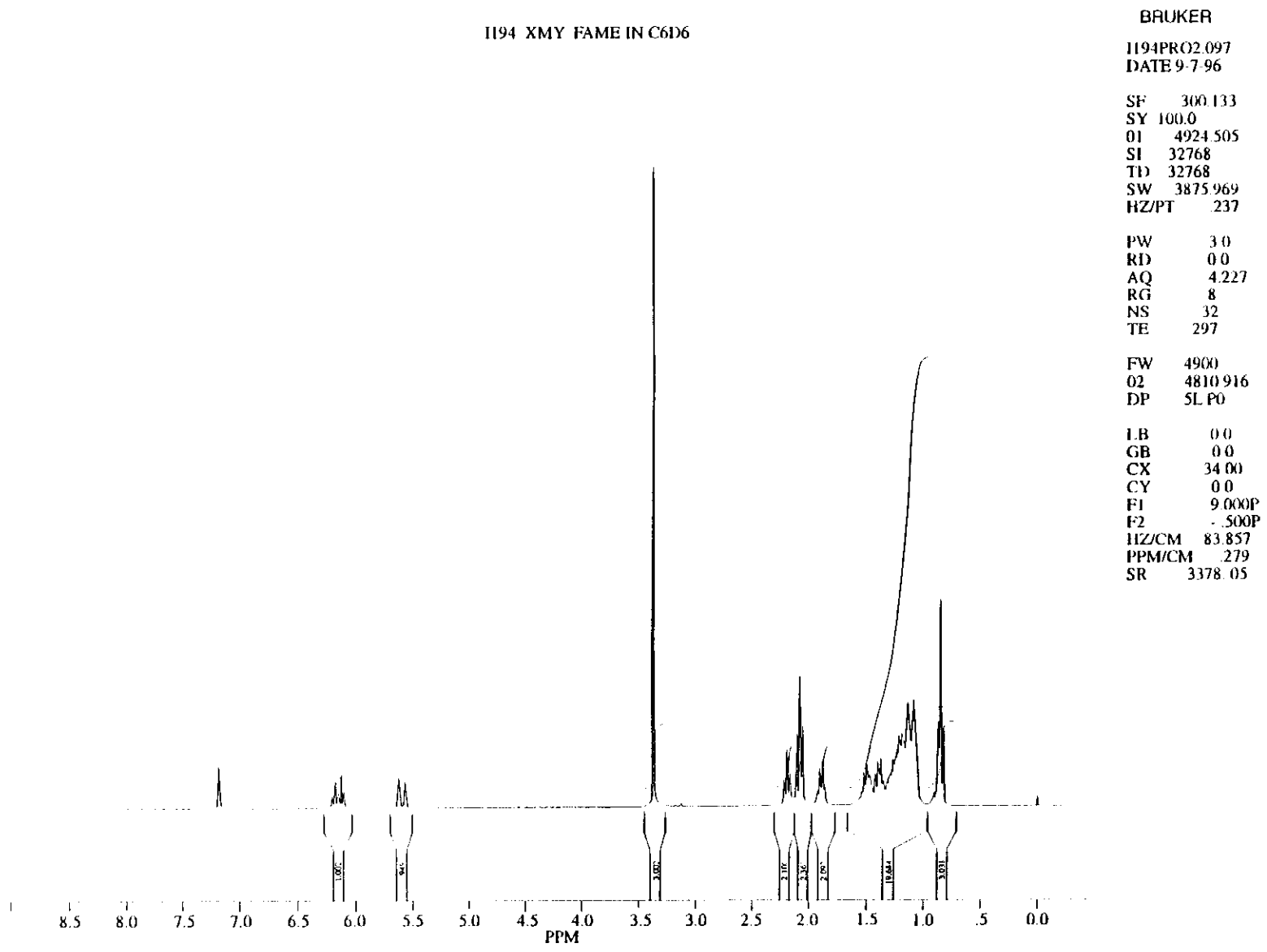


Figure 3.7 The ^{1}H-NMR spectrum of *trans* ximenynic acid methyl ester.

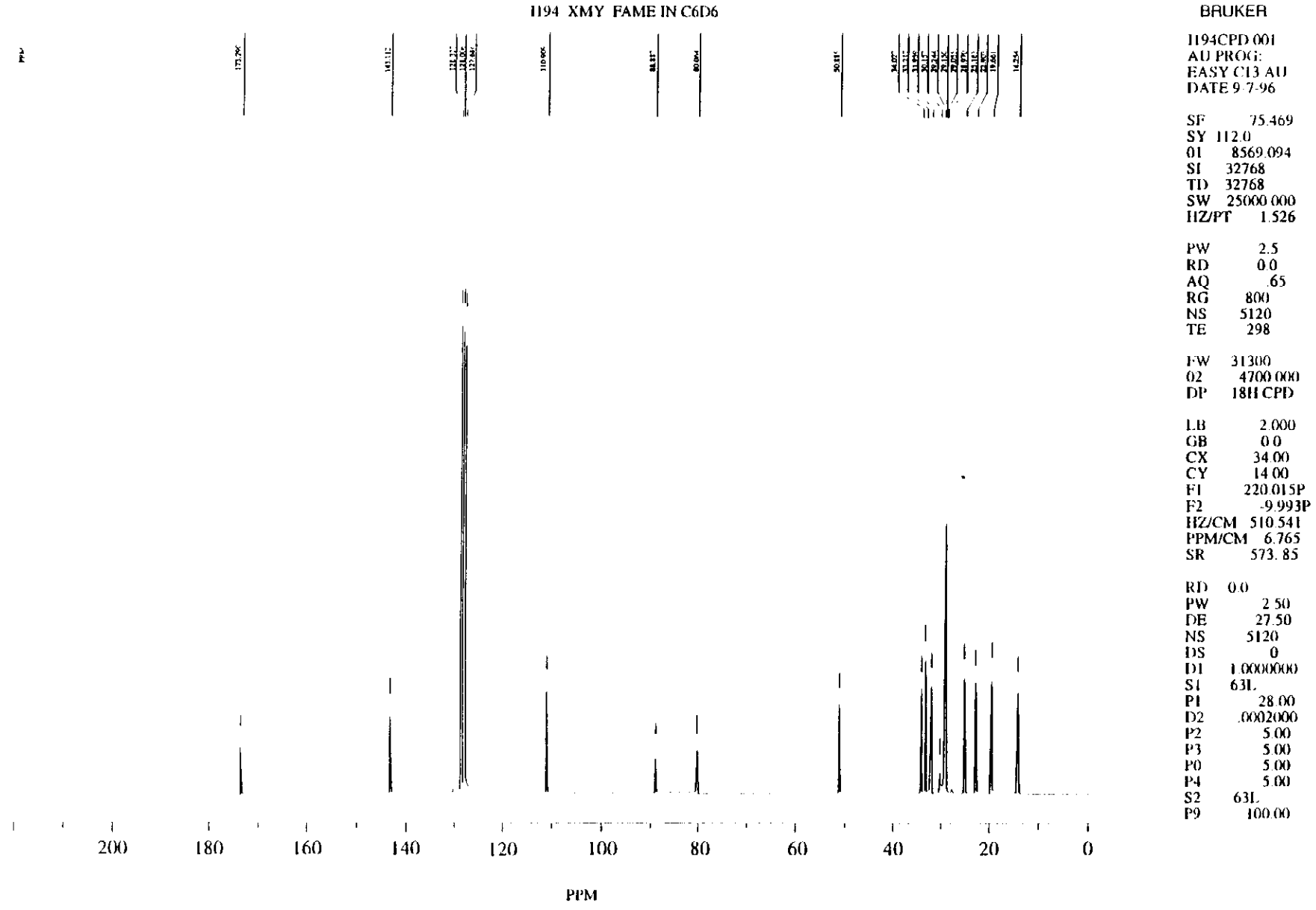


Figure 3.8 The ^{13}C-NMR spectrum of *trans* ximenynic acid methyl ester.

3.2.2 Fatty acid methyl esters of sandalwood seed oil

A typical gas chromatogram of *Santalum spicatum* FAME obtained using a capillary DB 23 column showed an excellent separation, as shown in Figure 3.4. The identity of the GC peaks of the FAME fraction was based on a consideration of their retention time relative to a 17:0 internal standard and was confirmed by examination of their electron ionisation mass spectra in conjunction with the electronic library assignment of peak identification.

The oleic acid peak occurs at retention time 32.39 min. (page 56) under the running conditions described previously (page 39), and shows a molecular ion (M^+) at *m/z* 296 (calculated $C_{19}H_{36}O_2$), and a peak *m/z* 264 (M-32) originates from loss of methanol from the molecular ion. Other typical peaks occur at *m/z* 222 $(M-74)^+$ and *m/z* 180 $(M-116)^+$. The characteristic ion at *m/z* = 74 is only represented in moderate abundance, and the fragment at *m/z* = 55 is the base peak.

In contrast, the methyl ester of XMYA at retention time 36.65 min (34%) displays a complex mass spectrum, as shown in Figure 3.9. In addition to the molecular ion at *m/z* 292, consistent with the molecular formula $C_{19}H_{32}O_2$, and minor, almost insignificant fragments at m/z 261 $(M-31)^+$ and *m/z* 221 $(M-71)^+$, there were additional, fragments at *m/z* 164 $[H\text{-}(CH{=}CH)_2\text{-}CH{=}CH\text{-}(CH_2)_5CH_3]^+$ and *m/z* 150 $[CH_2{=}C{=}CH\text{-}CH{=}CH\text{-}(CH_2)_5CH_3]^+$ indicative of a C18 eneyne structure with the triple bond between C9 and C10 (146). The most intense ion was at *m/z* 79 $(C_6H_7)^+$ which indicated that there were more double or triple bonds in the long chain (139). It was shown by a previous study that in the case of stearolic methyl ester [M^+ 294], the mass spectrum produced ions at m/z 166 $[H_2C{=}CH\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}CH_3]^+$ and 152 $[H_2C{=}C{=}CH\text{-}(CH_2)_7\text{-}CH_3]^+$ which are two characteristic ions arising from alkyl chain containing a triple bond (146). Similarly, the mass spectrum of methyl crepenynate shows a prominent peak at *m/z* 236 identified as $[H_3COOC\text{-}(CH_2)_7\text{-}CH{=}CH{=}CH_2\text{-}CH{=}C{=}CH_2]^-$ and the most abundant ion is at *m/z* 110 representing the hydrocarbon fragment of the 12-octadecynoate as$[H_3C\text{-}(CH_2)_4\text{-}CH{=}C{=}CH_2]^+$. Thus, the major peak at retention time 36.65 min. gave a mass spectrum consistent with that expected for *trans* XMYA methyl ester (42).

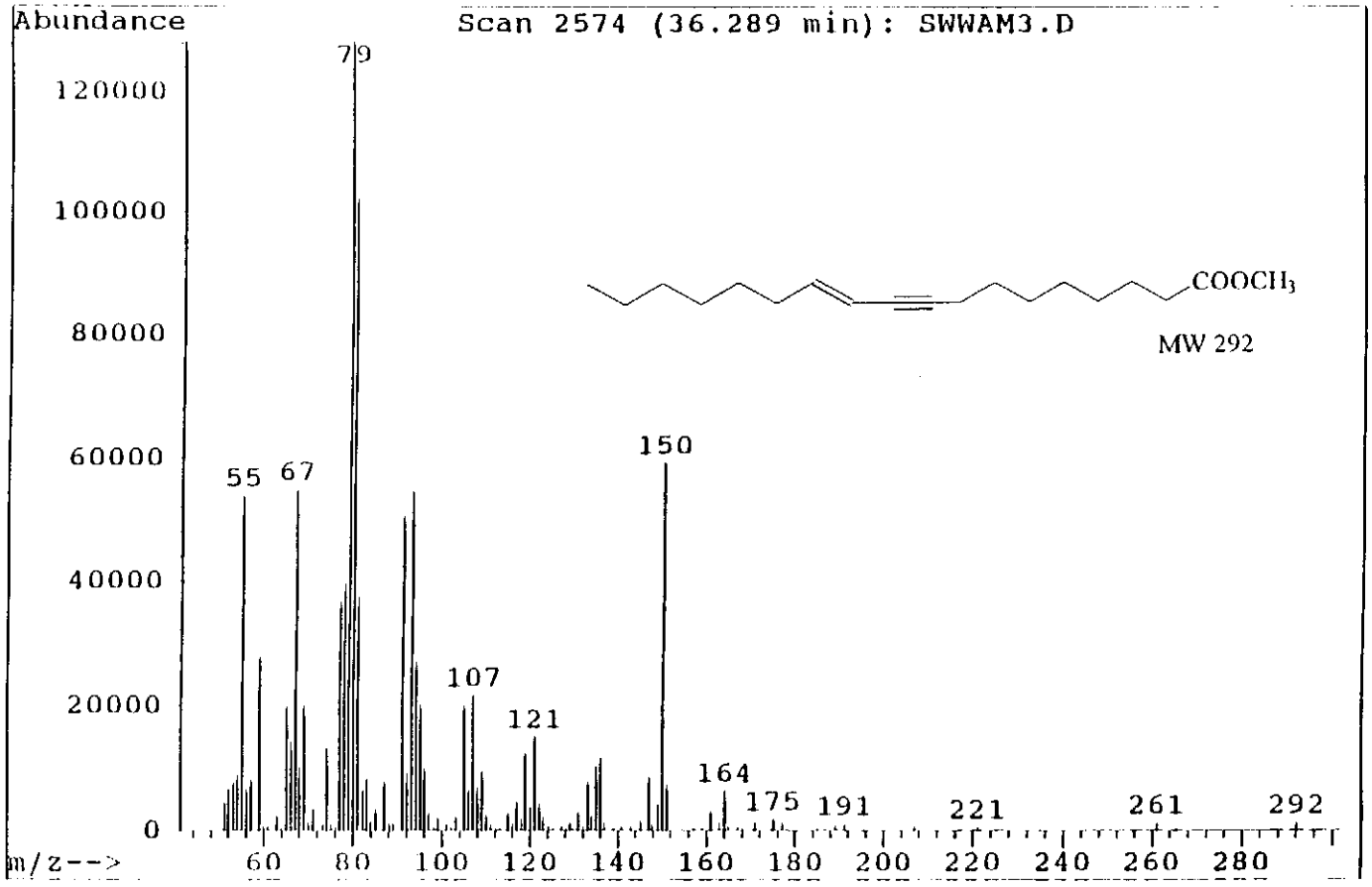


Figure 3.9 Electron ionisation (70 ev) mass spectrum of *trans* ximenynic acid methyl ester.

A minor peak at retention time 34.60 min. in the GC of *Santalum spicatum* seed oil gave a mass spectrum similar to that of *trans*-XMYA methyl ester confirming its general identity as an isomer of XMYA. Inspection of the mass spectrum revealed the base peak at *m/z* 79 as observed in the spectrum of XMYA methyl ester. Unfortunately, the mass spectrum of the minor peak could not be used to fully substantiate the identity of this as the *cis* isomer due to its low abundance and corresponding lack of fine detail and also due to the possibility of rearrangement of FAME derivatives.

3.2.3 4,4-Dimethyloxazoline derivatives of fatty acid of sandalwood seed oil

In the present study, the GC/MS of the fatty acid DMOX mixture also showed an additional minor peak at retention time 36.33 min. with a mass spectrum characteristic of the principal XMYA DMOX derivative peak at retention time 38.51 min. The mass spectra of both minor and major peaks of the XMYA DMOX are shown in Figure 3.10 and Figure 3.11. The spectrum of the more abundant *trans*-ximenynic acid (retention time 38.51 min.) shows a molecular ion at *m/z* 331 (calculated $C_{22}H_{37}NO$), and a characteristic fragment at 113 and 126 (base peaks), as shown in Scheme 3.1.

Examination of the typical fragmentation pattern of the DMOX-*trans*-ximenynic derivative shows that the even-mass homologous series of ions at *m/z* 126 + (14)n, deriving from cleavage at each saturated methylene bond, was interrupted by a mass separation of 10 amu between those at *m/z* 206 (C9) and 196 (C8), indicating an acetylenic bond at C9. The position of the double bond position of the conjugated system was indicated by a 12 amu difference between the low intensity fragments at *m/z* 232 (C11) and 220 (C12). In addition, these characteristic ion pairs were accompanied by a strong intensity fragment at *m/z* 182 (C7) and a minor fragment at *m/z* 220 (C10) giving a mass separation of 38 amu (79) (Figure 3.10).

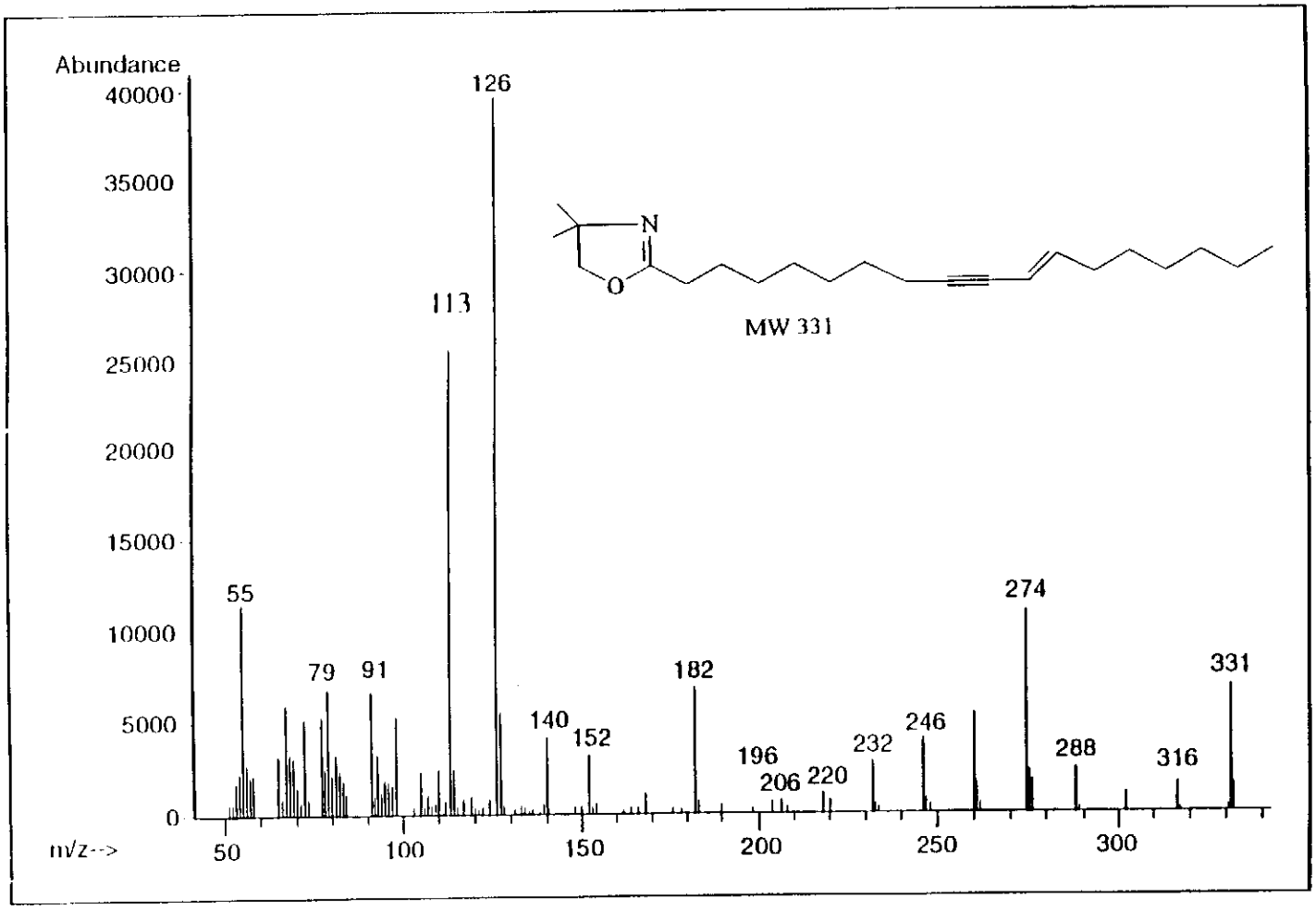


Figure 3.10 Electron ionisation (70 ev) mass spectrum of *trans* ximenynic acid 2-substituted-4,4-dimethyloxazoline derived.

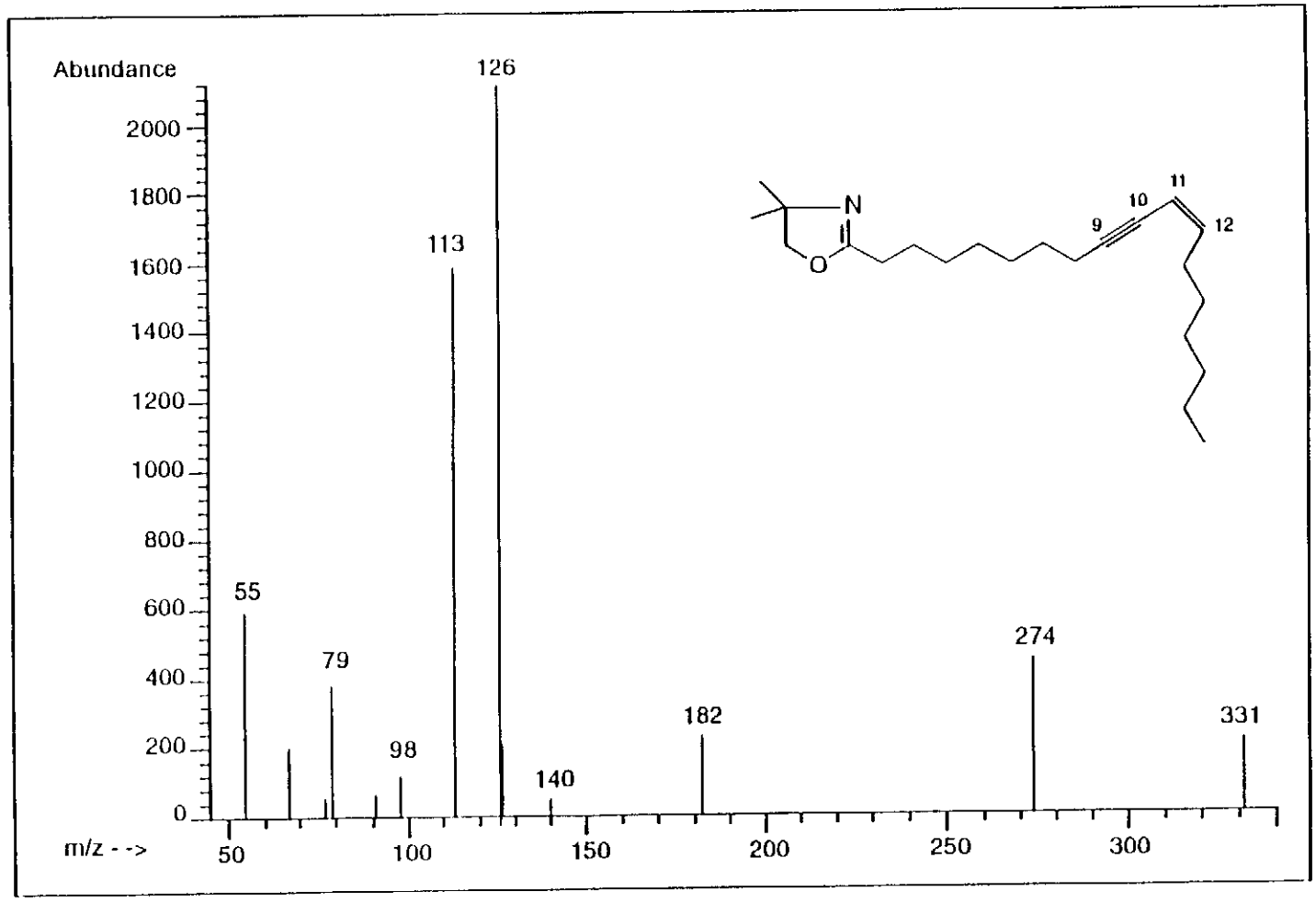


Figure 3.11 Electron ionisation (70 ev) mass spectrum of *cis* ximenynic acid 2-substituted-4,4-dimethyloxazoline derived.

Examination of the mass spectrum of the minor peak (retention time 36.33 min.) also shows a molecular ion at *m/z* 331, and characteristic fragments at 113, 126, 140, 182 and 274. The fragment at *m/z* 274, produced by fission at C14-C15, was found to be specific to ximenynic acid DMOX by comparison with the identified peak XMYA and by tests of peak homogeneity. Certain fragments such as *m/z* 232 (C11) and 220 (C12), and *m/z* 206 (C9) and 196 (C8) were not seen (Figure 3.11). According to previously published data (45, 79) the mass spectrum observed for the minor peak at retention time 36.33 min. confirms the conclusion that the unknown peak is the *cis*-isomer of ximenynic acid [*cis*-11-octadecen-9-ynoic acid, 18:2(9a,11c)] (42).

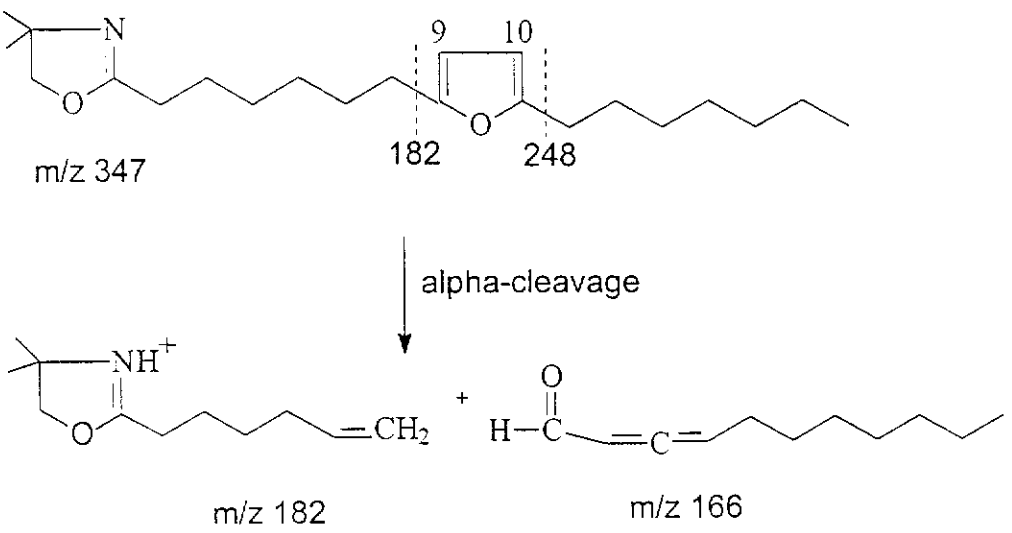
There is the possibility that the detected *cis*-XMYA may have been formed as an artefact from *trans*-XMYA during these chemical reactions. To resolve this problem, further analysis using a one-step conversion of the seed oil fatty acid to their DMOX derivatives was carried out without saponification (133). The GC of the resulting DMOX derivative mixture was analysed using a selected ion monitoring technique. Selection of characteristic ions occurring at *m/z* 232 and 274 confirmed the presence of only two geometrical isomers of XMYA as previous discussed, and the absence of any other fatty acid with that characteristic fragment. It was therefore concluded that the *cis*-XMYA detected was not an artefact but a natural feature of *Santalum spicatum* seed oil.

The natural co-occurrence of geometrical isomers of fatty acid in plant fixed oil as a product of specific metabolic processes is not uncommon, being particularly exemplified by oleic (*cis*-18:1 n-9) and elaidic (*trans*-18:1 n-9) acids. The present analysis of sandalwood seed oil routinely demonstrated that *cis*-XMYA appears to be a normal component occurring at low concentration (about 0.3%). During detection and characterisation of *cis*-XMYA in *Curupira tefeensis* (45), the urea adduct concentration technique was used to enrich the polyunsaturated FAME of the total lipid (70), and the findings substantiated the fact that the *cis* isomer (0.16%) may also occur naturally in other plants.

3.2.4 The identity of a ximenynic metabolite in the mouse liver lipid fraction

Mass spectrometry has been used in the study of eicosanoids, the oxygenated active metabolites of arachidonic acid to determine the involvement of these metabolites in various physiological and pathological conditions (139). Extensive literature research on this topic has demonstrated the absence of any information on mammalian biotransformation of XMYA. In this study, the DMOX technique provided an interesting insight into the biotransformation of XMYA. Examination of the DMOX derivatives of the resulting liver lipid fraction of mice fed an XMYA-enriched diet, showed the presence of a minor exotic fatty acid compound in addition to the expected major and minor fatty acid components such as palmitic, stearic, oleic and XMYA, as shown in Figure 3.12B. A minor peak (0.2-0.3%) at retention time 36.3 min. shown in Figure 3.12B gave the mass spectrum shown in Figure 3.13. A consideration of the fragmentation pattern has determined the probable identity as a furanoid fatty acid: 8,11-epoxy-8,10-octadecadienoic ($F_{8,11}$) acid. Details of the mass spectrum of the DMOX derivative of $F_{8,11}$ and proposed fragmentation scheme are shown in Scheme 3.2.

Scheme 3.2



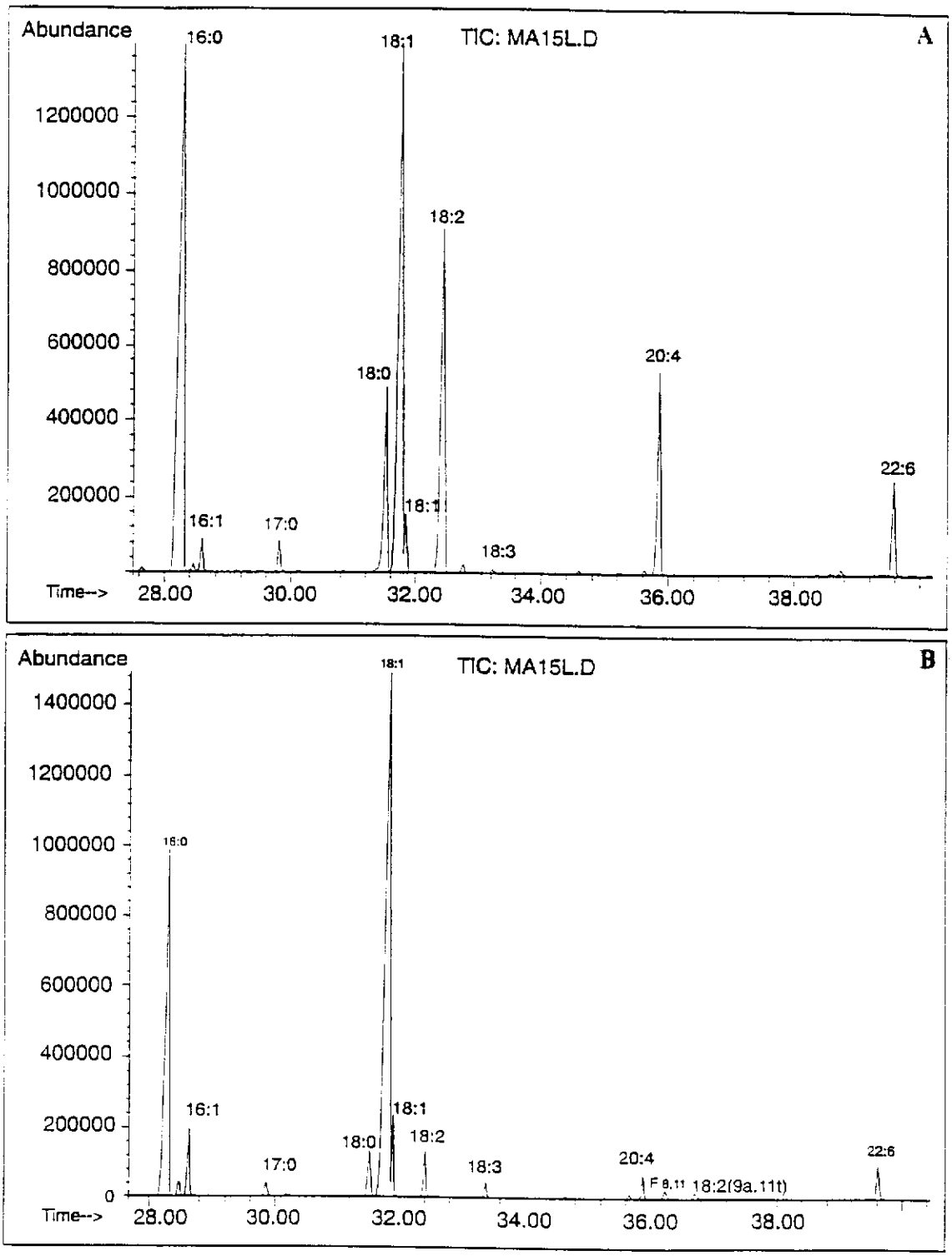


Figure 3.12 Typical capillary gas chromatography (GC) of separated mouse liver fatty acids. These chromatograms are from one mouse of each group fed the different diets, where A) standard lab diet (5% fat w/w) *vs* B) sandalwood seed oil diet (15% fat, w/w). GC conditions as described on page 39.

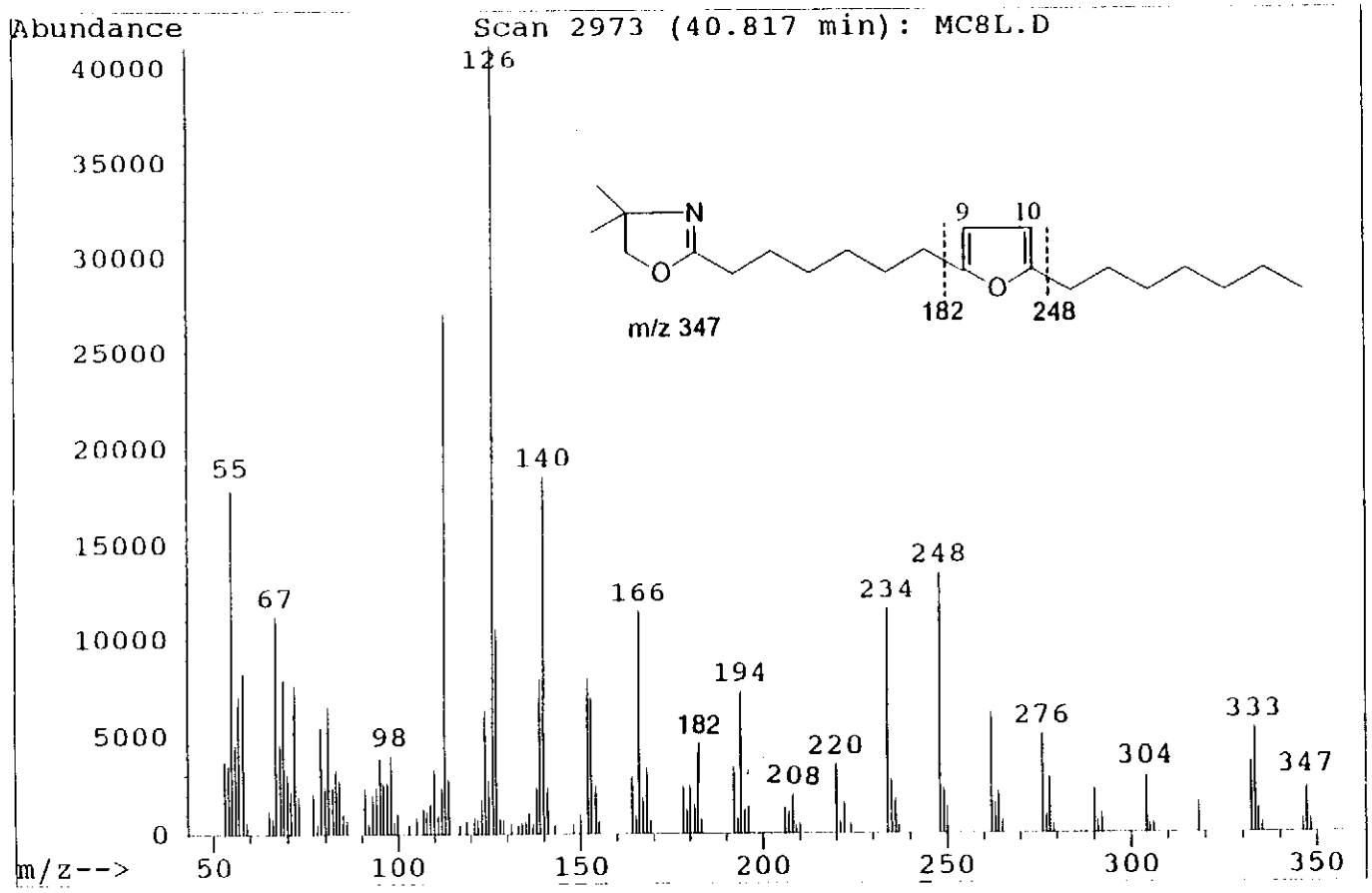


Figure 3.13 Electron ionisation (70 ev) mass spectrum of 8,11-epoxy-8,10-octadecadienoic acid 2-substituted-4,4-dimethyloxazoline derived.

Examination of the mass spectrum shows the molecular ion [M^+] to be 347 (calculated $C_{22}H_{35}NO_2$). The base peak at *m/z* 113 and *m/z* 126 are the usual characteristic fragments of DMOX derivatives (79). The intense peak at *m/z* 248 results from α-cleavage because of the stabilisation effect of the heterocyclic ring and indicates the position of the furan group (75). The intense ion at *m/z* 234 may be explained by loss of the oxygen atom from furan ring with formation of a conjugated diene (146-147). The differences of 12 amu between *m/z* 182 and *m/z* 194, and *m/z* 208 and 220 indicate double bonds to be located between C8 and C9, and between C10 and C11, respectively (Figure. 3.13). The mass spectrum was best explained as being that of the proposed F8,11 furanoid fatty acid and to be inconsistent with that of an oxiran or hydroxy fatty acid such as have been detected as FAME in other investigations (81).

3.2.5 Mass spectrum of conjugated linoleic acid

Since conjugated linoleic acid [18:2(9c, 11t)] was presumed to be an intermediate during the biosynthesis of XMYA in developing seed (later in Chapter 4), and in the biohydrogenation of XMYA in animal studies (later in chapter 6), and it is unavailable commercially, a synthesis of conjugated linoleic acid from XMYA was carried out according to the method of Gunstone and Russell (14) with selective hydrogenation of the XMYA triple bond producing the required *cis* double bond.

During FAME analysis of the above product, conjugated linoleic acid (61%) was found to be the predominant product together with 18:1(n-9) (18.5%), 18:1(n-11) (14.21%), and residual XMYA (5.8%). In addition, the UV spectral analysis of conjugated linoleic acid in hexane showed a λ_{max} at 231 nm, which is indicative of a conjugated system (14).

Comparing the mass spectrum of conjugated linoleic acid with that of linoleic acid [18:2(n-6)], it was found that the DMOX derivatives of conjugated linoleic acid exhibits a more intensive molecular ion at *m/z* 333, and also shows two intense peaks in the low mass range at *m/z* 113 and 126. The even-mass homologous series *m/z* 126 + (14)n is interrupted in the region of the double bonds by a mass difference of 12 amu

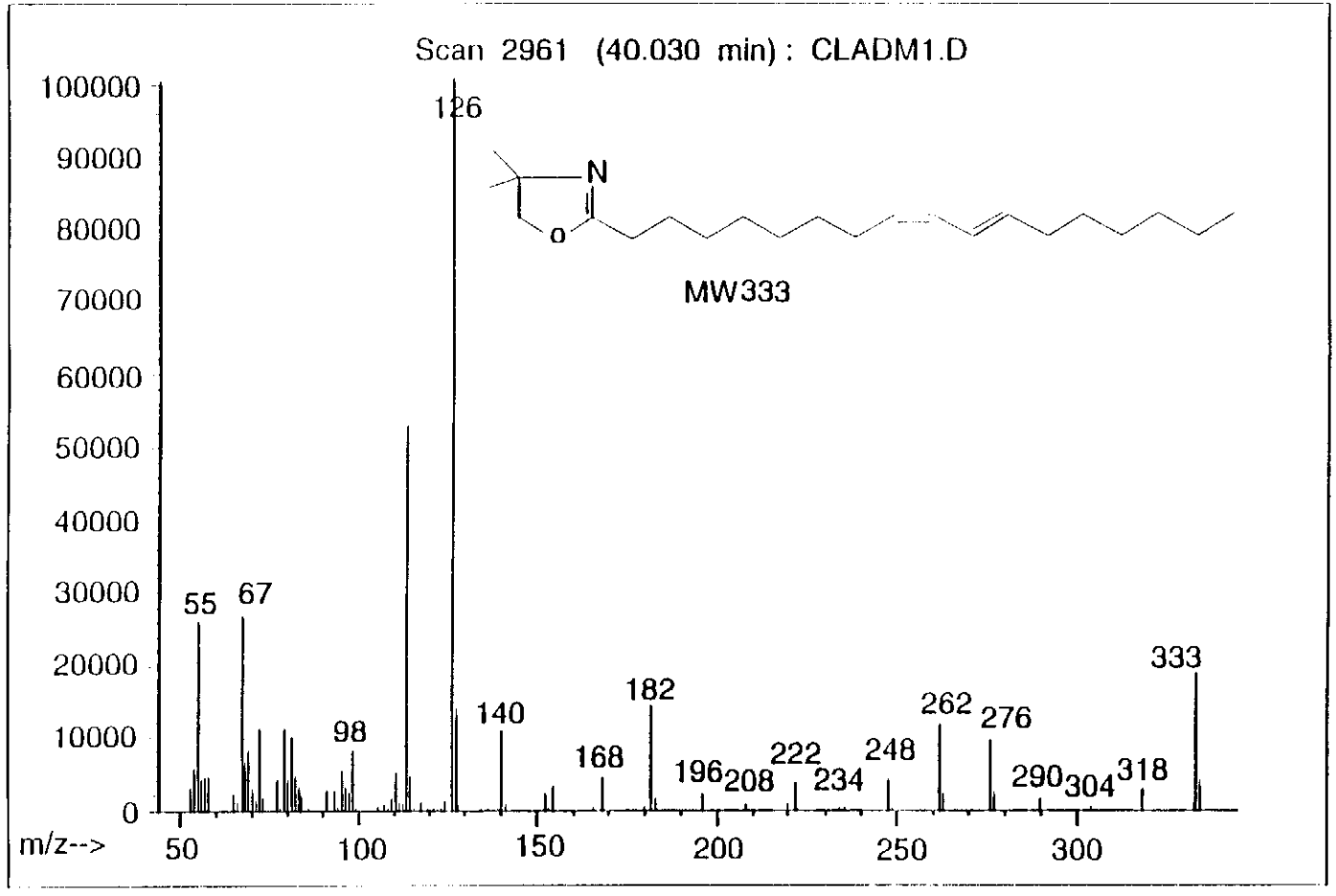


Figure 3.14 Electron ionisation (70 ev) mass spectrum of conjugated linolenic acid 2-substituted-4,4-dimethyloxazoline derived.

instead of the 14 amu between the low intensity fragment at *m/z* 208 and 196, and *m/z* 234 and 222 resulting from the presence of conjugated double bonds at position 9 and 11. This conclusion was further confirmed by two prominent fragments derived from a formal allylic cleavage at *m/z* 182 and 262. The fragments above *m/z* 234 again show the expected regular 14 amu difference (Figure 3.14).

3.3 CONCLUSION

GC/MS provides an excellent method for the separation and identification of most naturally occurring fatty acids. Oil and lipid fractions are quantitatively converted into the methyl esters and DMOX derivatives of the constituent fatty acid under different reagents and procedures. Methyl esters remain the most popular derivatives used for GC/MS quantitative analysis of fatty acids. However, they may undergo bond migration hindering absolute identification of polyunsaturated fatty acids.

4,4-Dimethyloxazoline derivatives of fatty acids can be used to locate the position of double bonds and other functional groups in the fatty acids by analysis of the mass spectral fragmentation pattern. This technique was applicable to samples of naturally occurring isomeric ximenynic acids, and the oxide metabolite of ximenynic acid in mouse liver lipid fraction. It has proven to be a very useful technique for the general GC/MS analysis of fatty acid in fats and oils.

CHAPTER 4: PROXIMATE ANALYSIS

4.1 INTRODUCTION

The seed kernels of *Santalum spicatum* are spherical and contained singly within a hard endocarp shell. The kernel weights generally occur within the range 0.6-1.3 g, and measure 0.4-1.46 cm in diameter, as determined by Fox and Brand (3). Fatty acid analyses of the seed lipid have been reported previously (25-26), as shown in Table 1.1. While a considerable amount of data has been previously reported on such characteristics as moisture and lipid content (4, 25), there are no studies on the broad nutritional suitability of the seed kernels.

The aim of this part of the study is to obtain basic information on the metabolic changes in selected primary constituents during seed development of *Santalum spicatum*, and to study the influence of geographical origin on the lipid content, moisture and fatty acid composition of *Santalum spicatum* seeds from different Western Australian locations.

Details of materials and methods have been presented in Chapter 2, page 30-39.

4.2 RESULTS AND DISCUSSIONS

4.2.1 Proximate composition

4.2.1.1 Characteristics of the developing seed of Santalum spicatum

Primary observations by Barrett indicated that *Santalum spicatum* flowering commenced at different times on different trees (148). For example, some trees bore flowers from early January, but others did not commence flowering until March or

April. In addition, flowers could be found on each tree for about four months although individual blossoms did not persist more than about two weeks (148). Therefore, a large number of flowers were tagged at flower stage, and the fruit age was then defined in terms of 'days after flowering' (DAF).

The biochemical characteristics of the developing fruit and kernel of *Santalum spicatum* determined by proximate analysis of samples collected from seven trees in 1993 season, and three trees in 1994 season are shown in Table 4.1A and 4.1B. Changes in the proximate composition of the seed during maturation were observed. As the seeds developed, the relationship between DAF and percentage oil content was established as shown in Figure 4.1. The findings of similar curves confirmed the relation between percentage oil production and DAF in the trees. Indeed, inspection of 1993 and 1994 data shows a reasonable comparison in all parameters, demonstrating the fundamental characteristics of fruit development in the trees. The kernel appeared as defined tissue around 91 to 98 DAF, however the fruits did not mature until 154 DAF by which time the epicarp changed from green to brown in colour, and commenced to dehydrate. The quantitative results at 154 DAF when the seed was almost mature, show that the kernel contained on average 41.8% of lipid and 15.6% of crude protein.

Inspection of the proximate composition data of *Santalum spicatum* from Table 4.1A and 4.1B revealed that the chemical composition of the fruit and later kernel was found to undergo considerable change during seed formation and maturation. In the early fruit development stages, ranging from 14 DAF to 112 DAF, the chemical composition of the whole fruit showed little variation, with the majority being moisture (65%) and carbohydrate (30%), and the lipid content at only about 0.2%.

Lipids are known to be present in nearly all the organs of a plant. In general, for oil-bearing plants they only accumulate in the cotyledons of the seed (e.g., 40% in sunflower) or in the mesocarp of certain fruit (e.g., 48% in oil palm) (149). According to Hitchcock and Nichols: in general, immature seeds contain far less lipid than the corresponding mature tissue, and the small amount of lipid fraction is chiefly composed of phospholipid and glycolipid together with a small proportion of

Table 4.1A Proximate Percentage Composition in Developing Sandalwood Seeds (results based on dry weight; trees grown at Curtin University, 1993)

DAF*	Moisture	Lipid	Ash	Protein	Carbohyd*
14	59.17 ± 1.32	0.26 ± 0.05	1.62 ± 0.20	3.78 ± 0.67	35.26 ± 1.27
21	64.07 ± 2.01	0.32 ± 0.06	1.45 ± 0.22	3.22 ± 0.29	30.95 ± 1.76
28	64.36 ± 1.43	0.19 ± 0.03	1.41 ± 0.19	3.53 ± 0.27	30.51 ± 1.47
35	64.54 ± 1.13	0.14 ± 0.02	1.37 ± 0.17	3.41 ± 0.34	30.66 ± 1.11
42	66.09 ± 1.33	0.13 ± 0.03	1.44 ± 0.13	3.39 ± 0.39	28.95 ± 1.47
49	67.30 ± 1.40	0.24 ± 0.10	1.33 ± 0.10	3.12 ± 0.30	27.87 ± 1.41
56	66.02 ± 2.63	0.15 ± 0.04	1.25 ± 0.22	3.21 ± 0.49	29.35 ± 2.32
63	64.40 ± 1.05	0.09 ± 0.03	1.37 ± 0.08	3.28 ± 0.36	30.86 ± 1.01
70	68.77 ± 1.11	0.07 ± 0.04	1.21 ± 0.08	2.58 ± 0.24	27.36 ± 1.23
77	70.95 ± 3.35	0.12 ± 0.02	0.99 ± 0.11	2.45 ± 0.41	25.48 ± 3.34
84	70.42 ± 7.62	0.26 ± 0.09	0.95 ± 0.24	2.57 ± 0.38	26.33 ± 7.48
91	74.06 ± 5.12	0.42 ± 0.55	0.93 ± 0.27	2.05 ± 0.39	22.54 ± 4.89
98	66.76 ± 7.30	1.41 ± 0.83	1.03 ± 0.23	3.82 ± 1.00	27.05 ± 6.74
105	63.49 ± 6.72	4.00 ± 3.84	1.02 ± 0.21	7.17 ± 4.00	24.32 ± 6.86
112	53.71 ± 6.25	13.32 ± 2.64	1.21 ± 0.17	10.63 ± 1.86	19.12 ± 4.48
119	55.65 ± 3.95	15.33 ± 2.70	1.50 ± 0.22	10.41 ± 2.17	19.12 ± 2.92
126	51.58 ± 5.04	18.48 ± 3.64	1.51 ± 0.34	12.56 ± 1.34	15.87 ± 3.86
133	34.75 ± 6.70	27.06 ± 4.54	1.90 ± 0.30	14.52 ± 4.06	21.77 ± 3.01
140	32.42 ± 2.49	29.65 ± 4.55	1.90 ± 0.30	15.90 ± 3.06	19.25 ± 3.45
147	28.51 ± 1.73	39.63 ± 3.46	1.83 ± 0.31	14.79 ± 3.16	15.24 ± 2.10
154	19.26 ± 4.79	40.63 ± 3.47	2.33 ± 0.37	16.95 ± 3.46	19.36 ± 4.55

In this Table, each value represents the mean ± S.D., n = 7.

DAF* - days after flowering.

Carbohyd* - carbohydrate calculated by difference.

Table 4.1B Proximate Percentage Composition in Developing Sandalwood Seeds (results based on dry weight; trees grown at Curtin University, 1994)

DAF*	Moisture	Lipid	Ash	Protein	Carbohyd*
7	55.86 ± 0.15	0.28 ± 0.03	1.83 ± 0.05	3.06 ± 0.31	38.97 ± 0.46
14	55.47 ± 0.25	0.35 ± 0.06	1.92 ± 0.19	2.76 ± 0.33	39.51 ± 0.53
21	56.87 ± 1.03	0.27 ± 0.04	1.84 ± 0.12	3.43 ± 0.68	37.58 ± 0.48
28	57.47 ± 0.29	0.49 ± 0.08	1.78 ± 0.05	2.60 ± 0.10	37.65 ± 0.37
35	65.57 ± 1.25	0.22 ± 0.14	1.52 ± 0.09	1.99 ± 0.04	30.70 ± 1.13
42	63.77 ± 0.92	0.19 ± 0.33	1.46 ± 0.15	2.11 ± 0.06	32.47 ± 0.78
49	65.14 ± 1.56	0.26 ± 0.05	1.36 ± 0.12	2.12 ± 0.16	31.12 ± 1.35
56	68.25 ± 1.21	0.16 ± 0.03	1.25 ± 0.12	2.13 ± 0.03	28.21 ± 1.07
63	69.93 ± 1.23	0.13 ± 0.01	1.14 ± 0.11	2.69 ± 0.24	26.11 ± 1.34
70	72.49 ± 0.93	0.18 ± 0.01	1.20 ± 0.11	2.13 ± 0.09	24.00 ± 0.76
77	78.84 ± 1.47	0.09 ± 0.02	1.01 ± 0.10	1.86 ± 0.09	22.20 ± 1.28
84	73.68 ± 1.53	0.14 ± 0.02	0.92 ± 0.05	1.80 ± 0.18	23.46 ± 1.39
91	70.60 ± 1.18	0.51 ± 0.20	1.15 ± 0.07	2.35 ± 0.24	25.40 ± 0.91
98	75.20 ± 4.86	1.57 ± 0.28	0.84 ± 0.19	1.77 ± 0.43	20.62 ± 4.54
105	78.22 ± 3.45	3.00 ± 0.48	0.72 ± 0.09	1.86 ± 0.33	16.20 ± 2.86
112	80.29 ± 3.42	4.75 ± 1.60	0.67 ± 0.16	1.98 ± 0.05	11.96 ± 1.32
119	78.26 ± 4.79	5.16 ± 1.04	0.62 ± 0.03	2.35 ± 0.34	13.62 ± 4.49
126	53.06 ± 2.38	20.53 ± 0.51	1.61 ± 0.07	6.79 ± 0.51	18.01 ± 1.97
133	50.42 ± 3.94	24.06 ± 2.94	1.61 ± 0.25	7.76 ± 0.78	16.15 ± 0.04
140	37.64 ± 7.74	32.56 ± 4.38	1.87 ± 0.24	10.21 ± 0.77	17.72 ± 2.88
147	27.40 ± 1.34	39.40 ± 2.21	2.17 ± 0.16	13.02 ± 1.26	18.01 ± 1.65
154	22.94 ± 1.54	41.84 ± 0.58	2.34 ± 0.22	15.62 ± 0.61	17.60 ± 1.86

In this Table, each value represents the mean ± S.D., n = 3.

DAF* - days after flowering.

Carbohyd* - carbohydrate calculated by difference.

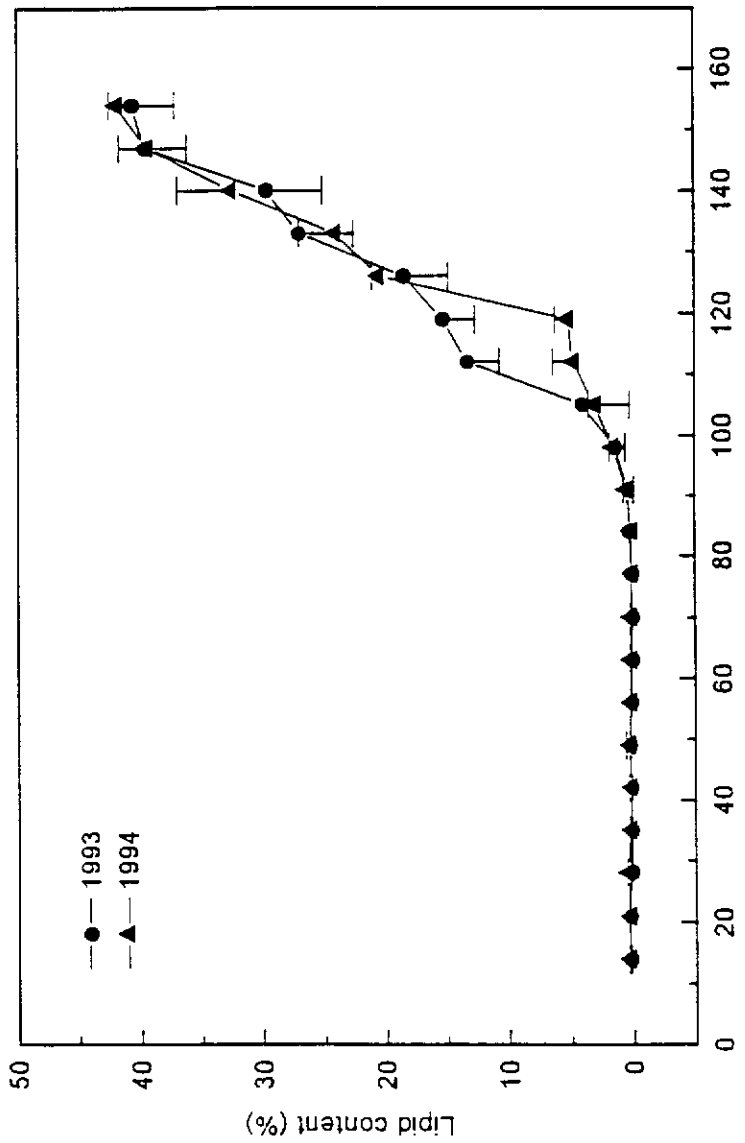


Figure 4.1 Accumulation of total lipids in developing *Santalum spicatum* seed.

triacylglycerols, and the levels of triacylglycerols increase markedly with seed maturation (30). Therefore, once the kernels were clearly differentiated, from 91 to 154 DAF, they were chosen for analysis, rather than the previous whole immature fruit (DAF 7-84).

Much of the weight increase during fruit development would appear to be due to the accumulation of lipid in the kernel. The results obtained by cold-solvent extraction showed that lipid content increased from *ca.* 0.4% (DAF 91) to 40.6% (DAF 154) in the 1993 season, and 0.5% (DAF 91) to 41.8% (DAF 154) in the 1994 season. Very little change in lipid content was observed in the final week of fruit maturation (DAF 147-154), although moisture content continued to decrease and protein content continued to increase. This finding reflects the early studies on the development of seeds of rapeseed (*Brassica napus*), and soybeans (*Glycine max*) (149-152).

The accumulation of lipid in developing seed typically follows three different phases (83, 150), however different plant seeds have markedly different lipid accumulation rates. For example, it takes about seven days for safflower (*Carthamus tinctorius*) to develop its total content of seed lipid whereas it takes seven months for full lipid content production in the fruit of olive (*Olea europaea*) (86). The three phases of development may be explained by studies done by Appleqvist (150), and Gurr and Harwood (39), which simply characterised them as: 1) seeds typically have a very high moisture content, 2) rapid accumulation of lipid, and 3) only further minor amounts of lipid accumulate and seed moisture content decreases. In developing *Crame abyssinica* seeds the three periods are: 0-8 DAF, 8-30 DAF, and 30 DAF to mature (83). In developing oilseed rape (*Brassica napus*), it was also found that the three phases of seed development were from *ca.* 0-18 DAF, 18-40 DAF, and 40-65 DAF (151). Rapid deposition of lipids began around 16 DAF and continued till about 40 DAF, thereafter a decline in fresh weight corresponds to the final dehydration phase of seed development. Thus, applying this staging rationalisation to data from the present study, the three distinct phases of *Santalum spicatum* seed development are from *ca.* 0-91 DAF, 91-147 DAF and 147 to 154 DAF (mature) (Figure 4.1). In general, the particular fatty acid pattern found in the final oil becomes prominent during the second

phase of rapid deposition (83, 150). Clearly, these findings also indicate that any study of lipid metabolism in connection with oil deposition in this species would need to be concentrated in the period 98-147 DAF, when the relevant enzymes appear to be most active. In fact, earlier studies have shown that for a number of important lipid-synthesis enzymes such as acetyl-CoA carboxylase and enol reductase, their activities markedly rise during the second phase in developing rape-seeds (*Brassica napus*) (153). Generally, in the developing seed the active oil deposition largely occurs only over a short period (149).

The protein content of *Santalum spicatum* seed has been investigated by Jones and coworkers (25). It was found that the de-oiled meal contained aspartic acid (19.3%), glutamic acid (15.6%) and glycine (13.3%) as the major component amino acids; and the de-oiled meal was also described as a rich source of essential amino acids such as lysine and threonine (25). Because the seed of *Santalum spicatum* has both high protein and lipid content in contrast to the low protein content of other common oilseeds, for example, sunflower seeds (16.5-19.6% protein) (23), they may be of use as a protein supplement for low-protein foods and feeds such as cereal grains. However, before they can be used in this way feeding studies are needed to demonstrate the lack of inherent toxicity.

The mineral ash content of *Santalum spicatum* seeds remained relatively low and unchanged during development as shown in Table 4.1A and 4.1B. Slight increases observed at DAF 154 may reflect maturation of the kernel testa. X-ray diffraction studies demonstrates the presence of K, Cl, P and Mg as shown in Figure 4.2, however the levels of each mineral constituent (K_2O, MgO, P_2O_5 and Cl salts) were not determined.

The carbohydrate content was calculated by difference. Thus, for 100 g seed: carbohydrate% = 100 g seed - (lipid% + protein% + ash% + moisture%). The percentage of carbohydrate represents real values modified by changes in the other components. Inspection of Table 4.1A and B, representing data for 1993 and 1994

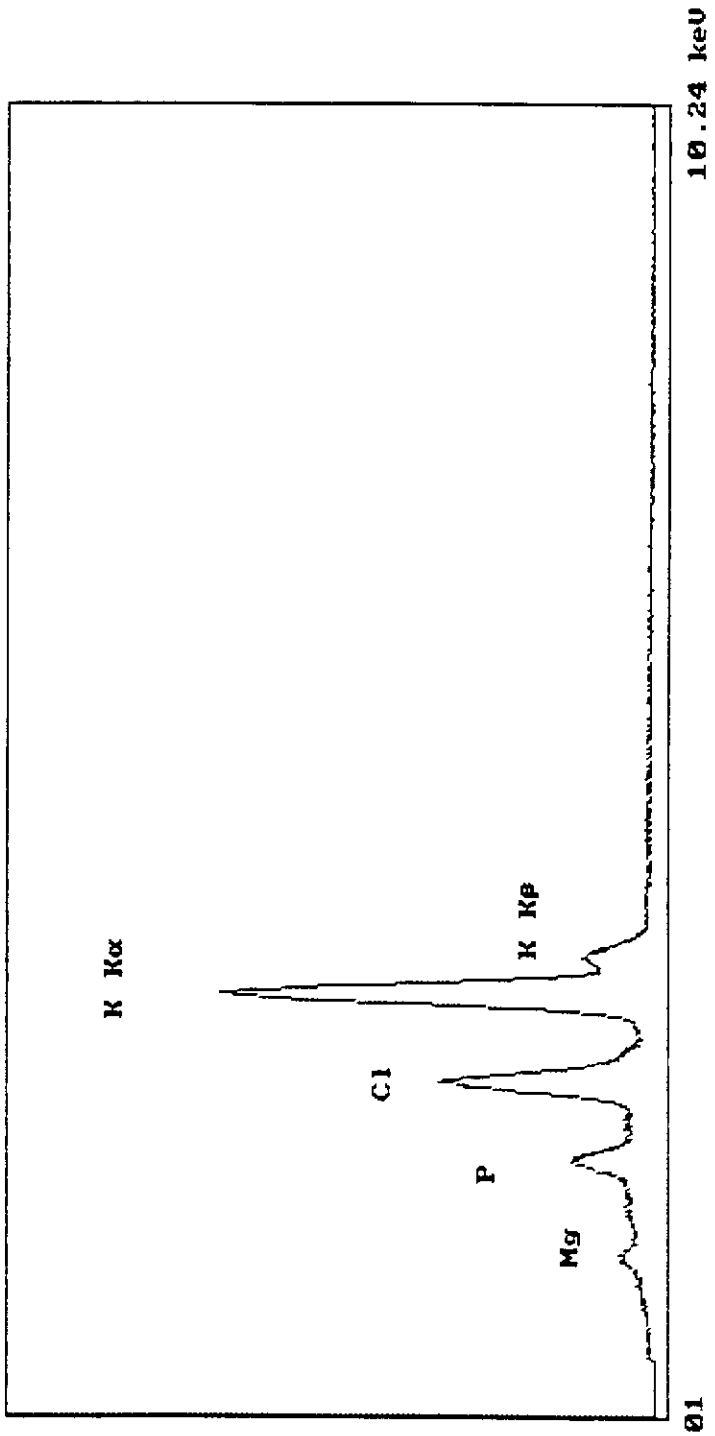


Figure 4.2 Mineral content of *Santalum spicatum* seed.

respectively, shows a general decrease in carbohydrate (by difference) as the fruit mature and produce increased levels of lipid and protein. No attempt was made to identify individual carbohydrates, however a few samples of mature kernel were analysed for soluble fibre according to the method of AOAC 1990 (55). The soluble fibre content of *Santalum spicatum* meal was on average 9.9% (based on dry de-oiled meal) higher than that of other oil-bearing plants such as soybean meal which contains 4.8% fibre (23), reflecting a lower value of metabolisable energy. The level of moisture content is inversely proportional to the content of other constituents including proteins, lipids, carbohydrates and ash, and only constitutes one of the characteristics of the developing seed. Maturation of the seed is characterised by an accumulation of proteins and other constituents at the expense of moisture *per se* (154).

4.2.1.2 Characteristics of the seeds from different Western Australian locations

From the published data on the variability of *Santalum spicatum* seed size, germination time and seedling growth rate, it is clear that these data bear relationship to environmental factors such as rainfall, soil type, temperature or latitude (3). The authors found that there were significant difference in seed size between different *Santalum spicatum* ecotypes. These seed samples were provided for chemical analysis, in order to investigate the influence of geographical origin on proximate composition of the kernel from a number of locations in Western Australia as listed in Table 2.2. The results of proximate analysis are shown in Table 4.2. Analysis of moisture, lipid, and ash were carried out on duplicate samples from each kernel, while nitrogen analysis to provide protein content was performed on single samples only due to sample size restrictions.

One-way ANOVA analysis of variance was conducted on moisture, lipid and ash level results. Inspection of Table 4.2 showed that of the moisture results, the sample from Everade was significantly different from that of Koorda ($p = 0.0062$). This conclusion arises partly because the Everade and Koorda results represent opposite outliers of the

observed range of values, and may merely reflect different sample ages and storage conditions. Similar statistical treatment of the lipid and ash results showed that none were significantly different within their categories at the tested level ($p<0.05$). Protein results could not be tested using this procedure due to their derivation as single values. Overall inspection of the results shown in Table 4.2 suggest that the major proximate components such as lipid content showed no significant differences ($p < 0.05$), and that in consequence there was little or no ecotypic variation in the samples. The results indicate that the seeds from different Western Australian location were found to be similar in their general chemical make up, which are not strongly influenced by environmental factors (155). If sandalwood is to be cultivated as a potential oil crop, it would be advantageous to obtain trees which produce high content of seed oil.

Table 4.2 Proximate Percentage Composition of Stored Ripe *Santalum spicatum* Seed Kernels from Different Western Australian Locations

Location	Moisture	Lipid	Ash	Protein	Carbohyd*
Dryandra	3.61 [ab]*	52.33 [a]	1.83 [a]	18.13	24.10
Everade	4.15 [a]	48.43 [a]	1.73 [a]	20.23	25.46
Kalgoorlie	3.73 [ab]	44.40 [a]	1.86 [a]	23.38	26.63
Koorda	3.16 [b]	50.27 [a]	1.59 [a]	23.31	21.67
Marvel Loch	3.56 [ab]	52.65 [a]	1.64 [a]	22.43	19.72
Mt. Wilkinson	3.51 [ab]	52.87 [a]	2.00 [a]	24.60	16.96
Nanga	3.43 [ab]	51.36 [a]	1.84 [a]	20.84	22.53
Norseman	3.23 [ab]	54.55 [a]	1.96 [a]	19.32	21.24
Shell Beach	3.98 [ab]	49.17 [a]	1.95 [a]	25.21	19.69
Average	3.60	50.67	1.82	21.94	22.00

ab* same letters indicate samples which are not significantly different ($p<0.05$) within a column using the One-Way ANOVA analysis.

Carbohyd* - carbohydrate calculated by difference.

4.2.2 Fatty acid composition

4.2.2.1 Changes in fatty acid composition during seed development

The TIC (Figure 3.3) shows the presence of a range of common fatty acids: palmitic (16:0), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), α-linolenic (18:3n-3) together with XMYA. Stearolic acid was only observed during the later stages of seed development. Table 4.3A and B show the fatty acid compositions of developing seed from 7 to 154 days after flowering in 1993 and 1994. These results are further represented in Figure 4.3.

Inspection of Tables 4.3A and 4.3B show that the fatty acid content underwent little change in the initial stages of the fruit development (14-84 DAF). The results indicated that 16:0 (24%), 18:1(n-9) (30%), 18:2(n-6) (9%), and 18:3(n-3) (33%) were generally present in a higher proportion than 18:0 (max. 5%) and XMYA (max. 2.6%) acids. The most abundant fatty acid at this stage was 18:3(n-3). Similarly, evidence from earlier studies have reported that 18:3(n-3) was observed as the predominant fatty acid in the initial period of development of *Crambe abssinica* seed (39). The biosynthesis of 18:3(n-3) in plant tissue has been assumed to take place by desaturation of 18:2(n-6), and suggests a simple precursor-product relationship between the 18:1(n-9), 18:2(n-6) and 18:3(n-3) (30). Previous studies carried out by Appelqvist suggested that there were two pathways of 18:3(n-3) biosynthesis in higher plants, one involving 16:3 as substrate and the other using 18:2(n-6) (150). However, FAME analysis of *Santalum spicatum* fruit and seed has failed to identify the presence of 16:3 in oil samples.

Oleic acid, 18:1(n-9), started increasing from DAF 84 in 1993 and DAF 91 in 1994 samples. Rapid synthesis of this acid has been suggested to occur by desaturation of 18:0 in plant tissue, as in developing safflower (*Carthamus tinctorius*) seeds (150).

XMYA did not appear as a lipid component in *Santalum spicatum* until 21 DAF in 1993 and 14 DAF in 1994, and only as a minor component during the initial stages of seed development.

Table 4.3A Changes in Fatty Acid (% of total) in Developing Sandalwood Seed (1993)

DAF*	16:0	16:1	18:0	18:1	18:2	18:3	18:1(9a)	XMYA	C22:0
14	23.67	tr	4.95	28.04	15.98	25.62	0	1.42	tr
	(1.44)		(1.27)	(3.71)	(2.14)	(2.76)		(1.75)	
21	23.67	tr	4.21	30.43	10.89	27.81	0	2.93	tr
	(2.03)		(0.57)	(3.13)	(2.16)	(3.63)		(1.84)	
28	23.67	tr	3.83	30.24	9.59	29.92	0	2.74	tr
	(1.95)		(0.89)	(2.33)	(1.30)	(2.69)		(3.21)	
35	23.40	tr	5.04	28.58	10.33	31.11	0	1.53	tr
	(0.72)		(1.73)	(1.90)	(1.25)	(3.04)		(3.99)	
42	23.71	tr	5.03	26.81	11.83	31.93	0	0.67	tr
	(2.20)		(1.38)	(2.02)	(0.89)	(2.44)		(1.40)	
49	24.24	tr	1.72	26.43	10.52	36.30	0	0.77	tr
	(1.95)		(0.87)	(3.17)	(2.20)	(3.66)		(0.65)	
56	24.31	tr	0.75	27.36	9.57	37.54	0	0.36	tr
	(2.35)		(0.34)	(3.68)	(1.12)	(3.19)		(0.41)	
63	24.40	tr	1.12	27.48	10.24	36.20	0	0.54	tr
	(2.65)		(0.73)	(3.16)	(2.13)	(3.40)		(1.09)	
70	25.72	tr	1.03	27.74	8.85	36.27	0	0.37	tr
	(2.17)		(0.39)	(1.41)	(1.72)	(2.83)		(0.67)	
77	24.87	tr	0.98	24.22	9.95	39.17	0	0.33	tr
	(1.90)		(0.46)	(1.69)	(1.85)	(1.87)		(0.56)	
84	23.85	tr	1.63	31.38	10.02	32.28	0	1.26	tr
	(4.65)		(0.52)	(10.48)	(3.47)	(5.98)		(2.16)	
91	18.38	tr	1.07	39.43	8.64	29.27	0	2.59	tr
	(6.17)		(0.20)	(10.97)	(3.50)	(10.32)		(5.54)	
98	8.59	tr	1.09	56.81	4.52	17.72	0	11.25	tr
	(6.60)		(0.18)	(12.82)	(3.77)	(7.29)		(5.24)	
105	6.44	tr	0.88	58.59	2.16	12.74	0.24	18.03	tr
	(2.39)		(0.31)	(10.04)	(0.81)	(5.89)	(0.39)	(8.93)	
112	3.81	tr	0.87	56.42	1.62	6.53	0.90	29.83	tr
	(0.64)		(0.45)	(4.35)	(0.74)	(1.76)	(0.61)	(4.11)	
119	3.04	tr	0.86	61.71	0.75	3.61	0.59	29.42	tr
	(0.40)		(0.17)	(4.25)	(0.13)	(0.97)	(0.15)	(3.52)	
126	2.89	tr	0.86	61.04	1.03	3.48	0.82	29.86	tr
	(0.69)		(0.25)	(4.38)	(0.43)	(1.12)	(0.27)	(5.02)	
133	3.57	tr	1.07	54.96	1.26	3.36	1.28	34.49	tr
	(0.37)		(0.33)	(7.78)	(0.34)	(0.91)	(0.59)	(3.04)	
140	4.10	tr	1.20	53.53	1.57	3.81	0.98	34.79	tr
	(0.84)		(0.40)	(2.47)	(0.52)	(0.87)	(0.31)	(2.20)	
147	4.35	tr	1.74	52.85	1.54	3.56	1.08	34.87	tr
	(0.61)		(0.81)	(5.03)	(0.42)	(0.71)	(0.39)	(3.74)	
154	4.72	tr	1.58	48.75	1.91	4.65	1.57	35.72	tr
	(0.52)		(0.43)	(3.50)	(0.72)	(0.93)	(0.37)	(3.32)	

In this Table, each value represents the mean (with S.D. in parentheses) of samples collected from seven trees. DAF* = days after flowering. tr = trace (<0.01%).

Table 4.3B Changes in Fatty Acid (% of total) in Developing Sandalwood Seed (1994)

DAF*	16:0	16:1	18:0	18:1	18:2	18:3	18:1(9a)	XMYA	C22:0
7	23.10	1.47	5.41	29.84	7.48	30.53	0	0	2.17
	(0.63)	(0.27)	(0.56)	(1.77)	(0.51)	(3.04)			(0.99)
14	24.13	1.22	3.59	28.51	7.37	32.78	0	0	2.40
	(2.62)	(0.22)	(1.13)	(2.09)	(0.48)	(1.27)			(0.79)
21	22.17	1.34	3.26	30.10	8.32	33.96	0	0.04	0.81
	(1.06)	(0.30)	(0.41)	(1.46)	(0.14)	(0.96)		(0.04)	(0.58)
28	21.66	1.73	2.21	26.95	9.55	35.37	0	2.49	0.04
	(1.46)	(0.35)	(0.37)	(1.73)	(1.39)	(4.65)		(3.51)	(0.04)
35	19.57	1.67	2.06	35.57	7.95	29.33	0	3.81	0.04
	(0.93)	(0.72)	(0.36)	(1.88)	(0.45)	(3.65)		(1.96)	(0.04)
42	20.50	1.78	2.28	28.46	9.32	33.56	0.31	2.06	1.73
	(1.73)	(0.45)	(0.25)	(1.96)	(0.41)	(2.19)	(0.43)	(1.46)	(0.37)
49	21.05	1.75	1.89	23.18	11.16	35.47	1.48	2.35	1.67
	(0.64)	(0.26)	(0.24)	(1.93)	(0.55)	(1.63)	(0.54)	(1.68)	(0.32)
56	22.13	1.80	1.94	24.45	10.88	35.59	0.70	0.74	1.77
	(0.59)	(0.36)	(0.32)	(1.84)	(0.72)	(1.65)	(0.13)	(0.06)	(0.09)
63	23.04	3.86	2.67	26.58	9.88	29.54	1.06	1.33	2.04
	(0.38)	(1.29)	(0.28)	(2.20)	(0.56)	(3.17)	(0.04)	(0.90)	(0.20)
70	22.00	2.86	2.48	29.37	10.23	28.47	1.42	0.90	2.27
	(1.74)	(0.72)	(0.03)	(1.60)	(0.59)	(1.12)	(0.43)	(0.60)	(0.51)
77	20.78	2.47	2.52	31.50	10.97	26.71	1.63	1.59	1.83
	(0.57)	(0.31)	(0.30)	(2.37)	(1.28)	(3.45)	(0.61)	(1.08)	(0.21)
84	21.57	2.06	2.77	30.31	11.20	26.60	1.14	2.33	2.02
	(0.62)	(0.50)	(0.83)	(3.53)	(1.03)	(6.10)	(0.06)	(2.38)	(0.48)
91	18.50	tr	1.67	32.95	9.68	31.82	1.54	2.94	0.89
	(2.20)		(0.61)	(1.77)	(1.63)	(1.05)	(0.37)	(2.53)	(1.24)
98	7.52	tr	2.42	51.82	8.20	19.50	1.61	8.91	tr
	(2.22)		(0.43)	(7.89)	(0.55)	(2.66)	(0.71)	(2.63)	
105	6.38	tr	3.05	55.06	4.97	15.03	1.64	13.85	tr
	(1.30)		(0.93)	(6.42)	(1.66)	(3.38)	(0.45)	(4.61)	
112	4.56	tr	3.28	51.28	4.25	14.60	1.57	20.44	tr
	(0.90)		(0.49)	(2.52)	(0.85)	(2.32)	(0.65)	(5.47)	
119	4.08	tr	3.50	52.75	3.36	9.85	2.06	24.38	tr
	(0.73)		(0.85)	(4.50)	(0.29)	(3.81)	(0.21)	(2.36)	
126	3.74	tr	1.99	54.44	1.86	6.83	1.88	29.23	tr
	(0.38)		(1.48)	(4.02)	(0.54)	(3.01)	(0.38)	(0.76)	
133	3.30	tr	3.42	55.12	1.50	4.02	2.03	30.60	tr
	(0.24)		(0.63)	(1.56)	(0.48)	(1.52)	(1.35)	(3.06)	
140	3.49	tr	2.87	52.68	1.76	3.67	1.66	33.85	tr
	(0.54)		(0.65)	(3.92)	(0.73)	(0.60)	(0.10)	(4.46)	
147	3.68	tr	3.32	49.45	2.18	4.65	1.27	35.43	tr
	(0.54)		(1.30)	(2.82)	(0.71)	(1.79)	(0.14)	(4.55)	
154	3.87	tr	1.46	52.49	1.29	3.15	0.70	37.02	tr
	(0.88)		(0.38)	(2.57)	(0.78)	(0.66)	(0.31)	(2.92)	

In this Table, each value represents the mean (with S.D. in parentheses) of samples collected from three trees. DAF* = days after flowering. tr = trace (<0.01%).

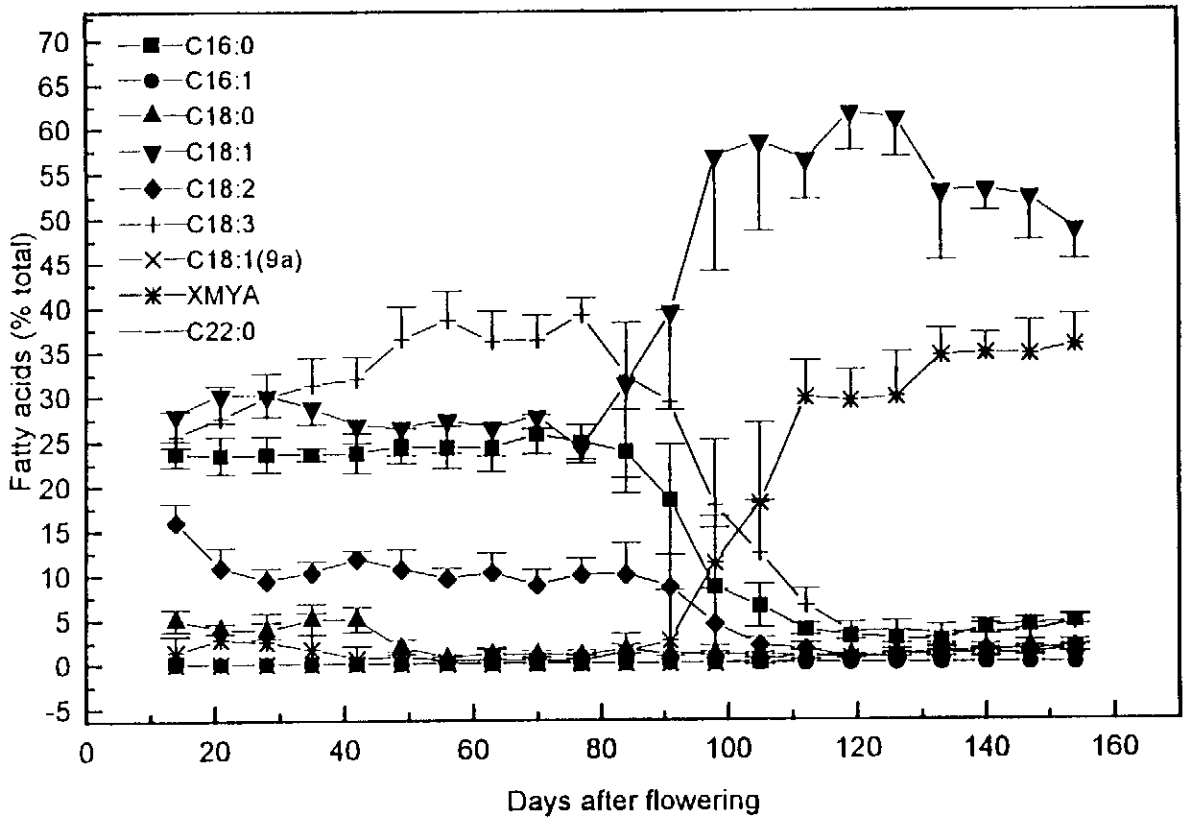


Figure 4.3 Average changes in fatty acid composition of developing seeds of *Santalum spicatum* (1993 season).

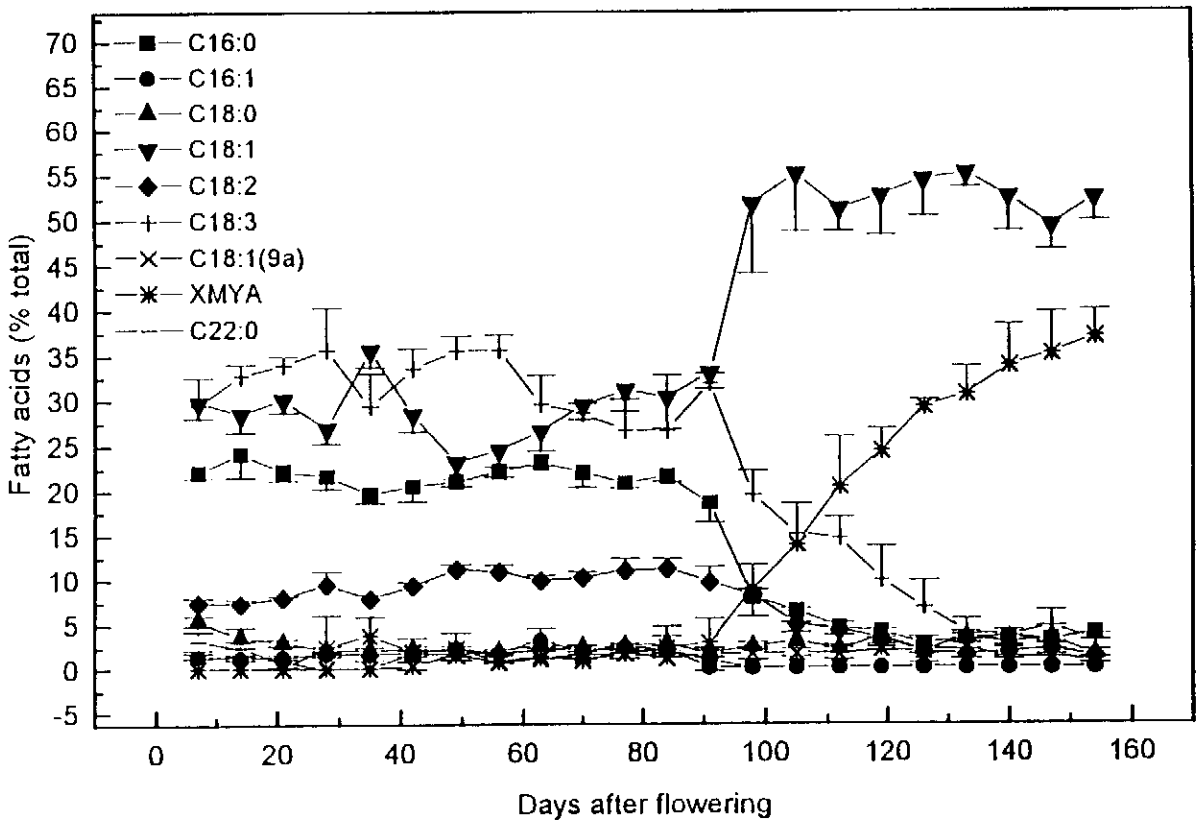


Figure 4.3 (continued, 1994 season).

Large changes occurred in the fatty acid compositions of the seed during the second phase (DAF 91-147) of seed development. In this stage the clearly differentiated kernel was used for analysis and results demonstrated a large increase in lipid content. The marked 'appearance' of XMYA (from DAF 91) was accompanied by significant decreases in the relative percentage of other fatty acids such as 16:0, 18:2(n-6) and especially 18:3(n-3), together with a major increase in 18:1(n-9).

During this period, 18:1(n-9) reached its highest percentage (58%), while XMYA content increased rapidly with the advancement of seed development, from 3% to an average 34% in 1993, and 35% in 1994. On the other hand, the small changes of other fatty acids such as 18:0 and 18:1(9a) were considered insignificant. In a corresponding study, it was shown that levels of crepenynic (cis-9-octadecen-12-ynoic) acid increased rapidly between 14 and 28 DAF to became the major fatty acid in the developing seed of *Crepis rubra* (92).

As noted previously, XMYA as the major component and characteristic fatty acid of *Santalum spicatum* seed oil, is found only in traces during the early stages of seed development. Other workers have found that, for example, ricinoleic acid (12-hydroxy-9-octadecanoic acid), was not found in very young castor bean (*Ricinus communis*) embryos (156); crepenynic acid was not detected in four day old embryos of *Crepis rubra* (92), and no erucic acid was produced before six days after petal fall in *Crambe abyssinica* (157). In addition, the above reports may suggest that oleic acid may serve as a precursor for several unusual fatty acid in different plants but only at a late stage of seed development.

In the present study, the pattern of fatty acid synthesis varies even during the period in which XMYA was present in trace amounts. In the first stage of seed development (DAF 7-84), the fatty acid produced most rapidly was 18:3(n-3). This increased from 25% to 35%, in contrast to 18:2(n-6) which decreased from 25% to 15%. Previous studies by Privett *et al.* have provided convincing evidence that 18:3(n-3) in soybean also increases rapidly in the initial stages of seed development commencing with 16:0 and 18:0 as the predominant fatty acid, while 18:2(n-6) and 18:3(n-3) occur only in

lower proportions; with maturation the proportion of 16:0, 18:0 and 18:3(n-3) decreased markedly while 18:1(n-9) and 18:2(n-6) increased significantly (152).

The data from the present study provides strong evidence for the interrelationship of certain fatty acids during seed development. While it is the relative percentage composition which is being reported here, nevertheless in absolute terms there appear to be strong relationships between the loss of 16:0, 18:2(n-6) and 18:3(n-3), and the gain of 18:1(n-9) and XMYA. This would support the understanding that 16:0, might be a substrate for the synthesis of the products 18:1(n-9) and XMYA. The origin of XMYA, as an acetylenic fatty acid is further discussed in section 4.2.2.3.

An analysis of the fatty acid contents of *Santalum spicatum* leaves was also carried out in the course of this study. The leaves contained a relatively low content of lipid at about 0.5-1.5% and showed a low content (4.5-7.3%) of XMYA around the 119 DAF to maturation. It was demonstrated that the leaf lipids contained 16:0, 18:1(n-9) and 18:3(n-3) as the major constituents, as shown in Table 4.4.

Table 4.4 Relative Percentage Composition of Sandalwood Leaf Lipid Fatty Acids (around 119 to 154 days after flowering; trees grown at Curtin University)

Code	C16:0	C18:0	C18:1	C18:2	C18:3	XMYA
93Y02	16.07	1.48	26.81	2.07	47.30	6.27
93Y03	17.24	3.09	22.38	8.66	44.13	4.50
93Y08	14.36	1.52	27.21	3.87	45.86	7.18
93Y10	14.74	2.85	24.37	3.38	47.34	7.32
Average	15.60	2.24	25.19	4.46	46.16	6.32

4.2.2.2 Fatty acid composition of seed oil from different Western Australian locations

The results of FAME analysis of *Santalum spicatum* seed oil, collected from several different Western Australian locations are shown in Table 4.5 (155). Inspection of the

individual fatty acid using one-way analysis of variance demonstrates some significant differences between samples. Whether these differences have any real meaning is debatable. However, examining each fatty acid in turn, it can be seen that with regard to 16:0, samples from Koorda and Nanga are significantly different from the other samples; the Nanga 16:0 content is significantly different from that of Mt. Wilkinson and Marvel Loch. The 16:1(n-7) contents are low and show few differences, however Norseman, Mt. Wilkinson and Dryandra are significantly different from Everade.

The 18:0 contents are also normally low. The main observation is that Koorda and Mt. Wilkinson samples are significantly different to those from Shell Beach, Everade, Dryandra, Nanga, Norseman, and Kalgoorlie. Oleic acid, 18:1(n-9) is the major component in sandalwood seed oil occurring at about 54% average content. Marvel Loch and Mt. Wilkinson representing the low value outlier of the range, are significantly different to that from Dryandra.

The 18:2(n-6) content is low and (with respect to the range extremes) shows significant differences between samples from Shell Beach, Marvel Loch, and Nanga in comparison to those from Koorda, Everade, Mt. Wilkinson, Dryandra, and Klagoorlie.

α-Linolenic acid, 18:3(n-3) showed significant differences between Everade and the majority of the other samples, excluding Kalgoorlie, Dryandra, and Shell Beach.

Finally with regard to the acetylenic acid 18:1(9a) and XMYA, the Koorda 18:1(9a) relative content is significantly different from the other samples, while Nanga, Everade, Kalgoorlie, and Dryandra show significant similarity to each other. The second most abundant fatty acid is XMYA which occurs to about 32.5% over a range in the seed oil. Dryandra, representing the low value outlier of the range, is significantly different from those from Mt. Wilkinson and Marvel Loch which represent the highest values in the range.

A more specific examination of the relative proportions of individual fatty acid again demonstrates some significant differences between samples. However, it should be expected that a lower level of a particular fatty acid will be compensated by a higher value or values elsewhere in the fatty acid profile. Simple correlations, for example with particular regard to the most abundant acids, 18:1(n-9) and XMYA, occurring at an average of 54.1% and 32.1%, respectively, and constituting 86.2% of the whole,

Table 4.5 Fatty Acid Composition of Stored Ripe *Santalum spicatum* Seed Oil from Different Western Australian Locations

Location	16:0	16:1	18:0	18:1	18:2	18:3	C18:1(9a)	XMYA
Dryandra	4.36 ±0.19 abc*	0.63 ± 0.09 b	2.73 ± 0.07 bcd	58.74 ± 1.24 a	1.65 ± 0.10 ab	2.73 ± 0.25 cd	1.28 ± 0.01 bc	27.91 ± 1.22 b
Everade	5.41 ± 0.15 abc	1.32 ± 0.02 a	2.47 ± 0.12 cd	55.23 ± 1.31 ab	1.47 ± 0.08 ab	2.41 ± 0.10 d	0.93 ± 0.01 dc	30.51 ± 0.37 ab
Kalgoorlie	4.30 ± 0.15 abc	0.79 ± 0.01 ab	3.29 ± 0.04 a	56.49 ± 0.67 ab	1.73 ± 0.05 a	2.92 ± 0.01 cd	1.03 ± 0.18 dc	28.79 ± 0.26 ab
Koorda	5.50 ± 0.32 ab	0.86 ± 0.25 ab	1.83 ± 0.09 e	53.64 ± 1.10 ab	1.41 ± 0.08 bc	3.28 ± 0.15 abc	2.34 ± 0.13 a	31.66 ± 1.41 ab
Marvel Loch	3.97 ± 0.71 bc	0.90 ± 0.16 ab	2.29 ± 0.12 de	50.57 ± 3.11 b	0.94 ± 0.02 d	3.16 ± 0.08 abc	0.82 ± 0.03 d	37.27 ± 4.07 a
Mt. Wilkinson	3.90 ± 0.10 c	0.60 ± 0.03 b	1.90 ± 0.10 e	50.78 ± 0.04 b	1.48 ± 0.04 ab	3.77 ± 0.13 a	0.86 ± 0.04 d	36.71 ± 0.14 a
Nanga	5.75 ± 0.40 a	0.85 ± 0.18 ab	2.93 ± 0.00 abc	55.47 ± 0.72 ab	1.09 ± 0.01 d	3.12 ± 0.06 bc	0.90 ± 0.04 dc	29.90 ± 1.20 ab
Norseman	5.16 ± 0.04 abc	0.55 ± 0.02 b	3.13 ± 0.06 ab	54.17 ± 2.07 ab	1.17 ± 0.01 cd	3.67 ± 0.04 ab	1.53 ± 0.05 b	30.64 ± 2.02 ab
Shell Beach	4.67 ± 0.11 abc	0.79 ± 0.02 ab	2.44 ± 0.19 cd	52.21 ± 0.60 ab	0.92 ± 0.03 d	3.01 ± 0.11 cd	0.86 ± 0.05 d	35.13 ± 0.81 ab
Average	4.78	0.82	2.56	54.15	1.33	3.12	1.18	32.06

In this Table, each value represent the mean ± S.D., n = 3

ab* same letters indicate samples which are not significantly different ($p < 0.05$) within a column using the One-Way ANOVA analysis.

exhibit no particular comparative richness relatable to a geographic locality. Therefore, while the relative abundance of the fatty acid may vary, they may not be taken as indicators of a particular ecotype of *Santalum spicatum* (155).

The relationship between geographic location and fatty acid composition has been recognised for many oil seed crops (158). For example, in sunflower seed oil, it was found that percentage of linoleic, oleic and stearic acids present significant differences between growing areas. It was also found a correlation between the contents of linoleic and oleic, and the increase of one results in a similar decrease of the other (158).

An investigation of kernel composition of *Santalum spicatum* seeds from different Western Australian locations has demonstrated an apparent inverse relationship with associated polynomial regression analysis showing:

[r = 0.971, XMYA% = 385.0 - 11.87(oleic%) + 0.1(oleic%)^2] between the relative concentration of 18:1(n-9) and XMYA, as shown in Figure 4.4. The metabolic origin of XMYA is still unknown but this finding substantiates the suggestion that 18:1(n-9) could be the precursor of XMYA (90). Thus the fall in the relative proportion of 18:1(n-9) may indicate synthesis of the new product XMYA.

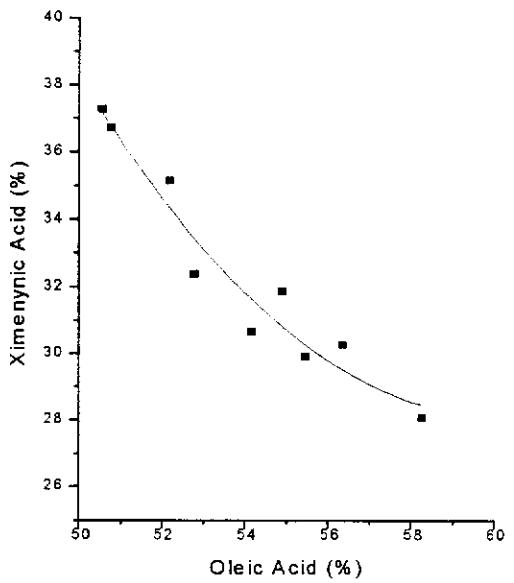


Figure 4.4 The relationship between oleic and ximenynic acids in sandalwood seed oil (155).

4.2.2.3 The biosynthesis of ximenynic acid

The biosynthesis of fatty acid in seeds has been extensively investigated in the past fifty years (84-88) as discussed previously (Chapter 1). Since most abundant unsaturated C18 fatty acids such as oleic acid occur naturally in major plant seed oil, the production of these acids has been extensively investigated. Using labelled [^{14}C] stearate as precursor, it was observed that certain plant enzymes introduced the first double bond in the Δ9 position to form 18:1(n-9) (87). In 1975, it was pointed out by Appelqvist that there is a simple precursor to product relationship in the biosynthesis of these acids, with the normal sequence of biosynthesis being 18:1(n-9) → 18:2(n-6) → 18:3(n-3), and involving an oxygen-dependant desaturation processer (150). Similarly, investigation of the formation of γ-linolenic acid [all *cis*, 6,9,12-octadecatrienoic, 18:3(n-6)] in developing evening primrose seeds (*Oenothera biennis* L.), showed that both 18:3(n-3) and 18:3(n-6) were synthesised in the early stages of seed development by Δ15 and Δ6 desaturation of 18:2(n-6), respectively, but that the activity of the Δ6 desaturase predominates in the seed during final maturation (159).

In addition, some interesting information has been obtained about the synthesis of many of the unusual fatty acids such as ricinoleic acid which constitutes about 85% of castor oil fatty acid contents (156). This acid was found to be formed by stereospecific hydroxylation at carbon 12 of 18:1(n-9) to give the D-isomer, the hydroxylation was catalysed by a microsomal-bound enzyme, and there was no evidence on the conversion of 18:2(n-6) to ricinoleic acid either in the intact or cell free systems (156). Thus, 18:1(n-9) was considered as the direct precursor of ricinoleic acid (156). This reaction was considered as the best characterised of the biosynthesis of 'unusual' fatty acids. As noted earlier an inverse relationship between the relative concentrations of 18:1(n-9) and XMYA has been observed. This result perhaps further supports the Bu'Lock and Smith suggestion that 18:1(n-9) is the XMYA precursor (33).

The co-occurrence of stearolic acid and XMYA in the seed oil of the *Santalaceae* family may indicate some biogenetic relationship, and it was speculated by Bu'Lock that 18:1(9a) may arise from 18:1(n-9) and is then converted to XMYA by desaturation at C11,12, replacing the usual conversion into 18:2(n-6) (90).

In a study of the biosynthesis of crepenynic acid, the authors indicated that there is no conversion either from *cis*, *cis*-linoleic acid, *cis*, *trans* (or *trans*, *cis*)-linoleic acid, or from *cis*-12,13-epoxy-oleic acid to crepenynic acid by a seed tissue preparation (92). However, as noted previously there is also no evidence for conversion of 18:2(n-6) to ricinoleic acid (156). Linoleic acid may be a intermediator during the formation of these unusual fatty acids.

A hypothesis arising from this study is that linoleic acid 18:2(n-6) could be the precursor during seed development for the production of conjugated linoleic acid [18:2(9c,11t)], which was detected in the seed fixed oil at very low concentration (0.1% relative total fatty acids content). This could occur by isomerisation of linoleic acid, and then the 18:2(9c,11t) further dehydrogenated to form XMYA. Thus, the conversion may be summarised as shown below:

18:1(n-9)	→	18:2(9c, 11c)	→	18:2(9c, 11t)	→	18:2(9a, 11t)
oleic		linoleic		conjugated linoleic		XMYA

Based on this hypothesis, the occurrence of isomers of XMYA may be explained as arising by isomerisation at C11, 12 during conversion of 18:2(n-6) to 18:2(9c, 11t). The presence of 18:2(9c, 11t) was not investigated in seedlings although XMYA occurs in *Santalum spicatum* leaf lipids at 4.5-7.3% of total fatty acids (Table 4.4) as this present study was only concerned with fatty acid changes in developing seed.

4.3 CONCLUSIONS

In this chapter data has been presented on the chemical composition of developing *Santalum spicatum* seeds at different stages of maturity. It has been demonstrated that the moisture content decreased significantly, in association with large increases in lipid and protein contents during seed development. Lipid deposition in significant amounts occurs during the period of 91 DAF to 154 DAF, seen as a rapid phase of oil accumulation. XMYA was apparently not synthesised in the early stage of seed development. With maturation after 91 days from flowering the proportions of 16:0, 18:2(n-6) and 18:3(n-3) fell markedly with corresponding increase in proportion for 18:1(n-9) and XMYA. The overall pattern of fatty acid change during seed development may be indicative of a precursor effect operating particularly regarding the relationship between XMYA and 18:1(n-9) as previously noted (page 95 and Figure 4.4). It would also appear that the biosynthesis of fatty acid in *Santalum spicatum* is unusual in its production of acetylenic fatty acids as end product rather than 18:2(n-6) and 18:3(n-3) as found in other plant seed oils. It suggests the presence of unusual fatty acid metabolising enzymes which are very active during the later stages of seed development. The geographical origins such as latitude, rainfall, mean temperature or soil types may have some influence over seed fatty acid composition with XMYA ranging from 27.9 to 37.3% of total fatty acids in the seed lipids, however the nature of this is unclear from these studies.

CHAPTER 5: LIPID ANALYSIS

5.1 INTRODUCTION

The changes of lipid content and fatty acid composition in developing *Santalum spicatum* seeds have already been reported in previously in Chapters 4. Fatty acid analysis gives a large amount of information on the seed oil. This chapter describes attempts to separate the seeds oil into various molecular species present in each triacylglycerol. This study also compares the results of total oil content between Soxhlet and cold solvent as the extraction methods.

5.2 RESULTS AND DISCUSSION

5.2.1 Extraction of oil from sandalwood seed

Lipid extracted from oil-seed consists mainly of triacylglycerols, together with small amounts of phospholipids, glycolipids, sterol esters and free fatty acids (less than 2%) (30, 56). It is known that the seed quality can affect the oil content. Therefore, selected *Santalum spicatum* seed was used for the comparative oil extraction. Oil was extracted from the mature seed kernels using both the Soxhlet and cold solvent methods (54, 55). The results of the Soxhlet method gave a significantly ($p = 0.0002$) higher yield of $55.3 \pm 1.2\%$ than the cold solvent procedure which yielded $52.2 \pm 0.9\%$ of oil. Both techniques display excellent precision, with low values of standard deviation. The better results of the Soxhlet method are probably due to the increased ability of a hot solvent to overcome hydrogen, ionic and Van der Waals forces which bind lipids within the sample matrix (23).

Compared with the Soxhlet method the major advantages of the cold solvent method of oil extraction are: 1) the solvent toxicity is relatively low, 2) it requires smaller

sample sizes (0.5 g) and smaller volumes of solvent (10 mL), and 3) heat is not required to facilitate the extraction. Due to the above advantages, the cold solvent method was used in this study as the general procedure to determine the fruit and seed lipid content.

The advantages of the Soxhlet method are that is efficient and easily adapted to large sample sizes to be extracted and a shorter time requirement. For example, 1.5 kg ground *Santalum spicatum* seed kernel was extracted using the Soxhlet apparatus in this study to produce oil suitable for the animal feeding experiment.

Hatt and Schoenfeld (4) observed that about 2% of a rubber-like resin is present in quandong (*Santalum acuminatum*) seed oil, and also indicated its presence in *Santalum spicatum*, but without estimation (4). As the oil extracts were to be used for animal feeding, the rubber-like resin was removed by dissolving the oil in acetone with consequent precipitation of the resin which could be determined by filtration and gravimetric determination of the residue. The resin content of *Santalum spicatum* seed oil was thus determined as 2.4 ± 0.2% (n = 10). The chemical nature of this resin is unknown. Therefore the seed oil was de-resinified to remove this unknown factor from the present considerations of sandalwood seed oil ingestion and fatty acid uptake in the test animals. This process involved the use of solvent acetone, which like many organic solvents, has been abandoned in food component treatments in many countries. Thus, solvent residues were removed as completely as possible using reduced pressure. Experiments on feeding whole seed oil to mice may be part of a future study.

5.2.2 Chromatographic separation of lipid classes

5.2.2.1 Lipid separation by thin layer chromatography

As has been noted TLC is a well established and reliable routine for the separation of individual lipid classes (56, 58). There are several excellent solvent systems that yield pure fractions for sterol esters, triacylglycerols, free fatty acids, diacylglycerols and monoacylglycerols.

The total lipid extract from *Santalum spicatum* seed may be separated into the lipid classes by TLC using a F_{254} (inorganic phosphor) plate with *n*-hexane/diethyl ether/acetic acid (70:30:1, by volume). With this solvent system, five neutral lipid fraction bands were present after TLC development, two minor with R_f values 0.26 and 0.90, and three major with R_f values 0.58, 0.67 and 0.75, respectively. A typical developed TLC plate profile is illustrated diagrammatically in Figure 5.1 using UV light at 254 nm to visualise the acetylenic fixed oil fractions.

The separation of the individual lipid classes by TLC depends upon the polarity of the eluting solvents. The neutral lipid can be isolated on the basis of polarity by means of adsorption chromatography (56). A previous study using a similar solvent system successfully separated a standard neutral lipid mixture of cholesterol ester (Rf = 0.91), triacylglycerol (Rf = 0.68), free fatty acid (Rf = 0.59), cholesterol (Rf = 0.35), diacyglycerol (Rf = 0.31) and monoacyglycerol (Rf = 0.03) by one-dimensional preparative adsorbent TLC (66). By use of this solvent system, the phospholipids are always present at the origin, i.e. Rf = 0. According to the above reference (66), the least polar compound migrates furthest, therefore in the separation of sandalwood seed oil components, the band near the solvent front (Rf = 0.90) comprises sterol esters, followed by three major triacylglycerols bands (Rf = 0.75, 0.67 and 0.58) and then diacylglycerols (Rf = 0.26). The phospholipid (Rf = 0.00) and neutral lipid classes are well resolved (66).

The bands representing phospholipid (Rf = 0.00) and triacylglycerols (Rf = 0.75, 0.67 and 0.58) were scraped off, and the individual components were eluted quantitatively with chloroform/methanol (1:1, by volume). After adding 1 mL (1 mg/mL) heptadecanoic acid as an internal standard, the fatty acid composition of each band was determined by subsequent analysis of the methyl ester derivatives using GC/MS under the condition described previously. The results of FAME analysis of the triacylglycerol and phospholipid bands isolated by TLC are summarised in Table 5.1.

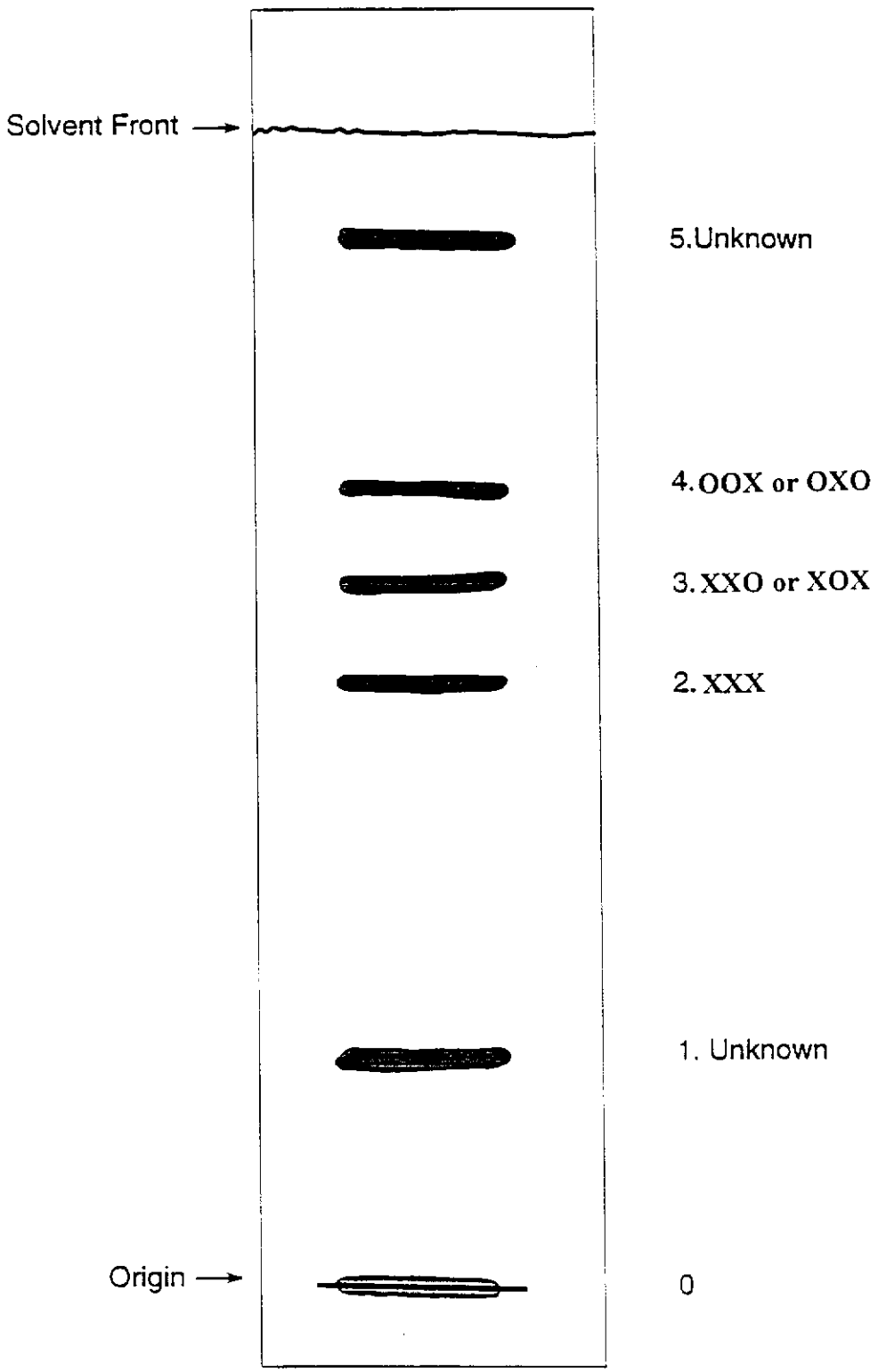


Figure 5.1 Schematic thin layer chromatography separation of *Santalum spicatum* seed oil on a silica G F_{254} plate. Solvent: hexane/diethyl ether/acetic acid (70:30:1, by volume) (where O = oleic acid and X = ximenynic acid).

Table 5.1 Fatty Acid Composition (mol%) of *Santalum spicatum* Seed Oil and Isolated Fractions.

Band	16:0	16:1(n-7	18:0	18:1(n-9)	18:2(n-6)	18:3(n-3)	18:1(9a)	XMYA
Band 4	5.76	1.31	3.14	55.93	3.47	4.09	1.85	24.45
Band 3	3.06	tr	1.65	33.30	1.36	3.03	2.01	55.62
Band 2	tr	tr	tr	tr	tr	tr	nd	99.90
Band 0	56.52	tr	nd	43.48	nd	nd	nd	nd
Oil	3.53	0.15	1.92	52.83	1.86	3.52	1.51	34.68

tr = trace (<0.01%); nd = not detected

It was thus demonstrated that oleic (O) and/or ximenynic (X) acids were the important constituents and that while other fatty acids were detected they were present in low concentrations only, probably representing other 'contaminant' minor triacylglycerols. Examination of the FAME results determined as described (2.3.3.1, page 35) indicated that band 4 (Rf = 0.75) was principally composed of two oleic acid residues and one XMYA residue, thus being the triacylglycerols OOX and/or OXO. Band 3 (Rf = 0.67) was composed of two XMYA residues and one oleic acid residue, suggesting the triacylglycerols XXO and/or XOX. Finally, band 2 (Rf = 0.58) was shown to be solely composed of XMYA and therefore XXX, triximenynin. Stearolic acid, a normal constituent of the seed oil (*ca.* 1%) was only detected at low concentrations in bands 4 (Rf = 0.75) and bands 3 (Rf = 0.67). Band 2 (Rf = 0.58) was surprisingly pure, showing only faint traces of other fatty acids. The 'XXX' determined by FAME could also represent free XMYA as indicated by the previously mentioned trial study (58), however further identification by HPLC and GC/MS confirmed the band as a triacylglycerol of triximenynin. Band 1 and band 5 are unknown compounds in low concentration which may be fractions of free fatty acids and sterol esters, respectively. GC/MS analysis failed to provide sufficient proof of specific identity.

The present experiment also revealed that both acetylenic acids, XMYA and stearolic acid are absent from the phospholipid. This observation is in agreement with an earlier report (44) that acetylenic acids present in mosses were not detected either in the glyco- or in the phospholipid fraction, but were all contained in the neutral lipid fraction. The origin-located phospholipid contained a higher concentration of palmitic acid and a low concentration of oleic acid in comparison to band 3 and band 4 the neutral lipid fractions.

5.2.2.2 Lipid separation by high performance liquid chromatography

The chromatogram of the seed oil triacylglycerols by non-aqueous reverse-phase HPLC is shown in Figure 5.2. Three major peaks were revealed using a μ-Bondapak™ C18 column with refractive index detection.

The separation of triacylglycerols by reverse-phase HPLC with various high-efficiency packed columns and mobile phase has been well documented (134, 160-161). In 1981, El-Hamdy and Perking proposed that under isocratic conditions, the logarithm of the elution volume of a triacylglycerols is directly proportional to the total number of carbon atoms (CN) and inversely proportional to the total number of double bonds (n) in the three fatty acyl chains of the triacylglycerols molecule (62). This elution behaviour of triacylglycerols molecular species is described by the 'equivalent carbon number (ECN),' which may be defined as:

$$ECN = CN - 2n$$

where CN = actual number of carbon atoms, n = number of double bonds per molecule. In general, triacylglycerols having the same ECN value such as tripalmitin (ECN = 48) and triolein [ECN = 54 - (2 × 3) = 48] tend to elute close together.

Applying this system, a previous study using acetone/acetonitrile (63.6:36.4, by volume) as mobile phase on a C18 column, eluted olive oil triacylglycerols by reverse-phase HPLC in the sequence of LOL, LOO, PLO, OOO, POO, POP, SOO and SOP (L, linoleic; O, oleic; P, palmitic; S, stearic) with ECN of 44, 46, 46, 48, 48, 48, 50 and

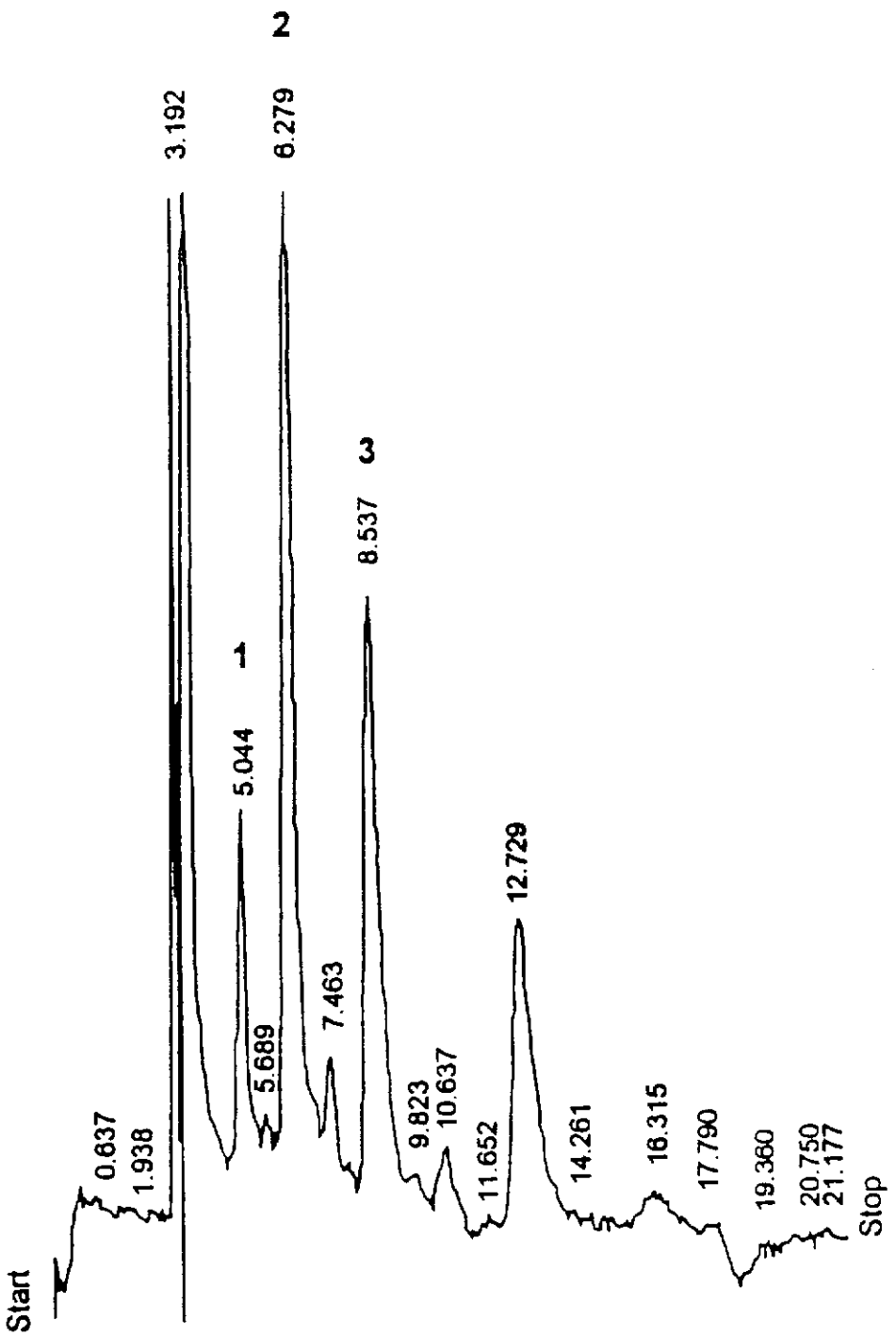


Figure 5.2 Reversed-phase high performance liquid chromatography (HPLC) of *Santalum spicatum* seed oil. HPLC conditions as described on page 38. Triacylglycerols: 1 = XXX (13.3%); 2 = XXO or XOX (51.5%); 3 = OOX or OXO (35.3%) (where O = oleic acid and X = ximenynic acid).

50, respectively (62). It was observed that the pattern of triacylglycerols elution sequence starts with the lowest ECN triacylglycerol and finishes with the highest one. OOO and PPP are triacylglycerols in which the individual fatty acid, oleic and palmitic are critical pairs with the same ECN: [18 - (2 × 1) = 16], and hence the ECN of the triacylglycerols are both 48. triacylglycerols having the same ECN do not necessarily co-elute but appear in the sequence with the more unsaturated and hence more polar fatty acid eluting first. Hence OOO would elute before PPP and in fact the elution of the total 'sequence' of O and P triacyglycerides, all with ECN of 48, would be: OOO, OOP, OPP and PPP.

Recently, the triacylglycerols of conjugated fatty acid in reverse-phase HPLC have been studied by Chang and coworkers (162). In the study of Tung oil, these authors gave a value of ECN = 36 for trieleostearin, in which eleostearic acid (*cis*-9, *trans*-11, *trans*-13-octadecatrienoic acid) has three conjugated double bonds and therefore has the same ECN as linolenic acid (162).

Unfortunately, there have been no reverse-phase HPLC studies on acetylenic acids. In fact, mono-acetylenic acids are isomeric with the corresponding diethylenic acids and therefore, the ECN of a mono-acetylenic acid may be equal to its corresponding isomeric diethylenic acid. Thus, a hypothesis raised in this study is that XMYA and linolenic acid are critical pairs [XMYA ECN: 18 - (2 × 3) = 12] according to their ECN value. Similar calculations would give, XXX (ECN = 36), XOX (ECN = 40) and OXO (ECN = 46). According to the elution order, triacylglycerols with the lowest ECN elute first, therefore it may be predicated that the HPLC fractions 1, 2 and 3 represent XXX, XOX/OXX and OXO/XOO, respectively. This was confirmed by FAME analysis of these fractions described below.

Generally, triacylglycerols separated by HPLC can be identified either by correlation between the chromatographic behaviour and structure of a compound, or on the basis of fatty acid analysis. In this study, the assignment of peak identities was supported by the intermediate use of TLC which also yields three major triacylglycerol bands. HPLC analysis of each major band showed a direct comparison with the TLC results.

In TLC the major band of higher Rf values (0.75) was equivalent to peak 3 of the HPLC spectrum. Similarly, the middle TLC bands at Rf values (0.67) and Rf (0.57)

were equivalent to HPLC peak 2 and 1, respectively.

These findings are in contrast to a previous study by Jones and his coworkers on the related species quandong (*Santalum acuminatum*) which contains *ca.* 40% XMYA in the seed oil (25). The significant triacylglycerols were determined, using HPLC, to be XOX (or XXO), and OOX and OXO only, however, the presence of triximenynin (XXX) was not demonstrated in *Santalum acuminatum* (25).

5.2.2.3 Lipid separation by gas chromatography

Triacylglycerols may be directly analysed with a high degree of resolution by high-temperature capillary GC (66). On a polar column, triacylglycerols are separated mainly according to fatty acid chain length with further separation being based on the different combinations of saturated and unsaturated fatty acids (70). In principle, the polarity of a triacylglycerol increases with the degree of unsaturation in fatty acid. For example, the typical polarity sequence for C18 fatty acids increase from stearic, through oleic, linoleic to linolenic acids (146). The elution sequence for a triacylglycerols of 54 carbon number (C54), e.g., typical stearyl (S)/oleyl (O) triacylglycerols was reported as SSS, SOS/SSO, SOO/OSO and finally OOO, that is the less polar triacylglycerols elute before the more polar triacylglycerols (67). Accordingly, capillary column on a Wide-bore (WBOT, 0.53 mm, i.d.) analysis of the C54 triacylglycerols from palm oil has separated them in the sequence of SOS, SOO/SLS, OOO/SLO, OLO, OLL (67).

Generally, triacylglycerols separated by GC may be identified through chromatographic retention characteristics, or using the interpretation of triacylglycerol profiles obtained from other techniques such as HPLC or TLC. In the present study, a mixture of authentic standard triacylglycerols tripalmitin (PPP) and triolein (OOO) was used in order to estimate the proportional distances between C48 and C54 triacylglycerols, and the results are shown in Figure 5.3.

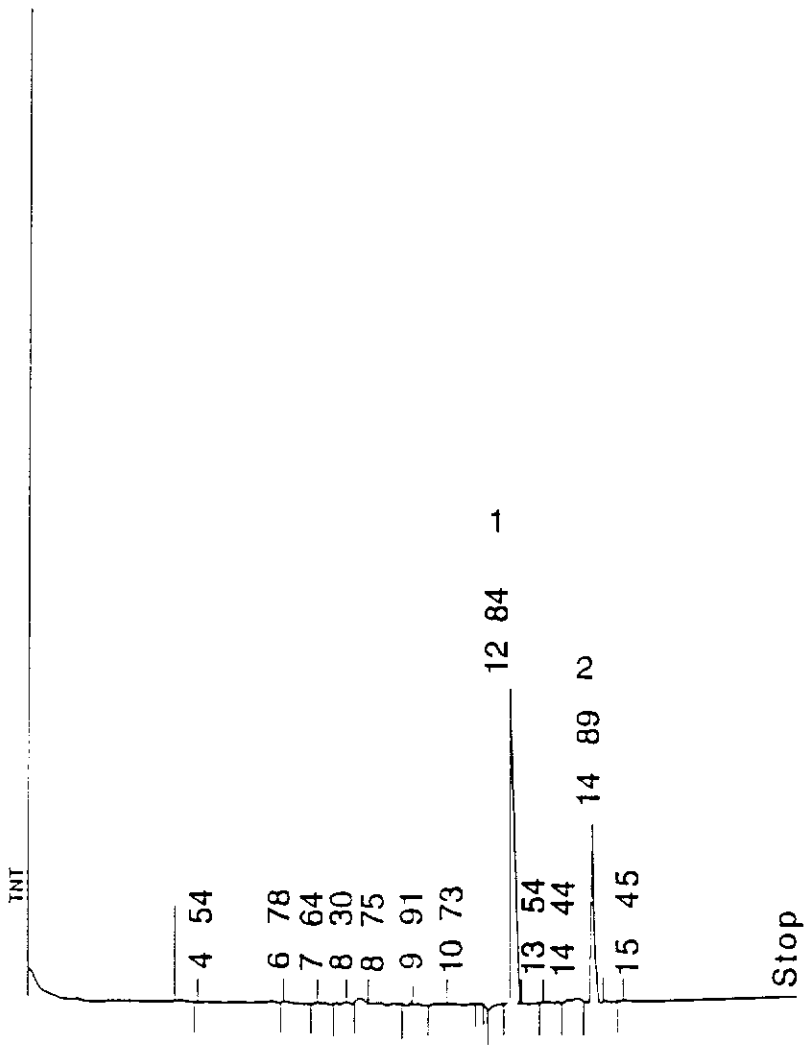


Figure 5.3 Gas chromatography analysis (GC) of triacylglycerol standard mixture. GC conditions as described on page 38. Triacylglycerol: 1 = tripalmitin 2 = triolein).

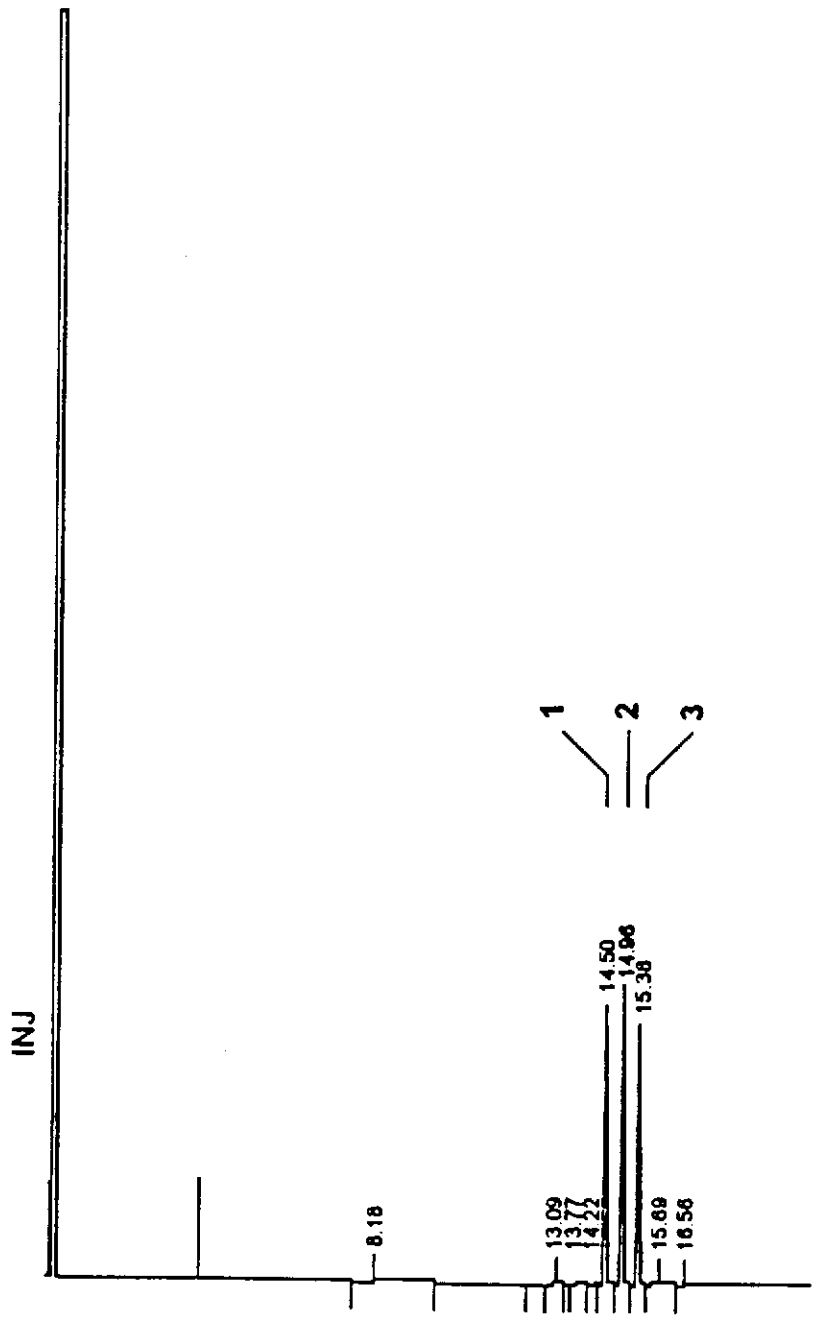


Figure 5.4 Gas chromatography analysis (GC) of *Santalum spicatum* seed oil. GC conditions as described on page 38. Triacylglycerols: 1 = XXO or XOX (37.1%); 2 = OOX or OXO (34.9%); 3 = XXX (28.1%) (where O = oleic acid and X = ximenynic acid).

The chromatographic analysis of sandalwood seed oil triacylglycerols on a HT-5 column by GC/FID over 20 min. is shown in Figure 5.4, with three distinct peaks being produced.

The three major bands at Rf = 0.58, Rf = 0.67 and Rf = 0.75 isolated by TLC were further analysed by GC/FID and demonstrated to be relatively pure, and were confirmed to be present as triacylglycerols by comparison with standard PPP and OOO (Figure 5.3) and consideration of their retention times. Finally, FAME analysis using GC/MS confirmed the three major peaks separated by GC/FID, HPLC and TLC to be XOO/OXO, XOX/OXX, and XXX, respectively.

5.3 CONCLUSION

Lipids can be completely extracted from sandalwood seeds using different extraction procedures. The extracts contain a complex mixture of different lipid classes which are best analysed using chromatographic procedures, i.e., TLC, HPLC, GC/FID and GC/MS. TLC is most useful for the separation and identification of intact lipid classes. The individual fractions may be further analysed using other chromatographic techniques. A reverse-phase HPLC with RI has been used for the analysis of triacylglycerols. The recently developed detection methods such as UV, FID and light scattering detection systems overcome the compatibility problems of the refractive index detectors for HPLC, e.g., the use with gradient elution. GC/FID and GC/MS have also been used to separate and quantify lipid classes as molecular species. The fatty acid compositions of each triacylglycerol isolated from *Santalum spicatum* seed oil was investigated by using GC/MS and three major triacylglycerols: OOX/OXO, OXX/XOX and XXX (where O = oleic acid; X = ximenynic acid) were identified in the seed oil.

CHAPTER 6: NUTRITIONAL AND TOXICITY ANALYSIS

6.1 INTRODUCTION

Chemical analyses of *Santalum spicatum* seed oils showed that the oil contains significantly high amounts of XMYA, an unusual fatty acid which does not occur in other common edible oils (Chapter 3, 4 and 5). Although kernels of the sandalwood seeds have been eaten in the past without apparent harm, concern has been expressed about the possible long term toxicity of the acetylenic fatty acid, which have been found to inhibit enzymes of prostaglandin synthesis (117-118, 128-130). Recently, some aspects of the metabolism of XMYA, present in quandong (*Santalum acuminatum*) seed oil, were studied by Jones *et al* (100). It was demonstrated that XMYA was incorporated into different tissues only to a minor extent (0.5-5.4%). Little specific information is currently available about mammalian biochemical pathways utilising XMYA.

This chapter describes the effects of feeding sandalwood seed oil on mice growth rate, serum aspartate aminotransferase (ASAT) activity (limited availability of mouse serum to be collected on a regular basis without killing the animal), tissue lipid content and fatty acid composition and histopathology, that may provide information on the metabolic utilisation of XMYA and its potential for anti-inflammatory activity.

6.2 RESULTS AND DISCUSSION

6.2.1 Fatty acid composition of dietary fats

Dietary fat contains a mixture of three types of saturated, monounsaturated and polyunsaturated fatty acids. The quantity and type of dietary fatty acid ingestion by human and animal has considerable influence on health and disease. For example,

intake of saturated fatty acid increases the risk of coronary heart disease, while consumption of monounsaturated fatty acid is associated with relatively low incidence of coronary heart disease (163).

The fatty acid compositions of the standard lab diet, canola oil-enriched and sandalwood seed oil-enriched diets as measured by GC/MS are shown in Table 2.4 (pp. 45). The control diet standard lab diet is particularly rich in saturated fatty acids (16:0 and 18:0 represent 41.8%, by weight). Canola oil is characterised by the presence of C18 unsaturated fatty acids, especially 18:1(n-9) and 18:2(n-6), which represent 89.9% (by weight) of total fatty acids, while the sandalwood seed oil diet contains high levels of 18:1(n-9) (represents 52.8%, by weight), and is significantly enriched in XMYA (34.7%, by weight).

The ratio of saturated fatty acid to monounsaturated fatty acid in standard lab diet, canola oil and sandalwood seed oil diets are 1:0.9, 1:10 and 1:7.9, respectively. It was because of the general similarity of the fatty acids in canola oil and sandalwood seed oil diets, that the canola oil diet was used as a high fat control diet. The quandong diet used by Jones *et al.* (100), incorporated a mixture of quandong and canola oil in 6:1 ratio giving relative fatty acid contents of 18:1(n-9) 51%, and XMYA 34%. Such contents are similar to the general fatty acid character of the sandalwood seed oil diet in this study. In addition, they also used canola oil-enriched feed as the high fat control diet (100), and provided an interesting parallel for comparison with this study.

In effect, we are comparing standard lab diet (5% fat, normal control) with high fat (15% canola oil, high fat control) diet, with the high fat canola oil diet then becoming a second control for sandalwood seed oil (15% by weight, test group C). This justifies the use of canola oil diet as a control for the sandalwood seed oil diet. A comparison of fatty acid tissue changes was made between mice fed sandalwood seed oil for eight weeks and these fed sandalwood seed oil for four weeks (group D).

6.2.2 Animal growth and food intake

Changes in food intake and mouse weight are shown in Table 6.1. Significant diet-related differences were observed between the mice fed standard lab diet, canola oil and sandalwood seed oil, and are displayed in Figure 6.1.

The starting body weight of animals in groups A, B and C were not statistically different. The animals were randomly chosen when setting up the diet groups and the apparent observed variation is not due to any specific selection process.

The mice (initial weights of about 26 g) gained weight whether they were fed on standard lab diet, canola oil or sandalwood seed oil diets. Thus the growth rates on average were group A, standard lab diet = 0.51 ± 0.21, group B, canola oil = 0.79 ± 0.44 and group C sandalwood seed oil = 0.38 ± 0.16 (g/week) over eight weeks feeding. The group D (subset of eight mice fed the standard lab diet for the first four weeks and then the sandalwood seed oil diet for the remaining four weeks) showed a weight gain of 0.27 ± 0.15 (g/week) in the final four weeks. In contrast, mice in the standard lab diet group gained 0.36 ± 0.32 from week four to eight on the standard lab diet. Statistical analysis indicated that there was no significant difference in growth rate (g/week) between the standard lab diet and sandalwood seed oil-fed groups over the whole eight week period.

Although the sandalwood seed oil-diet contained 15% fat, it neither promoted nor suppressed the growth of mice. Clearly, the growth rate of the sandalwood seed oil-fed group was comparable to those on standard lab diet-feed even though it was a high fat diet. However, when growth rates are compared between sandalwood seed oil- and canola oil-fed groups, significant differences were found in weeks 1, 4, 5, 6, 7 ($p<0.05$). The growth of canola oil-fed group is significantly greater than both the standard lab diet- and sandalwood seed oil-fed groups in weeks 1, 5 and 7.

Table 6.1 Mean Body Weights of Mice Fed Test or Control Diets for Eight Weeks

Diet	AFI[#] (g/day)	Animal number	Body weight (g)									Change g/week
			0	1	2	3	4	5	6	7	8	
SLD*	3.85	15	25.67 ± 1.74[a]	26.29 ± 1.54[a]	26.42 ± 2.02[a]	27.97 ± 2.00[a]	28.23 ± 2.25[ab]	27.36 ± 1.78[a]	28.39 ± 2.89[ab]	27.97 ± 2.16[a]	29.72 ± 2.60[a]	0.51 ± 0.21
CO*	3.00	15	27.32 ± 2.06[a]	27.75 ± 2.55[b]	28.90 ± 2.90[b]	29.71 ± 3.45[a]	30.98 ± 3.79[a]	30.12 ± 4.40[b]	31.90 ± 4.17[a]	31.07 ± 4.28[b]	32.68 ± 4.89[a]	0.79 ± 0.44
SWO*	2.24	15	26.46 ± 2.79[a]	25.77 ± 2.41[a]	27.11 ± 2.20[ab]	27.62 ± 2.39[a]	28.21 ± 2.85[b]	27.73 ± 2.42[a]	28.17 ± 2.69[b]	28.58 ± 2.28[a]	29.49 ± 3.11[a]	0.38 ± 0.16
SWO (4 wks)	nd	8	nd	nd	nd	nd	28.16 ± 2.30	27.97 ± 2.38	28.41 ± 2.44	28.20 ± 1.92	28.49 ± 2.15	0.27 ± 0.15

In this Table, each value represents the mean ± S.D.; nd = not determined.

Same letters (a, b and ab) indicate samples which are not significantly different ($p<0.05$) within a column using the One-Way ANOVA analyses.

AFI[#] = Average food intake g/day per animal over test period.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

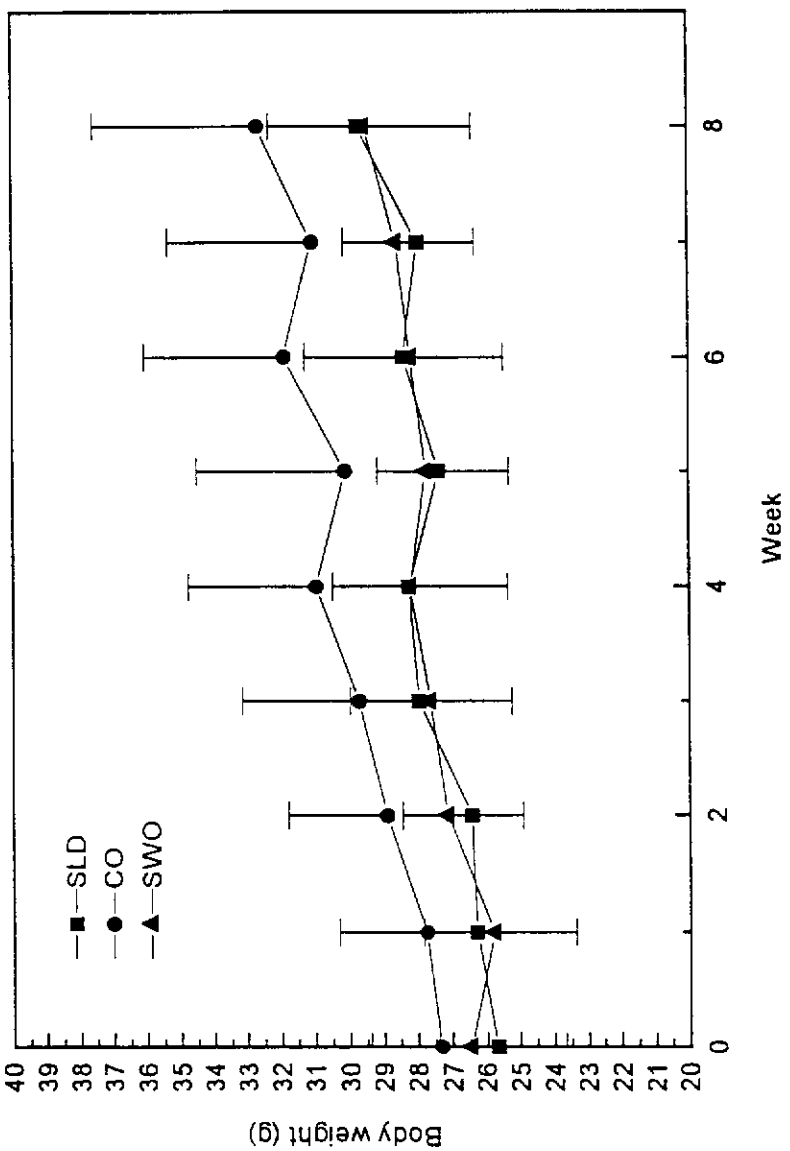


Figure 6.1 The growth rate of mice receiving standard lab (SLD), canola oil (CO) or sandalwood seed oil (SWO) diets.

The standard lab diet has been tested and refined over many years and is considered an adequate diet for healthy mouse growth (135). Based on the fat content of the three diets, such data may indicate that growth rates are depressed in mice receiving the sandalwood seed oil diet when compared with mice in the canola oil-fed group. The decreased growth rates of the sandalwood seed oil group C (compared with group B canola diet mice) may be due to poor utilisation of the diet. Possibly, by some mechanism, the XMYA content may counteract the otherwise high fat content of the diet. Although there is some evidence in the literature on the metabolism of acetylenic acid (98-100), no clear data are available on actual changes that occur in animal body after either short term or long term digestion of these acids.

Appreciable differences in feed intake were observed. Over the full eight weeks experimental period, the average daily food intake of mice fed sandalwood seed oil and canola oil diets were 58% and 78% of the average standard lab diet intake, respectively. However, the available energy for each diet suggests that the mice on standard lab diet, canola oil and sandalwood seed oil consumed 0.056, 0.054 and 0.040 MJ respectively of available energy per day through the various diets (page 42, Table 2.3). Inspection of Table 2.3 demonstrates that there appears to be no limitation on the calculated energy level of the sandalwood seed oil-enriched diet. As noted in Table 2.4 (page 43), the sandalwood seed oil diet exhibits low level of 18:2(n-6) [1.9%; (18.9% in canola oil)], but has equivalent levels of 18:3(n-3) [3.5%; (3.5% for canola oil)] and is relatively rich in 18:1(n-9) [52.8%; (71.1% in canola oil, and 30.24% in standard lab diet)]. In fact, group C (15% sandalwood seed oil diet) mice failed to display any obvious signs of essential fatty acid deficiency, at least in the short term feeding, such as hair loss or obvious dermatological symptoms. However, it cannot be assumed that the sandalwood seed oil diet was totally satisfactory in term of energy supply or provision of essential fatty acid.

To a certain extent the reduced food consumption was balanced against the higher energy content of the diet. This was particularly evident when comparing the consumption data with those mice on the standard lab diet and canola oil diets. These

effects are consistent with a previous study (164), in which bovine steers fed a high-fat diet containing approximately 10% fat from rape seed, sunflower seed oil, or tallow, reduced their feed intake when compared with a 3% fat control diet. Despite the species differences in this study, it has been demonstrated that mice fed either sandalwood seed oil or canola oil had reduced feed intake, and differential feed efficiencies were also found. For example, mice gained body weight slowly in the sandalwood seed oil-fed group and fast in canola oil-fed group.

In growing animals, growth retardation is probably due to inadequate intake of food. A direct reason for the relative lower growth rate (compared with CO fed mice) on sandalwood seed oil-fed mice may be lower food consumption. Preliminary studies by other workers have shown that the unpleasant aromatic taste factor in quandong kernel was methyl benzoate, which occurred in concentration of about 32-1294 mg/kg of fresh weight (165). Organoleptic examination of the sandalwood seed oil diet showed the presence of a subtle, slightly aromatic taste which may also have acted as an appetite depressing factor. However the pellets were essentially odorless and retained their general physical structure. The graphical representation (Figure 6.1) suggests that mice on the sandalwood seed oil diet showed little significant difference in weight gain from the group A (standard lab diet) mice.

A previous report demonstrated that rats fed on diets providing 50% of energy requirement from rapeseed oil compared with the effect of a diet containing butterfat for six weeks, had reduced rates of weight gain (166). An appetite-depressing factor in rapeseed oil was considered to be important. In another case (167), rats fed rapeseed oil had slightly decreased food intake and a smaller weight gain compared to those fed on diets containing lard and soybean oil. Indeed, erucic acid which is present in high levels in rapeseed seed oil-enriched diet has been reported to be the substance responsible for the growth retardation (166).

On the other hand, the growth-retarding action or effect may be attributed to an unbalanced ratio of saturated fatty acid to monounsaturated fatty acid in dietary fat. A previous feeding study has shown the optimum ratio of saturated fatty acid to

monounsaturated fatty acid in diet fat to be 1:0.7 to 1:0.2. When the ratio was decreased to 1:1.8, a lower growth rate was observed (166). Accordingly, the canola oil and sandalwood seed oil diets used in the present study have a similar ratio of saturated fatty acid to monounsaturated fatty acid, and both of them are far less than the optimum ratio. However, different growth rates were observed, and the relative lower growth rate of sandalwood seed oil-fed mice is probably due to its high content of XMYA.

If XMYA was considered as a saturated fatty acid, the ratio of saturated fatty acid to monounsaturated fatty acid would become 1:0.8 in sandalwood seed oil diet, being then approximately equal to that of the standard lab diet. However, XMYA is an unsaturated fatty acid and its physical and chemical characteristics do not appear to reflect saturated fatty acid features. Therefore, it seems unreasonable to explain the similar growth rate (compare with standard lab diet fed mice) of sandalwood seed oil-fed mice as being due to the ratio of saturated fatty acid to monounsaturated fatty acid. The results of this present study may actually suggest that there are significant differences between erucic acid and XMYA in the mechanism of growth retardation. Erucic acid has low digestibility and absorption, only 55% of this acid is absorbed from a test diet, whereas 85% of 18:1(n-9) was absorbed by rats during 8 hours (166). Jones *et al.* found XMYA to be readily absorbed and metabolised (100). However, it is important to recognise that the rates and mechanism of absorption, distribution, metabolism and excretion are different in rats and mice. These differences with respect to the various metabolic pathways are a subject for further study.

In addition, the double bond of XMYA is present in the *trans* configuration, and thus the sandalwood seed oil diet is a major source of *trans* fatty acid. It is known that naturally occurring *trans* fatty acid are present in small amounts in food such as milk and butter, but more generally in hydrogenated vegetable oil (43, 123). The dietary *trans* fatty acids are readily absorbed. For example, the absorption of *trans* 18:1 is around 95%, and the trielaidin is absorbed as readily as triolein (168). The position of the double bond in fatty acids has been shown to be quite important in some metabolic reactions. Recently, Emken has indicated that although there are no differences in the

absorption of *cis* and *trans*-fatty acid, the latter are not biochemically equivalent to either the 'corresponding' saturated or *cis* unsaturated isomers (43).

In support of these conclusions, Kunch *et al.* reported that cessation of growth occurred earlier when rats were fed on a diet containing 28% hydrogenated oil, however supplementation with 100 mg ethyl linoleate/animal/day ensured a normal growth rate by comparison with the control group (169). However, the nutritional effects of *trans* fatty acid are still not clear. In essential fatty acid deficiency, *trans* fatty acids accentuate dermal symptoms and suppress growth (170). In 1964, Privett and Blank showed that 18:2(9t, 12t), as a 5% dietary calorific supplement, has no ability to cure essential fatty acid-deficient rats, while rats fed fat-free or 18:2(9c, 12t) and 18:2(9t, 12t)-enriched feeds had similar growth rates, but lower than those fed on a 18:2(n-6)-enriched diet (171). Overall, it could be concluded that the geometrical isomers of 18:2(9c, 12c) do not have essential fatty acid activity.

In the present study, the relative percentage content of essential fatty acid, as 18:2(n-6), in sandalwood seed oil, canola oil and standard lab diet diets are 1.9%, 18.9% and 24.9%, respectively. Clearly, the amount of essential fatty acids in the sandalwood seed oil diet is far less than that of canola oil and standard lab diet, however may still be adequate for mice growth.

Finally an interesting observation made during the feeding trial was that those mice fed a 'total' sandalwood seed oil diet (group C) all showed loss of snout whiskers, but not fur, after the third week of feeding. However, no specific essential fatty acid-deficiency symptoms such as definite dermal symptoms were observed in the sandalwood seed oil group. This unusual and unexpected phenomenon was not observed in group D mice which had received sandalwood seed oil diet from the fourth week for four weeks.

Photographs of standard lab diet and sandalwood seed oil mice are shown in Figure 6.2. No explanation for whisker-loss has been readily forthcoming. Histological

examination of snout sections of mice selected from each of sandalwood seed oil, canola oil and standard lab diet group showed no pathological differences in these organs, as described further in section 6.2.8.

6.2.3 Serum aspartate aminotransferase

Aspartate aminotransferase (ASAT) is an enzyme that catalyses transfer of an amino group from L-aspartic acid to α–ketoglutaric caid (172). ASAT occurs in relatively low concentrations in the blood of healthy animals, but is present in much larger quantities in heart, liver, and muscle tissue, being a more organ specific enzyme (172). When the liver is damaged such as by traumatic injury, chemical poisoning (e.g., carbon tetrachloride) or acute hepatitis, ASAT is released into the blood in high concentrations and its activity can be detected for several days thereafter. Thus assay of this enzyme may be of clinical value in the early diagnosis of myocardial infarction, liver diseases and muscle dystrophy (172). Due to the limited blood samples (max. 0.3 mL) which can be collected each time, to allow the mice to survive after sampling, only one suitable monitoring test can be made as an index of potential liver damage caused by any test diets (136). Other liver function tests which could have been selected include hepatic cytochrome *P*-450 activity assay and serum γ-glutamyl transpeptidase activity assay (172).

Blood samples were obtained from five individual ear-coded mice, e.g., A1-A5, randomly selected from each feeding group, and each blood sample provided three samples for the determination of serum ASAT activity. The measurement of serum ASAT did show some variability which is not due to the small sample 0.2 mL of diluted serum used. The determination of ASAT activity was performed at three different stages, at the commencement of the feeding experiment (week 0), at week 4, and finally at week 8. For group D, when feeding sandalwood seed oil commenced in week 4, the three occasions of determination were: week 4, week 6 and week 8.

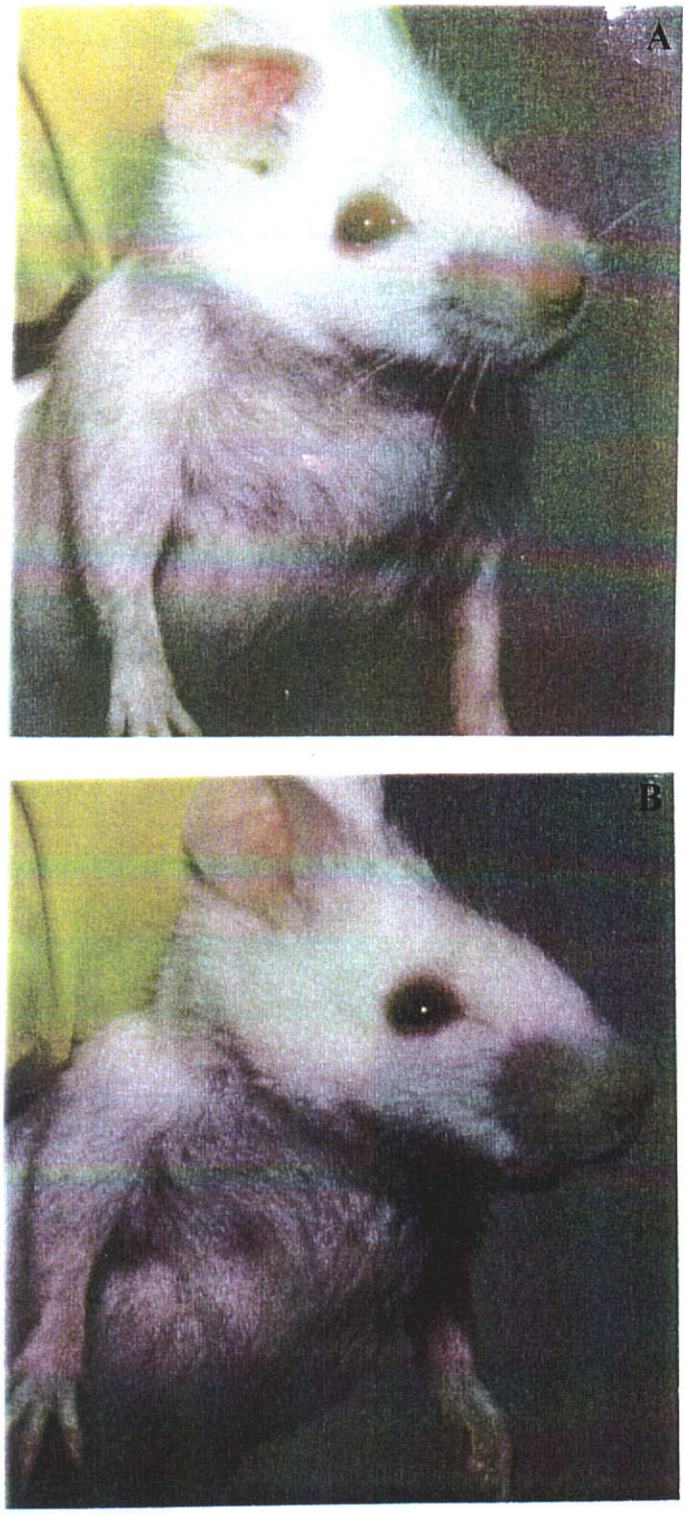


Figure 6.2 Effect of diet on the whiskers of female mice of the ARC(S) strain. Animal fed a diet supplemented with 15% (by weight) fat A) canola oil; B) sandalwood seed oil, nose whiskers lost.

The results shown in Table 6.2 reveal that mice fed on standard lab diet show some variation in ASAT levels over the total collection period, however without any overall trend. The level of ASAT generally remained within the normal range expected which has been stated to be 23.2-48.4 IU/L (173). Thus, the test is quite accurate under the stated condition.

Mice fed on the canola oil diet showed a general increase of plasma ASAT over the whole feeding period with a marked increase between week 4 and 8. For example, in mouse B2, the serum ASAT levels increased from 43.3 IU/L at 4th week to 72.4 IU/L at 8th week, displaying an almost two-fold elevation, which may then indicate fatty liver damage.

In contrast, mice fed on sandalwood seed oil diet showed the greatest increase in serum ASAT activity in the first 4 weeks of feeding, and maintained high ASAT levels from week 4 to 8. Group D mice, which had been converted from standard lab diet (which had been supplied from week 0 to week 4) to a sandalwood seed oil diet (from week 4 to week 8) also demonstrated a similar response in ASAT levels after two weeks feeding an sandalwood seed oil diet. Thus, it may be that the effect of sandalwood seed oil diet on the enzyme system achieves a relative stability after two weeks feeding. On the basis of these findings, it would appear that XMYA or high dietary fat in the sandalwood seed oil diet has affected liver function quite markedly with consequent elevation in serum ASAT levels.

Earlier studies have found that cytochrome *P*-450, a liver mixed-function mitochondrial oxidase, plays a vital role in the biotransformation of a wide variety of compounds including drugs, environmental chemicals, dietary fatty acid and natural food toxicants, and of some endogenous substances such as steroids and eicosanoids (174). It is well recognised that nutritional factors and certain dietary lipids are necessary to maintain the function of this enzyme systems (175). For example, saturated fat can decrease the enzyme systems activity, while polyunsaturated fats increase the activity. Recently, Jones *et al.* observed that dietary quandong seed oil,

fed to rats for 20 days significantly increased liver cytochrome *P*-450 levels and *P*-450 reductase activities, and suggested that XMYA is an hepatic inducer of this enzyme system (100).

In accord with the above study, the results of the present study show that ASAT levels were elevated which may be indicative of liver damage, or as a consequence of XMYA being an ASAT activator. These results suggest the possibility that more than one liver enzyme (such as Δ9-desaturase enzyme, described in later in section 6.2.5.1) may be affected by dietary XMYA.

In the traditional view, lipids were considered as simply sources of energy. Recently, it has been shown that dietary lipids also play an important role in modulation of membrane-dependent cell function (37, 39, 111). For example, essential fatty acid deficiency has been associated with a decreased growth rate, skin symptoms, impairment of lipid transport, and alteration in prostaglandin biosynthesis. Locnisker *et al.* have found that feeding rats a diet containing 24% polyunsaturated fat depresses lymphocyte transformation response compared with those receiving 5% mixed fat, 24% saturated fat or 24% partially saturated fat for two and half months (176). In 1996, Chandra reported that dietary fat is a major modulator of immune function, e.g., influencing metabolic activity, altering cell membrane composition, and affecting the synthesis of several lipid derived mediators such as prostaglandins and leukotrienes (177).

Table 6.2. Changes in ASAT Activity of Selected Mice (IU/L)

Diet	Mouse Code	Start (week 0)	Middle (week 4)	Final (week 8)
SLD*	A1	28.03 ± 3.84	36.4 ± 2.27	36.40 ± 1.09
	A2	48.78 ± 8.34	49.87 ± 3.30	40.40 ± 7.88
	A3	40.04 ± 1.67	31.36 ± 2.66	45.14 ± 11.78
	A4	36.76 ± 3.83	43.68 ± 3.94	31.30 ± 2.50
	A5	30.94 ± 3.15	47.68 ± 2.27	46.02 ± 3.07
CO*	B1	40.04 ± 0.63	42.95 ± 4.54	58.97 ± 2.89
	B2	34.94 ± 3.94	43.32 ± 2.27	72.43 ± 5.60
	B3	25.48 ± 3.51	40.49 ± 14.44	69.53 ± 4.42
	B4	55.32 ± 1.67	59.33 ± 6.01	78.63 ± 1.10
	B5	26.94 ± 1.26	54.23 ± 2.27	49.87 ± 5.04
SWO*	C1	38.22 ± 9.46	68.14 ± 8.39	36.04 ± 7.16
	C2	54.24 ± 3.15	75.90 ± 6.95	54.96 ± 11.15
	C3	44.77 ± 0.00	64.43 ± 13.24	65.52 ± 3.94
	C4	46.59 ± 1.67	64.43 ± 7.65	63.70 ± 11.98
	C5	28.75 ± 0.63	50.59 ± 13.5	52.57 ± 8.41
SWO* (4 wks)		week 4 (start)	week 6	week 8
	D1	36.04 ± 2.19	50.23 ± 6.65	58.60 ± 6.30
	D2	50.60 ± 8.82	59.69 ± 5.50	63.68 ± 5.40
	D3	44.04 ± 8.83	93.93 ± 7.18	76.08 ± 3.84

In this Table, each value represents the mean ± S.D., n = 3 of each blood sample.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

6.2.4 Lipid content of mouse tissues

Practical investigation of test samples indicated that the Bligh and Dyer (53) method could be modified for use in the present study. Total lipids were extracted according to the above method from five mouse organ samples of each group. The results of the lipid extraction are shown in Table 6.3.

Table 6.3. Total Lipid Content of Tissues after Completion of Feeding Trials [g/100g tissue (wet weight), mg/mL in plasma sample]

Samples	SLD*	CO*	SWO*	SWO (4 wks)
Adipose	53.08 ± 1.43^{a}	57.00 ± 1.68^{a}	55.98 ± 1.74^{a}	47.54 ± 1.14^{a}
Plasma	12.31 ± 2.20^{a}	11.40 ± 1.43^{a}	9.20 ± 1.92^{a}	12.20 ± 0.91^{a}
Brain	8.74 ± 1.87^{a}	6.90 ± 0.97^{a}	6.60 ± 1.61^{a}	7.59 ± 1.17^{a}
Heart	8.15 ± 2.03^{a}	6.28 ± 1.49^{a}	5.41 ± 1.83^{a}	7.23 ± 1.21^{a}
Kidney	5.59 ± 1.42^{a}	3.96 ± 0.39^{a}	4.18 ± 1.19^{a}	5.19 ± 1.98^{a}
Liver	4.75 ± 0.84^{a}	6.22 ± 0.81^{b}	5.78 ± 0.88^{ab}	5.4 ± 1.49^{a}
Muscle	5.65 ± 1.53^{a}	5.15 ± 1.05^{a}	3.93 ± 1.86^{a}	4.75 ± 1.65^{a}

In this Table, each value represents the mean ± S.D., n = 5.
Same letters (a, ab) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.
SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil

Each of the total lipid content of plasma, brain, heart, kidney, and muscle tissues of canola oil and sandalwood seed oil-fed mice yielded less lipid than those of standard lab diet-fed mice. The opposite trend was noted in adipose tissue and liver. However, a significant difference was only observed in the liver lipid among the four groups. An earlier study reported that adipose tissue is known to synthesise 24% of the total body

lipids, while the liver synthesises 37% lipid products which are then transported to adipose tissue synthesis and storage (179). Clearly, adipose tissue is the site of lipid accumulation. In the present study, it may be suggested that most of the body weight gained in canola oil-fed mice were in the sites of adipose tissue and liver. This conclusion is supported by the observation of a previous study (180), in which rats were fed a high-fat diet for 31 weeks after weaning, showed marked increased in body weight, and the weight of the fat pad was three times as high as that of controls.

The effect of dietary fat on plasma lipids concentration has been observed. Feeding mice the sandalwood seed oil diet for eight weeks decreased the plasma lipids levels when compared with the effect of the standard lab diet or canola oil diet. Evidence from epidemiological studies showed that populations such as in Mediterranean regions which consume larger quantities of olive oil, have relatively low rates of coronary heart disease (181). The low incidence of atherosclerosis and heart disease in Greenland Eskimos has also been attributed to the high intake of (n-3) polyunsaturated fatty acids in fish oil (182, 183). The present study indicates that long term feeding of sandalwood seed oil at 15% levels may be beneficial in terms of a decrease in plasma lipid concentration in mice. Therefore, sandalwood seed oil may have clinical significance as a potential hypolipidemic therapeutic substance.

The lipid concentration in muscle was lower in the sandalwood seed oil group than in standard lab diet or canola oil groups (no significant difference). In addition, mice fed the sandalwood seed oil diet showed higher values of monounsaturated fatty acids (16:1 + 18:1, 68.6%, by weight) than did those fed the standard lab diet (57.5% by weight) or canola oil diet (47.4% by weight), and a lower concentration of saturated fatty acid (21.3% by weight) was also demonstrated in the sandalwood seed oil group. Nutritional research has suggested that the unsaturated fat of some animal muscle tissue may be potentially beneficial to health when used as food (184). Dietary sandalwood seed oil was shown to alter the fatty acid composition of mouse muscle in this study, producing a higher degree of unsaturated fatty acids (Table 6.6, page 138).

In conclusion, sandalwood seed oil did not appear to have caused any major changes in lipid content of the organs examined, except that generally speaking, sandalwood seed oil fed mice showed a low lipid content overall and also an altered fatty acid composition. Such results translated into the human condition may suggest a beneficial effect, arising by some anti-metabolic action.

6.2.5 Fatty acid composition of selected tissues

6.2.5.1 Subcutaneous adipose tissue

The adipose tissues of land animals usually contain 60-85% lipids, and the fatty acid components of the lipids are present largely (90-99%) in the form of triacyglycerols (49). It is known that adipose tissue is a dynamic 'organ' for a number of metabolic processes. For example, triacyglycerols in adipose tissue play a major role towards energy storage during caloric excess acting as a reservoir, and toward the release of free fatty acid to meet energy needs, especially by muscle tissue (180). On the other hand, adipose tissue also contains lipid oxidising system in which the triacyglycerols are subject to lipolysis by enzymes to ultimately form CO_2 providing energy for other fatty acid and glyceride synthesis (180). The triglycerols-fatty acid cycle in adipose tissue is known catalyse by enzymes, and this cycle provides a subtle mechanism for feedback regulation of the rate of fatty acid mobilisation (185) (Figure 6.3).

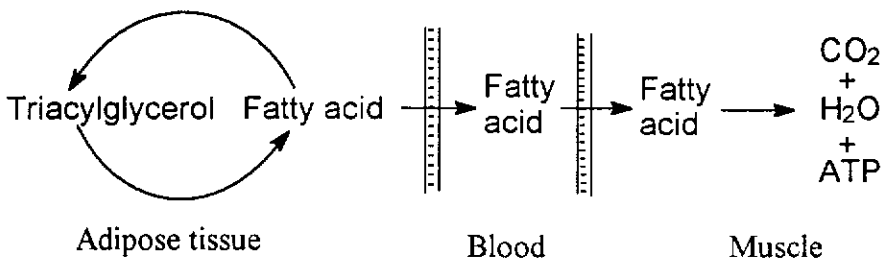


Figure 6.3 Feedback control of fatty acid mobilisation from adipose tissue via the triacylglycerol-fatty acid (185).

Early studies have indicated that many factors, although not always well understood, are important in determining the triacylglyceride fatty acid pattern of a given species. Phylogenetic and dietary factors appear to be the most important, whereas ambient temperature, site of adipose tissue within a given organism, age, etc. are less important. In addition, several reports have provided convincing evidence that the adipose tissue fatty acid profile usually reflects dietary fatty acid composition, particular for 18:2(n-6) and saturated fatty acid with 14 or less carbon atoms (180, 186-187). It has been demonstrated that when wild rabbits were switched from their natural diet containing 60-70% 18:2(n-6) to a low-fat diet, then the percentage of this acid in adipose tissue decreased from 40% to 27% (180). This may be due to the slow turnover of adipose tissue. It is known that turnover is an important concept which envisages continual renewal, involving synthesis and breakdown, among body constituents (39). Thus, the slow turnover of body lipid and the resulting fatty acid composition may provide a recent history of dietary intake and provide a reasonable index of long term modification of dietary fats (39).

In the present study, adipose tissue fatty acid analysis may provide important information on the utilisation of sandalwood seed oil *in toto* and specifically with regard to its fatty acid composition. The effects of dietary fat on adipose tissue fatty acid composition from mice fed the different diet are presented and compared in Table 6.4.

The major long-chain saturated fatty acid present was 16:0, of which the greatest accumulation occurred in adipose tissue of standard lab diet-fed mice (24.7%), and the lowest content appeared in canola oil-fed mice (14.4%). Of the unsaturated fatty acid, 18:1(n-9) was present in very high proportions in the adipose tissue of all groups of mice. Since the triacyglycerols in animal tissue usually include ten or more different fatty acids, the presence of any one fatty acid to the extent of more than 10% usually means that this one represents the major component acid (49). Oleic acid, 18:1(n-9) and 16:0 were thus the predominant fatty acids with additional varying amounts of

Table 6.4 Relative Percentage Composition of Fatty Acid in Mouse Adipose Tissue Lipid

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks)
14:0	0.81 ± 0.07^{b}	tr	0.61 ± 0.05^{a}	0.61 ± 0.11^{a}
16:0	24.66 ± 2.00^{b}	14.41 ± 2.28^{a}	14.80 ± 1.05^{a}	15.63 ± 2.74^{a}
16:1(n-7)	4.85 ± 1.70^{b}	2.36 ± 0.70^{a}	4.75 ± 0.81^{b}	5.22 ± 1.00^{b}
18:0	1.88 ± 0.52^{b}	1.34 ± 0.33^{b}	0.54 ± 0.20^{a}	0.8 ± 0.17^{a}
18:1(n-9)	53.28 ± 2.69^{a}	65.75 ± 1.29^{b}	70.71 ± 1.03^{c}	68.22 ± 2.82^{bc}
18:1(n-7)	2.38 ± 0.39^{b}	1.90 ± 0.24^{ab}	1.54 ± 0.29^{a}	1.58 ± 0.38^{a}
18:2(n-6)	12.14 ± 1.16^{b}	12.65 ± 1.29^{b}	2.49 ± 0.58^{a}	4.19 ± 1.19^{a}
18:3(n-3)	tr	1.59 ± 0.21^{c}	0.45 ± 0.11^{a}	0.81 ± 0.19^{b}
18:1(9a)	nd	nd	0.82 ± 0.19	tr
XMYA	nd	nd	3.29 ± 0.86^{a}	3.07 ± 0.54^{a}
Subgroups				
Total SFA*	27.35 ± 2.59	15.75 ± 2.61	15.95 ± 1.30	17.04 ± 3.02
Total MUFA	60.51 ± 4.78	70.01 ± 2.23	77.00 ± 2.13	75.02 ± 4.20
16:1/16:0	0.19 ± 0.07^{a}	0.12 ± 0.04^{a}	0.32 ± 0.07^{b}	0.34 ± 0.09^{b}
18:1/18:0	29.78 ± 7.29^{a}	51.35 ± 11.89^{b}	118.2 ± 14.29^{c}	97.93 ± 15.77^{c}

In this Table, each value represents the mean ± S.D., n = 5.

Same letters (a, b, ab and c) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

tr = trace (< 0.01%), nd = not detected

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

14:0, 16:1(n-7), 18:0, 18:2(n-6) and 18:3(n-3) in adipose tissue of all groups. Because these fatty acids are expressed here in terms of relative percent content, a change in the level of one component, particularly a major one, will alter the relative content of the remaining fatty acid. Hilditch and Williams have pointed out that 18:1(n-9) is certainly the most widespread fatty acid, representing more than 30% of the total in many fats (40). The fatty acid 18:2(n-6) and 16:1(n-7) are nearly as widespread; the corresponding 16:0 is also very widely distributed in depot fats, in which it may contribute from 15 to 30% of the total fatty acid content.

Mice fed the standard lab diet accumulated the highest concentrations of 16:0 and lowest of 18:1(n-9) when compared to a high-fat diet. An early study has found that large amounts of a saturated fatty acid tend to occur together with smaller amounts of its next higher and lower member in the homologous series of natural saturated acids (180). Accordingly, in this study, the highest level of 16:0 was in standard lab diet-fed group, at 24.7% of total fatty acid, and therefore its next higher acid, 18:0, occurred at 1.9%, was 29% and 71% higher than the corresponding level in canola oil- and sandalwood seed oil-fed mice, respectively. The next lower homologue, 14:0, at 0.8% also showed higher levels than those corresponding to the 14:0 level of canola oil-fed (trace) and sandalwood seed oil-fed (0.6%) mice. Clearly, these results strongly support the early finding, i.e., the saturated fatty acid pattern is normal in this feeding study.

In mice fed the canola oil-diet, more than 65% of the adipose tissue fatty acid content was 18:1(n-9). In contrast with the canola oil group, mice fed a sandalwood seed oil diet had the highest concentrations of 18:1(n-9) with levels of about 70.8%, which was 25% and 7% higher than that of standard lab diet-fed and canola oil-fed, respectively. This finding was supported by previous reports that mice fed a diet containing high levels of 18:1(n-9), show increased percentage of 18:1(n-9), and decreased percentage of 16:0 in the adipose tissue (180). Rule and coworkers found that in bovine adipose tissue, if the content of 16:0 decreased, the level of 18:1(n-9) would not have increased. This is because 16:0 was considered as an elongation/desaturation substrate

for synthesis of C18:1(n-9) (188). However, the present study revealed unexpected results, there were large decreases in 16:0 and 18:0 with corresponding large increases in 18:1(n-9) in adipose tissue of mice fed sandalwood seed oil diet. It should be noted that XMYA constitutes 34% of the total fatty acids in the sandalwood seed oil diet, however only about 3% could be detected in adipose tissue lipids. Therefore, it was presumed that the adipose tissue of sandalwood seed oil-fed mice contained high levels of 18:1(n-9) partly of dietary origin, such as reflected in the adipose tissue of standard lab diet-fed mice, and partly arising by synthesis from XMYA or stimulation of a desaturase (see page 130).

Mice fed the sandalwood seed oil-diet for four weeks after four weeks of the standard lab diet-diet showed a similar fatty acid profile to that of mice fed for a full eight weeks on the sandalwood seed oil-diet. For example, the levels of 18:1(n-9) is at about 68.2% of four weeks and 70.8% of eight weeks respectively, together with only minor appearances of XMYA at 3.1% and 3.3%, respectively. On the basis of the present data the relative percentage of XMYA incorporated into adipose tissue can be estimated to be approximately 10% of the sandalwood seed oil XMYA relative percentage (34.68%, Table 2.4) content. This relative content relationship appears to reflect rapid metabolism of XMYA after absorption. Similar findings have been reported by Jones *et al.* (100), in which XMYA was detected in rat adipose tissue at 5.2% and 5.4% when fed quandong seed oil for 10 and 20 days, respectively.

On the other hand, consideration should be given to the stereochemistry of the *trans* double bond and its effects on absorption and metabolism of XMYA. The deposition of *trans* fatty acid in adipose tissue has been shown to depend on the percentage of this acid in the dietary fat intake. Recently, Emken concluded that the relation between dietary *trans* fatty acid and adipose appears to be 2:1, which holds for several animal models, and may be considered valid for human subjects (43). The ratio of XMYA in dietary fat and adipose tissue appears to be 10:1, which may reflect rapid metabolism of XMYA after ingestion, or that XMYA was simply not absorbed.

In general, 16:1(n-7) occurred to only a low relative content in the diet fats e.g. 0.7% in standard lab diet, and only traces in canola oil and sandalwood seed oil diets. Thus, of considerable interest was the large increase of this acid in the body fat in all feeding groups. Adipose tissue is the important organ for fatty acid biosynthesis, for example saturated fatty acid such as 16:0 can be the precursor for conversion into the corresponding monounsaturated form, 16:1(n-7) by microsomal Δ9-desaturase enzyme (106, 192). This enzymatic reactions may be expressed by the 16:1/16:0 and 18:1/18:0 ratios, when a rise in the numerical value of these ratios would indicate a higher lipogenic activity.

During investigation of the Spanish Oil Poisoning Case, a case of poisoning induced by denatured industrial rape seed oil mixed with animal fat, an interesting study demonstrated that the 16:1/16:0 and 18:1/18:0 ratios, indicating lipogenic activity, were significantly low in adipose tissue of rats fed the toxic oil when compared to the effect of a control diet (192). By contrast, the important observation from this study is that the sandalwood seed oil-fed mice significantly ($p<0.05$) increased the 16:1/16:0 and 18:1/18:0 ratios. Such results may imply that sandalwood seed oil has not demonstrated a specific toxicity in mice. The higher ratios appear to be explained by the presence of XMYA in the sandalwood seed oil diet. Therefore, it may be suggested that XMYA could stimulate Δ9-desaturase enzyme activity to produce high levels of 18:1(n-9).

The *trans*-double bond is known to be the normal substrate for β-oxidation, e.g., *trans*-18:2 has been shown to be oxidised to CO_2 via β-oxidation about 1.5 times less than the all-*cis* isomer (190). Early studies in the 1960s by Dhopeshwarkar and Mead proved that in methyl elaidate-1-C^{14} fed fat-deficient guinea pigs, the amount of 18:0 was higher in adipose tissue than those of coconut-oil-fed animals (101). They concluded that the formation of 18:0 arose by direct biohydrogenation of *trans* 18:1(n-9). Accordingly, the higher percentage of C18:1(9c or 11t) in sandalwood seed oil-fed mice may have been produced from XMYA, by a biohydrogenation pathway.

In conclusion, the marked increase in 18:1(n-9) content in sandalwood seed oil-fed mice adipose tissue could be the result of two processes: the first is one in which XMYA stimulates the activity of Δ9-desaturase enzyme to convert 18:0 into 18:1(n-9) (Scheme 1), and secondly XMYA could be the substrate for 18:1(n-9) biosynthesis via biohydrogenation process (Scheme 2) as outlined below:

Scheme 1

$$18{:}0 \xrightarrow[\text{ximenynic acid}]{\text{9-desaturase}} 18{:}1(n\text{-}9)$$

Scheme 2

$$18{:}2(9a,\ 11t) \xrightarrow{\text{biohydrogenase}} 18{:}1(9c,\ \text{or}\ 11t$$

Although there is no vaccenic acid, [*trans* 18:1(n-7)] in the feeding diets, more than 1.5% of this acid was found in the lipid of adipose tissue, and this was observed in all the experimental groups. Vaccenic acid is a common fatty acid of laboratory animals being formed by microorganisms present in the gut of mice and it may also arise during coprophagy (102). The occurrence of *trans* 18:1(n-7) in animal tissue was reported in the 1940s by Nath *et al.*, who isolated this acid from sheep and beef lipids (193). It was suggested that the *trans*-vaccenic acid was derived from oleic acid by a process of *cis-trans* isomerisation in addition to a selective shift of the 9,10-double bond to the 11,12-position (194).

The essential fatty acids, 18:2(n-6) and 18:3(n-3), are an essential component of dietary fat intake. In 1970, Beare-Rogers suggested that the kinetics of turnover for different fatty acid change with intake (195). For example, mice appear to store linoleate in adipose tissue during periods of high intake and slowly release it when the dietary supply is low. In another study, pigs fed whole soya-beans contained more 18:2(n-6) in adipose tissue than those fed on soya-bean meal, and an increase of 18:2(n-6) in tissue

was accompanied in general by a decrease in the proportion of 16:0, 16:1(n-7), and 18:1(n-9) (196). Similarly, in the present study, the proportion of 18:2(n-6) reflected the concentration of dietary 18:2(n-6) for all three treatments. The increased levels of 18:2(n-6) in standard lab diet and canola oil groups was only accompanied by a decrease in the proportion of 18:1(n-9) compared with sandalwood seed oil fed mice.

Stearolic acid, which is present in sandalwood seed oil only in low concentration (1%), was also detected in low concentration (0.8%) in adipose tissue. However, evidence is not presently available regarding the metabolic activity of this acid or its possible production from XMYA after dietary intake.

As 18:1(n-9) is the precursor for other polyunsaturated fatty acid by Δ9-desaturase enzyme activity (39), the deposition of 18:1(n-9) in adipose tissue is not necessarily proportional to dietary supply. Even though the standard lab diet provides the lowest amount of 18:1(n-9) at 30.2%, there remains a tendency for this acid to reach higher specific levels of 53.3% in adipose tissue, which may represent source from endogenous synthesis. It is also interesting to examine the overall trend of 18:1(n-9) and 16:0 in diets relative to adipose tissue fatty acid content. In relative terms, an increase in 18:1(n-9) content in adipose tissue of canola oil-fed and sandalwood seed oil-fed mice appear to be compensated by a specific reduction in 16:0.

Overall, in addition to phylogenetic factors, diet is the main factor influencing fatty acid pattern of adipose tissue. Accordingly, the present data have demonstrated that the content of certain fatty acid in adipose tissue lipid does resemble the dietary intake to some degree such as with 18:2(n-6). However, the adipose tissue fatty acid composition should not be taken as a consistent indicator of diet fatty acid content. The relationship between dietary intake and adipose tissue fatty acid composition may be related to dietary fatty acid profile and relative content rather than the general quantity of fatty acid in the diet (195). Thus, fatty acid deposition does not appear to be a random process but instead may be a product of specific processes reflecting a particular tissue.

6.2.5.2 Plasma

A number of studies have investigated plasma lipids (49, 96). The measurement of lipids in plasma provides a relatively quick indication of a person's lipid levels which reflect responses to dietary or drug treatments, and aids in the assessment of risk of heart disease (182). The fatty acids in adipose tissue are known to be in the form of triacyglycerols. In contrast, the lipids of plasma contain a higher proportion of phospholipids (96). As a result of both dietary and endogenous triacyglycerol breakdown, a greater proportion of the free fatty acid bound to albumin is transported in plasma, and removed rapidly in various tissues to undergo oxidation or esterification, depending upon metabolic circumstances (Figure 6.3) (37, 95, 185). Consequently, if the free fatty acid concentration in the blood reaches a high level, it will stimulate the action of re-esterification enzymes to store these excess free fatty acids as triacyglycerols in adipose tissue. Indeed, plasma fatty acids serve in part as a transport system for the exchange of lipid in cells. Since these free fatty acids derive from both dietary and endogenous triacyglycerol, it is likely that plasma fatty acid patterns may also be an index of modification by dietary lipids. In the present study, changes in fatty acid composition in plasma as a result of consuming different diets are summarised in Table 6.5.

The major saturated fatty acid in plasma was 16:0 (44%), representing the greatest accumulation in 'tissues' despite the variation of this acid in the diets (as shown in Table 2.4). The high content of 16:0 in the control group may represent source from endogenous synthesis. In particular, the original low relative percentage content in the canola oil and sandalwood seed oil diets, at 4.2% and 4.7%, respectively, has 'resulted' in a 10-fold relative increase in plasma content of 16:0.

Recent information has shown that a large amount of 16:0 tends to occur in the *sn*-2 (where *sn* indicates carbon 2 of the glycerol moiety of the TAG) position in milk triacyglycerol rather than the *sn*-1 and 3 positions (196). Consequently, during triacyglycerol breakdown such as by pancreatic lipase, fatty acids are released from

Table 6. 5 Relative Percentage Composition of Fatty Acid in Mouse Plasma Lipids

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks)
16:0	45.44 ± 2.51[a]	44.47 ± 6.73[a]	43.54 ± 5.22[a]	42.92 ± 2.51[a]
16:1(n-7)	tr	tr	tr	tr
18:0	12.02 ± 1.29[b]	7.18 ± 8.03[a]	6.94 ± 2.27[a]	6.78 ± 1.90[a]
18:1(n-9)	21.11 ± 1.16[a]	26.79 ± 2.44[a]	35.53 ± 2.13[b]	34.46 ± 2.26[b]
18:1(n-7)	3.28 ± 2.69	tr	tr	2.09 ± 0.40
18:2(n-6)	6.88 ± 1.71[b]	10.97 ± 1.03[b]	3.74 ± 0.96[a]	4.75 ± 2.30[a]
18:3(n-3)	tr	tr	tr	tr
XMYA	nd	nd	tr	tr
20:4(n-6)	13.34 ± 2.39[b]	8.51 ± 2.54[a]	6.45 ± 2.41[a]	5.90 ± 2.84[a]
22:6(n-3)	tr	tr	3.30 ± 1.20[a]	3.10 ± 0.65[a]
Subgroups				
Total SFA*	57.46 ± 3.80	51.56 ± 14.76	50.48 ± 7.49	49.70 ± 4.41
Total MUFA	24.39 ± 3.85	26.79 ± 2.44	35.53 ± 2.13	36.55 ± 2.66
Total (n-3)	tr	tr	3.30 ± 1.20	3.10 ± 0.65
Total (n-6)	18.22 ± 4.10	21.48 ± 3.57	10.19 ± 3.37	10.65 ± 5.14
C22:6(n-3) /C20:4(n-6)	tr	tr	0.51	0.53

In this Table, each value represents the mean ± S.D., n = 5.

Same letters (a, b) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

tr = trace (<0.01%); nd = not detected.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

sn-1 and 3 positions to form 2-monoacylglycerols (95). The released fatty acids are ready to be utilised by various tissues, however the 2-monoacylglycerol in plasma is a substrate for re-esterification in the triacyglycerol-fatty acid cycle. In terms of relative percentage content of fatty acid, the retained 16:0 as 2-monoacylglycerol may be one of the reasons to best explain the high concentration of this acid in plasma lipids. Only trace amounts of 16:1(n-7) were detected in plasma lipids, a reduction on the dietary level intake. It may be surmised that the results of Δ9-desaturase enzyme activity were not displayed in the circulatory system.

It has been demonstrated that saturated fatty acid such as dietary 16:0 in plasma tend to elevate the serum lipid and plasma cholesterol content while unsaturated fatty acid such as 18:1(n-9) show opposite trends (197). For example, rats fed semi-purified diets containing 10% fat as corn oil, which is rich in 18:2(n-6), had lower levels of cholesterol than those fed on palm kernel oil, which is rich in 16:0, for three weeks.

A similar trend was observed for 18:0. The differences in 18:0 level in plasma does not reflect in similar degree the difference in levels of this acid in the diets. The relative percentage levels of 18:0 in plasma were about four or five fold higher than that in the diets, but were still present as a relatively minor fatty acid. In contrast to other saturated fatty acids, the biological activity of 18:0 has been considered as effective as 18:1(n-9), probably due to 18:0 being easily desaturated to form 18:1(n-9) (39). The percentage of 18:1(n-9) and 18:2(n-6) in plasma showed a similar pattern to that of adipose tissue in these mice. Only a trace of 18:3(n-3) was detected in plasma lipids. Both 18:2(n-6) and 18:3(n-3) may be considered as respective precursors of (n-6) and (n-3) long-chain polyunsaturated fatty acids, which may serve as precursors for the biosynthesis of prostaglandin and leukotriene (39). In this study, arachidonic acid was found in plasma lipids of all the groups, however only trace amounts of docosahexaenoic acid were found in group A and group B mice. The total amounts of long-chain polyunsaturated fatty acids in the three dietary groups were not significantly different in plasma lipids, being represented as 11.3%, 10.5% and 9.8% (9.0% in group D) (of total fatty acids) respectively for standard lab diet, canola oil and sandalwood

seed oil. In mice fed the standard lab diet and canola oil diets, plasma lipids contained relatively less docosahexaenoic acid (trace) and more arachidonic acid than did those fed the sandalwood seed oil diet. It is of interest to note that the increased amounts of docosahexaenoic acid detected seems to be directly related to reduced levels of arachidonic acid. Clearly, specific alteration in levels and distribution of arachidonic and docosahexaenoic acids in plasma lipids in sandalwood seed oil fed mice was observed, and the markedly low levels of arachidonic acid were compensated by a higher level of docosahexaenoic acid.

Docosahexaenoic acid is known to be very important in the membranes of brain cells, heart muscle cells, the rods and cones of the retina and spermatozoa (106). Increases of such (n-3) fatty acid in plasma lipids has been cited as a positive benefit in the reduction of plasma triacyglycerol and cholesterol concentrations, and was suggested to prevent cardiovascular disease, rheumatoid arthritis, diabetes and some types of cancer (112, 123). Epidemiological studies have shown that populations such as Greenland Eskimos, who consume large quantities of fish oils, have very low incidence of inflammatory and autoimmune disorders (198). In animal feeding studies, an increase of polyunsaturated fats from vegetable sources in the diet may cause changes that are associated with a decreased risk of heart disease and cholesterol levels in plasma (199).

In fact, the reduction of arachidonic acid in the sandalwood seed oil-fed group may be suggested to be a result of the conversion of 18:2(n-6) to arachidonic acid being inhibited by the presence of XMYA. An earlier study with 5,8,11,14-eicosatetraynoic acid has shown its affect on fatty acid metabolism (200). It was found that a decrease in arachidonic acid proportion and increase in eicosapentaenoic acid was observed in rat plasma lipids, indicating an inhibition in the conversion of linoleate to arachidonate. In addition, *trans*-18:1 positional isomers have also been reported to be specific inhibitors for desaturation-elongation of (n-6) fatty acid (43). To conclude, the findings presented in this study confirm the biological role of sandalwood seed oil, acting as a source of both as an acetylenic and *trans* fatty acid.

Overall, it would thus appear that the sandalwood seed oil diet shows a typical unsaturated fatty acids effect on plasma lipid content, and has in particular produced one major benefit in the elevation of docosahexaenoic acid content of the plasma. Since a deficiency in (n-3) polyunsaturated fatty acid has been proposed as a syndrome of modern society giving rise to a number of health problems (111), the results of this study suggest that sandalwood seed oil may be of interest to be explored in clinical investigations as a beneficial diet supplement for humans (201).

6.2.5.3 Muscle

In the present study, total lipids of muscle samples isolated from mouse leg were analysed. The fatty acid composition of lipids extracted from the muscle is shown in Table 6.6. Inspection of the results indicates a large influence of the dietary fatty acid profile on muscle fatty acid content.

The main fatty acids in all mouse muscle lipids were 18:1(n-9) and 16:0, as found for adipose tissue, however the proportion of these two acids had an opposite trend compared to plasma lipid. This finding may further support the concept that 16:0 preference appears to be for the *sn*-2 position as monoacylglycerol, where it is subject to less lipase-catalysed hydrolysis (196). Muscle 16:0 content is relatively high but is not necessarily maintained at a constant level in this tissue. The lower proportion of 16:1(n-7) is consistent with lowered levels of 16:0, because 16:1(n-7) primarily arises from Δ-9 desaturation of 16:0 (37, 106). However, there is no reasonable explanation for the lowest percentage of 16:1(n-7), and the highest of 16:0 observed in the canola oil fed group, where the desaturation mechanism may not appear to be operating to any major extent. In 1976, Newsholme and Crabtree pointed out that the activities of triacyglycerol lipase were much lower than those of the fatty acid oxidative enzymes in muscle, which would have implications in both metabolic-regulation and heat-production mechanism (185).

Table 6.6 Relative Percentage Composition of Fatty Acid in Mouse Muscle Lipid

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks)
14:0	0.96 ± 0.10^{b}	0.31 ± 0.04^{a}	0.67 ± 0.15^{b}	0.82 ± 0.10^{b}
16:0	24.47 ± 3.42^{b}	27.41 ± 2.64^{b}	18.52 ± 3.00^{a}	17.11 ± 3.06^{a}
16:1 (n-7)	7.38 ± 2.03^{b}	1.39 ± 0.44^{a}	5.91 ± 1.47^{b}	6.42 ± 1.17^{b}
18:0	2.97 ± 1.37^{a}	5.61 ± 0.99^{b}	2.07 ± 0.84^{a}	1.32 ± 0.58^{a}
18:1 (n-9)	47.25 ± 4.49^{a}	44.84 ± 3.38^{a}	60.92 ± 3.66^{b}	64.78 ± 3.59^{b}
18:1 (n-7)	2.42 ± 0.26^{c}	1.21 ± 0.20^{a}	1.76 ± 0.21^{b}	1.55 ± 0.17^{b}
18:2 (n-6)	11.90 ± 0.82^{b}	13.11 ± 3.76^{b}	3.38 ± 0.27^{a}	3.89 ± 0.72^{a}
18:3 (n-3)	0.25 ± 0.17^{a}	0.95 ± 0.38^{b}	0.86 ± 0.24^{b}	0.92 ± 0.23^{b}
XMYA	nd	nd	2.82 ± 0.56^{a}	2.89 ± 0.35^{a}
20:1 (n-9)	tr	tr	tr	tr
20:4 (n-6)	1.37 ± 1.10^{a}	2.87 ± 0.26^{b}	1.14 ± 0.50^{a}	0.63 ± 0.08^{a}
22:6 (n-3)	1.00 ± 0.71^{a}	2.15 ± 0.37^{b}	1.96 ± 0.83^{ab}	0.91 ± 0.18^{a}
Subgroups				
Total SFA*	28.40 ± 4.89	33.33 ± 3.67	21.26 ± 3.99	19.25 ± 3.74
Total MUFA	57.05 ± 6.78	47.44 ± 4.02	68.59 ± 5.34	72.75 ± 4.93
Total (n-3)	1.25 ± 0.88	3.10 ± 0.75	2.82 ± 1.07	1.83 ± 0.41
Total (n-6)	13.27 ± 1.92	15.98 ± 4.02	4.52 ± 0.77	4.52 ± 0.80
C22:6(n-3) /C20:4(n-6)	0.73^{a}	0.75^{a}	1.72^{b}	1.44^{b}

In this Table, each value represents the mean ± S.D. n = 5.

Same letters (a, b and ab) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

tr = trace (<0.01%); nd = not detected.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

The standard lab diet and canola oil-fed mice had a higher proportions of 18:2(n-6) than those fed sandalwood seed oil, suggesting that the incorporation of this acid was influenced by dietary treatment. Arachidonic acid was found in relatively low proportion in all mice groups. It may be inferred that muscle tissue is not a major site for arachidonic acid synthesis from 18:2(n-6), but is more occupied with oxidative breakdown of fatty acid to provide energy for fibre contraction. The most important effect of sandalwood seed oil in muscle is reflected in the 18:1(n-9) content, which was similar to that of adipose tissue. Clearly, the increase in percentage of 18:1(n-9) was accompanied by a decrease in the relative proportions of 16:0 and 18:0. The reduced levels of saturated fatty acids constituent have an important nutritional benefit (197). Despite the occurrence of lower concentrations of arachidonic and docosahexaenoic acids, fatty acid composition of muscle lipids generally corresponded to the intake dietary fatty acid content, and are similar to adipose tissue composition. In fact, previous biochemical studies have shown that fatty acid derived from adipose tissue triacyglycerol is transported *via* the bloodstream for the needs of muscle, and these fatty acid are then oxidised to CO_2 and water to provide energy (185).

Statistically significant differences in the proportion of arachidonic and docosahexaenoic acids were observed between mice fed the sandalwood seed oil diet for four and eight weeks. The percentage of arachidonic and docosahexaenoic acids in the eight weeks feeding trial were 81 and 115% respectively higher than those in the four weeks group. Similarly, this finding was also seen in plasma, heart, kidney and liver lipids fractions. However, the C22:6(n-3)/C20:4(n-6) ratios are similar in various tissues in mouse feeding on the sandalwood seed oil diet for four and eight weeks. Comparing such results with standard lab diet-fed mice, it is noted that the short time feeding sandalwood seed oil diet suppresses the formation of arachidonic acid, but has a higher docosahexaenoic acid to arachidonic acid ratio. This may probably be due to the presence of XMYA, which may be inhibiting the (n-6) fatty acid elongation and desaturation enzymes. The regulation of arachidonic and docosahexaenoic acids may suggest that XMYA inhibits the conversion of arachidonic acid, and promotes the formation of docosahexaenoic acid.

6.2.5.4 Brain

The brain has the second highest concentration of lipids (7.9g/100g) compared to adipose tissue. In a recent study, it has been reported by Bourre and coworkers that brain lipids have a structural function, and are not related to energy metabolism since they participate directly in the function of the membranes (202). In brain lipids, saturated and monounsaturated fatty acids are mainly produced by endogenous synthesis. The polyunsaturated fatty acids present in the membranes are not the dietary 18:2(n-6) and 18:3(n-3), but the long-chain polyunsaturated fatty acids such as arachidonic and docosahexaenoic acids. In fact, these two fatty acids of the brain lipids are derived only from dietary 18:2(n-6) and 18:3(n-3), respectively (202).

The fatty acid compositions of brain lipids in the three dietary groups is shown in Table 6.7. Inspection shows that there was no effect on brain fatty acid composition by feeding different diets, and indeed there was a remarkable degree of similarity between the samples. This suggested that mouse physiology controls brain lipid fatty acid composition to an exact degree, possibly almost as a protection mechanism for this centre of cerebral activity.

According to the present data, the major saturated fatty acids in brain total lipids were 16:0 and 18:0, and they represented 45% of total fatty acids. The predominant monounsaturated fatty acid is 18:1(n-9), which occurred in relatively higher proportions than other monounsaturated fatty acids, such as 16:1(n-7), 18:1(n-7), 20:1(n-7), 20:1(n-9), and 24:1(n-9). The total amount of monounsaturated fatty acid on average is 25.1%, and was the second largest group fatty acid in the brain total lipids. The precursor of (n-6) series fatty acid, 18:2(n-6) was generally present as a low average of 0.7% $\pm$ 0.25 in this tissue, whereas 18:3(n-3), the precursor of (n-3) series fatty acid was not detected in this study. It is well known that the essential fatty acids must be taken up by the brain from blood as free fatty acids, as components of triacyglycerols or as components of phospholipids (203). In developing rat brain, the synthesis of arachidonic acid from 18:2(n-6) and docosahexaenoic acid from 18:3(n-3)

Table 6.7. Relative Percentage Composition of Fatty Acid in Mouse Brain Lipid

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks
Unk-27.6*	2.30 ± 0.33	2.50 ± 0.13	2.45 ± 0.08	2.38 ± 0.09
16:0	26.76 ± 1.14	26.66 ± 1.01	27.48 ± 0.85	28.31 ± 2.47
16:1(n-7)	0.71 ± 0.26	0.48 ± 0.13	0.59 ± 0.14	0.77 ± 0.10
Unk-30.8	4.58 ± 0.37	5.01 ± 0.25	4.92 ± 0.24	4.40 ± 0.32
Unk-30.97	0.93 ± 0.16	1.01 ± 0.07	1.02 ± 0.08	1.01 ± 0.14
Unk-31.14	1.28 ± 0.37	1.48 ± 0.18	1.47 ± 1.13	1.49 ± 0.14
18:0	20.87 ± 0.87	20.72 ± 1.81	19.93 ± 0.52	20.17 ± 1.11
18:1(n-9)	19.14 ± 1.65	18.60 ± 1.52	19.03 ± 0.54	21.41 ± 2.76
18:1(n-7)	2.95 ± 0.15	2.62 ± 0.21	2.46 ± 0.13	2.89 ± 0.24
18:2(n-6)	0.92 ± 0.44	0.80 ± 0.23	0.37 ± 0.12	0.55 ± 0.10
20:0	0.31 ± 0.04	0.25 ± 0.08	0.26 ± 0.05	0.27 ± 0.06
20:1(n-9)	1.62 ± 0.08	1.54 ± 0.18	1.24 ± 0.26	1.82 ± 0.31
20:1(n-7)	0.28 ± 0.01	0.20 ± 0.03	0.23 ± 0.02	0.25 ± 0.03
20:4(n-6)	6.34 ± 0.52	6.36 ± 0.30	6.13 ± 0.46	4.88 ± 0.62
22:4(n-6)	1.60 ± 0.17	1.51 ± 0.07	1.46 ± 0.04	1.39 ± 0.18
22:6(n-3)	9.11 ± 0.85	10.22 ± 0.85	10.99 ± 0.51	8.03 ± 0.56
24:1(n-9)	0.50 ± 0.16	0.36 ± 0.12	0.29 ± 0.01	0.35 ± 0.03
Subgroups				
Total SFA*	47.94 ± 2.05	47.63 ± 2.90	47.67 ± 1.42	48.75 ± 3.64
Total MUFA	25.20 ± 2.05	23.80 ± 2.06	23.84 ± 0.96	27.50 ± 3.37
Total (n-3)	9.11 ± 0.85	10.22 ± 0.85	10.99 ± 0.51	8.03 ± 0.56
Total (n-6)	8.86 ± 1.13	8.67 ± 0.60	7.96 ± 0.62	6.82 ± 0.90

In this Table, each value represents the mean ± S.D., n = 5.

Unk*: unknown peak at retention time 27.60.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

has been reported by assaying desaturation and elongation, and the amounts of long-chain polyunsaturated fatty acid derivatives are substantially higher than that of the precursor essential fatty acids (41). According to early published data, the ratios of docosahexaenoic acid to 18:3(n-3) is 230:1 in adult human, and 36:1 in red deer (182). It was indicated that regardless of dietary source, animals can synthesise the 20- and 22-carbon fatty acids needed by their neural tissues from shorter-chain precursors from the diet (182).

XMYA was found to be absent from the brain lipid fraction, and this was in accordance with early studies which reported that acetylenic fatty acid such as crepenynic or stearolic acids were not incorporated into brain lipids (98-99). The absence of XMYA in brain lipids was also found in a previous study (100), but the authors put forward the suggestion that if it were fed during the critical period of brain development, this could possibly result in the incorporation of ximenynic acid into brain lipids.

The major polyenoic constituents of brain lipid were the long-chain polyunsaturated fatty acids which were generally present in the average proportions of 22:6(n-3) > 20:4(n-6) > 22:4(n-6). Both the long-chain (n-3) and (n-6) polyunsaturated fatty acid are important in nervous system lipids, especially in the brain and retina system. Docosahexaenoic acid has a more specific tissue distribution, especially those membranes of the nervous system, and it was the only (n-3) polyunsaturated fatty acid detected in brain total lipids. As shown in Table 6.7, the docosahexaenoic acid is highly controlled at a specific level. It was the fourth most abundant fatty acid in the brain (approximately 10%). Evidence has indicated that docosahexaenoic acid synthesised from dietary 18:3(n-3) by the liver is secreted into the bloodstream in lipoproteins, taken up by the brain, and incorporated into cell membranes. Recently, the relationship between dietary 18:3(n-3) and brain docosahexaenoic acid levels was demonstrated by Bourre *et al.* It was found that 0.26% of dietary energy was the minimal amount of dietary 18:3(n-3) which would maintain the maximal required level of docosahexaenoic acid in brain lipids (202). The replacement of

docosahexaenoic acid by (n-6) polyunsaturated fatty acid is a consistent finding in studying (n-3) polyunsaturated fatty acid deficiency. The (n-3) polyunsaturated fatty acid deficiency syndrome in fish and insects especially includes impaired growth and abnormal wing development, respectively. However it is difficult to produce such effects in mammals because (n-3) polyunsaturated fatty acids are conserved and neural tissues are difficult to deplete (182).

In the three diets of the present study, even with standard lab diet dietary fat containing the lowest 18:3(n-3) level at about 1.3%, all resulted in the same level of docosahexaenoic acid. The levels of docosahexaenoic acid were not affected when mice were fed diets containing different amounts of 18:3(n-3).

Arachidonic acid was the major polyunsaturated fatty acid of the (n-6) series detected in brain lipids, representing approximately 6% of the total fatty acids. Arachidonic acid plays a major role in the structure, enzyme activities and function of the membrane (37, 39). A previous study by Koletzko and Braun showed that there is a significant correlation between arachidonic acid and neonatal growth, and the growth-promoting effect of arachidonic acid could be a related to its function as a precursor of eicosanoids or to its structural roles in membrane phospholipids (204). Earlier data from a neonatal rat study published by Sinclair suggested that an efficiency ratio of 18:2(n-6) to 20:4(n-6) is 1 to 10 for the brain membranes during growth (205). Nervonic acid, 24:1 (n-9), was also found to occur at about 0.4%. It is an important constituent of the sphingosides, which have a specific distribution in cell membranes and serve as regulators of many cell functions (39).

The effect of dietary fats on brain fatty acid compositions in rats has been reported recently (206). A study using weanling rats fed with a basal diet supplemented with a 10% (by weight) of fat as coconut, olive or sunflower oil for six days, showed no differences in the saturated and monounsaturated fatty acids content among the different groups. The authors suggested that during the suckling period, the developing rat brain accumulates most of the final amount of polyunsaturated fatty acids,

including 22:6(n-3) which finally present in the adult rat (206). Accordingly, data presented in this study strongly supports the accepted concept that the brain does not modify its fatty acid compositions according to the diet. Thus, brain fatty acids may be highly preserved with their concentration maintained at a specific level, an important difference to other organs.

6.2.5.5 Heart

Under physiological conditions, fatty acids are one of the most important sources of energy for cardiac muscle. Fatty acid composition of the heart muscle may be altered by an external factor such as dietary fats thus earlier studies with laboratory rats have indicated that both the cardiac sarcolemma membranes fatty acid composition and functional properties are influenced by dietary fatty acid, especially (n-3) polyunsaturated fatty acid (207-209). It therefore seems that fatty acid composition in heart muscle may be a factor in health and disease. For example, intake of saturated fatty acid increases the risk of coronary heart disease, while consumption of unsaturated fatty acid produces a relatively low incidence of coronary heart disease (37). Research on the role of (n-3) polyunsaturated fatty acid in heart disease is of current interest, and its clinical significance has drawn great attention.

Changes observed in the fatty acid profile of heart total lipids in response to dietary fats intake, are shown in Table 6.8. The saturated and monounsaturated fatty acids compositions in heart lipid is similar to brain total lipids. Mice fed the standard lab diet had higher total saturated fatty acid (16:0 + 18:0, 41.8%) levels and lower total monounsaturated fatty acid [16:1(n-7) + 18:1(n-9), 26.6%] levels than those of the other groups. The opposite trend was observed for the mice fed the sandalwood seed oil diet. In the sandalwood seed oil fed group, the content of 18:1(n-9) in heart muscle tended to be similar to that of blood plasma samples. Monounsaturated fatty acid such as 18:1(n-9), has been shown to be as effective as polyunsaturated fatty acid in reducing heart cholesterol content (210, 211).

Table 6 8. Relative Percentage Composition of Fatty Acid in Mouse Heart Lipid

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks
Unk-27.60	2.07 ± 0.27	1.64 ± 0.29	1.90 ± 0.26	1.45 ± 0.31
16:0	23.88 ± 1.32^{b}	22.05 ± 1.89^{ab}	21.17 ± 1.38^{a}	23.72 ± 4.12^{b}
16:1(n-7)	1.88 ± 0.55^{a}	1.13 ± 0.33^{a}	1.47 ± 0.48^{a}	2.81 ± 1.01^{b}
Unk-30.80	2.07 ± 0.40	2.06 ± 0.68	1.19 ± 0.66	0.89 ± 0.21
Unk-30.97	tr	0.96 ± 0.03	0.93 ± 0.16	tr
18:0	17.94 ± 0.94^{b}	14.96 ± 1.52^{a}	15.95 ± 0.41^{a}	12.95 ± 3.67^{a}
18:1(n-9)	22.65 ± 2.69^{a}	28.84 ± 3.81^{b}	31.65 ± 3.30^{b}	39.99 ± 2.28^{c}
18:1(n-7)	2.31 ± 0.22^{b}	2.26 ± 0.23^{b}	1.80 ± 0.29^{a}	1.89 ± 0.26^{a}
18:2(n-6)	10.08 ± 1.77^{b}	9.49 ± 2.34^{b}	4.37 ± 0.71^{a}	4.19 ± 1.25^{a}
XMYA	nd	nd	0.51 ± 0.31^{a}	1.62 ± 0.42^{b}
20:1(n-9)	0.77 ± 0.12^{a}	0.93 ± 0.10^{a}	tr	tr
20:4(n-6)	5.50 ± 0.95^{c}	3.97 ± 0.58^{b}	3.49 ± 0.24^{b}	1.95 ± 0.65^{a}
22:4(n-6)	0.81 ± 0.26^{a}	0.60 ± 0.13^{a}	tr	tr
22:6(n-3)	11.08 ± 1.48^{a}	11.21 ± 1.46^{a}	15.58 ± 1.60^{b}	9.36 ± 6.81^{a}
Subgroups				
Total SFA*	41.82 ± 2.26	37.01 ± 3.41	37.12 ± 1.79	36.67 ± 7.79
Total MUFA	27.61 ± 3.03	33.16 ± 4.47	34.92 ± 4.07	44.69 ± 3.55
Total (n-3)	11.08 ± 1.48	11.21 ± 1.46	15.58 ± 1.60	9.36 ± 6.81
Total (n-6)	16.39 ± 2.98	14.06 ± 3.05	7.86 ± 0.95	6.14 ± 1.90
C22:6(n-3) /C20:4(n-6)	2.01^{a}	2.82^{b}	4.48^{c}	4.80^{c}

In this Table, each value represents the mean ±S.D., n = 5.

Same letters (a, b ab and c) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

tr = trace (<0.01%); nd = not detected.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

Ruiz-Gutierrez et al. fed diets containing 10% fat supplements 18:1(n-9) (olive oil), 18:2(n-6) (corn oil), 18:3(n-3) (soybean oil) and polyunsaturated fatty acids (fish oil) to rats for for six weeks and reported that olive oil is as effective as fish oil in reducing plasma cholesterol content, and the incidence of coronary heart disease is reduced by both olive oil as a source of 18:1(n-9) and fish oil as a source of (n-3) polyunsaturated fatty acid (210). Since 1988, the American Heart Association has recommended limiting the fat intake to 30 energy% of total calories with 10 energy% from saturated fatty acid, an increase of 15 energy% monounsaturated fatty acid and a reduction to 5 energy% polyunsaturated fatty acid (212).

The proportion of 18:2(n-6) in mouse heart lipid changed with diet, particularly reflecting the amount of 18:2(n-6) in the diet. However, the decrease in 18:2(n-6) in SWO-fed mice was much less than that in diet composition. It has been previously reported that rats feeding on both the olive oil and fish oil diets produced a significant decrease in heart 18:2(n-6) levels by comparison with those consuming corn oil, which is rich in 18:2(n-6) (210). The levels of arachidonic acid were lower in the heart muscle of mice fed the sandalwood seed oil diet, presumably due to the low level of 18:2(n-6), the precursor of arachidonic acid, in that oil. It is of interest to note that 18:3(n-3) was not present in detectable amounts in heart tissue of any individual groups. Similarly, no 18:3(n-3) accumulation was found in rat heart in a previously study (210).

The most important changes found in sandalwood seed oil fed mice were the marked increase of the levels of docosahexaenoic acid accompanied by a decrease in arachidonic acid when compared with standard lab diet or canola oil diets fed groups. As a consequence, a significant increase in the ratio C22:6(n-3)/C20:4(n-6) was found in the sandalwood seed oil group. This could lead to decreased formation of prostaglandins, because docosahexaenoic acid competes with arachidonic acid at the level of cyclo-oxygenase to inhibit formation of prostanoid (112, 115). It has been proposed that the increased C22:6(n-3)/C20:4(n-6) ratio in platelet membrane phospholipids may be responsible for decreased platelet aggregation (213). Available evidence has demonstrated that rats fed (n-3) polyunsaturated fatty acids from linseed

oil or fish oil can alter cardiac fatty acid compositions, such as replacement of 18:2(n-6) and arachidonic acid with docosahexaenoic acid (207). In contrast, at similar ratios of (n-3) to (n-6) dietary fatty acids, long-chain (n-3) polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids, which are rich in fish oils, appear to be more effective than purified 18:3(n-3) in suppressing arachidonic acid levels and the capacity of the tissues to synthesise eicosanoids. The mechanism for decrease in the concentration of arachidonic acid by (n-3) polyunsaturated fatty acid may be different from that apparent for 18:3(n-3).

In a previous study by Garg and coworkers (213), it has been demonstrated that rats fed diets containing (n-3) polyunsaturated fatty acids lowered the concentration of arachidonic acid in liver microsomes, with an accompanying increase in membrane eicosapentaenoic and docosahexaenoic acids content, when compared with the control group. This indicated that dietary (n-3) polyunsaturated fatty acid inhibits Δ6-desaturase activity of rat liver microsomes. It seems that eicosapentaenoic and docosahexaenoic acids appear to be effectively incorporated into membrane phospholipids not only because they displace arachidonic acid but also inhibit the enzyme Δ6-desaturase, thereby reduced the amount of 18:2(n-6) converting to arachidonic acid (194). In addition, it has also been shown that 18:2(n-6) and 18:3(n-3) compete for enzyme Δ6-desaturase, with 18:3(n-3) being the preferred substrate (213). Thus the (n-3) polyunsaturated fatty acid may conceivably act as analogues of arachidonic acid to inhibit Δ6-desaturation by a feedback mechanism. The presence of docosahexaenoic acid in cardiac tissue has also been reported to be involved with preventive effects on the development of coronary heart disease both relating to the coronary circulation and the myocardium (183). Therefore, docosahexaenoic acid is an important factor in determining the dietary fat influence on cardiac fatty acid composition.

Epidemiological data on coronary heart disease risk from Willett and coworkers indicated that *trans* fatty acid intake is associated with increasing low density lipoprotein cholesterol, decreasing high density lipoprotein cholesterol, and increased risk of coronary heart disease (214). By contrast, in numerous studies it has been

suggested that *trans* fatty acid intake and coronary heart disease are equivocal compared with extensive data from epidemiological observations and animal and human studies (43). In the present study, sandalwood seed oil did not appear to have caused any pathological or morphological changes or changes in mortality. This result is supported by a summary of the reports of the British Nutrition Foundation Task Force in 1995. This concluded that animal studies have not indicated adverse effects of *trans* fatty acid on longevity, reproductive performance or growth, or revealed any specific abnormalities in organs examined histologically (43).

In conclusion, the important findings were the increased levels of both 18:1(n-9) and docosahexaenoic acid in sandalwood seed oil-fed mice cardiac tissue. These results may imply an important benefit from the consumption of sandalwood seed oil. It may be suggested that sandalwood seed oil not only models the function of olive oil, but also produces similar effects to those of fish oil, and thus indicates a potential of this diet to reduce the incidence of heart disease and associated heart problems. However more research is definitely needed in this field, especially in the study of the effect of SWO on plasma and heart muscle cholesterol levels.

6.2.5.6 Kidney

Much kidney research has focused on kidney function in relation to its physiological role. There are few investigations which report renal fatty acid and their metabolism. Preliminary studies demonstrate that the major metabolic pathways of fatty acid in the kidney are esterification or incomplete oxidation to meet energy needs such as sodium reabsorption (215). It is likely that free fatty acids are major substrates for kidney. The uptake and oxidation of free fatty acid in the intact kidney may be regulated by both extra-renal factors such as the concentration of arterial free fatty acid, and intra-renal factors such as the renal blood flow rate or renal Na^+ reabsorption (215). Therefore, dietary fat can be an extra-renal factor to modulate the renal fatty acid composition. In this present study, the analysis of the kidney fatty acid composition is given in Table 6.9.

Table 6.9 Relative Percentage Composition of Fatty Acid in Mouse Kidney Lipid

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks)
Unk-27.60	1.52 ± 0.46	1.46 ± 0.19	1.42 ± 0.21	1.02 ± 0.51
16:0	29.25 ± 1.77^{b}	26.15 ± 2.63^{a}	26.22 ± 1.08^{a}	25.91 ± 1.38^{a}
16:1(n-7)	1.38 ± 0.57^{a}	1.21 ± 0.74^{a}	1.04 ± 0.24^{a}	2.20 ± 1.11^{a}
Unk-30.80	1.61 ± 0.30	1.07 ± 0.13	0.86 ± 0.21	0.57 ± 0.27
Unk-30.97	0.57 ± 0.15	0.82 ± 0.16	0.97 ± 0.15	0.57 ± 0.23
18:0	15.47 ± 1.87^{a}	12.60 ± 1.98^{a}	14.15 ± 1.08^{a}	10.74 ± 3.41^{a}
18:1(n-9)	16.43 ± 3.77^{a}	25.56 ± 4.36^{b}	24.86 ± 1.18^{b}	35.90± 10.98^{c}
18:1(n-7)	1.91 ± 0.20^{a}	1.66 ± 0.26^{a}	1.65 ± 0.14^{a}	1.44 ± 0.27^{a}
18:2(n-6)	7.98 ± 1.25^{b}	10.27 ± 2.51^{b}	5.45 ± 0.25^{a}	5.07 ± 1.42^{a}
18:3(n-3)	0.18 ± 0.05^{a}	0.72 ± 0.14^{c}	0.36 ± 0.18^{b}	0.49 ± 0.22^{b}
XMYA	nd	nd	0.59 ± 0.30^{a}	1.45 ± 0.79^{b}
$F_{8,11}$	nd	nd	1.24 ± 0.25	0.51 ± 0.28
20:4(n-6)	17.54 ± 1.79^{b}	11.91 ± 1.80^{a}	12.88 ± 0.22^{a}	8.98 ± 1.92^{a}
22:6(n-3)	6.16 ± 0.91^{a}	5.95 ± 0.85^{a}	8.61 ± 2.14^{b}	5.12 ± 3.03^{a}
Subgroups				
Total SFA*	44.72 ± 3.64	38.75 ± 4.61	40.37 ± 2.16	36.65 ± 4.79
Total MUF	19.72 ± 4.54	28.43 ± 5.36	27.55 ± 1.56	39.54 ± 12.36
Total (n-3)	6.34 ± 0.96	6.67 ± 0.99	8.97 ± 2.32	5.61 ± 3.25
Total (n-6)	25.52 ± 3.04	22.18 ± 4.31	18.33 ± 0.47	14.05 ± 3.34
C22:6(n-3) /C20:4(n-6)	0.35^{a}	0.50^{b}	0.67^{b}	0.57^{b}

In this Table, each value represents the mean ± S.D., n = 5.

Same letters (a, b and c) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

Un-27.60 = unknown peak at retention time 27.60. tr = trace (<0.01%);

nd = not detected. SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil. SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

The kidney lipid of mice fed on different diets had similar results to these of heart fatty acid profiles, with regard to total saturated fatty acids, total unsaturated fatty acids, 18:2(n-6) and arachidonic acid levels, and for XMYA levels for mice fed the sandalwood seed oil diet for four and eight weeks, respectively. The low concentration of renal 16:0 compared with plasma lipids may be due to the lowered rates of this acid uptake as free fatty acid by kidney. Such results could further support the concept that 16:0 occurs mainly as 2-monoacyglycerol in plasma lipids.

Differences in 18:1(n-9) occurrence were observed in mice fed the sandalwood seed oil diet for four and eight weeks. The mice fed for four weeks had the highest 18:1(n-9) levels at 35.9%, but a significant variation with standard deviation (±10.98) was observed. A minimum level of 24.9% was observed in two out of five mice and was similar to mice fed for eight weeks (24.9%). The most important finding, observed only in kidney, was that mice fed the canola oil diet had higher 18:1(n-9) content than those fed the 8 weeks sandalwood seed oil diet. Those fed the 'four weeks diet' showed an unusually high proportion of 18:1(n-9) after the eight weeks. Recently, the effect of dietary unsaturated fatty acid in each phospholipids class and the formation of prostanoids stimulation were studied using modified kidney cells (216). The authors found that C18 unsaturated fatty acids were preferentially incorporated into phosphatidylcholine over phosphatidylethanolamine. Arachidonic acid displayed an opposite trend. Supplementation with dietary (n-6) polyunsaturated fatty acid caused a significant increase in the synthesis of prostaglandin F_2 (216). Accordingly, the higher level of arachidonic acid in standard lab diet fed mice is believed to be biosynthetically related to the higher level of 18:2(n-6) in the dietary fat. Although the concentration of 18:2(n-6) in the sandalwood seed oil diet was 10% of the average of the canola oil diet, mice fed the sandalwood seed oil diet had similar levels of arachidonic acid compared with those in the canola oil group. In addition, the (n-3) to (n-6) ratios remained at the highest value in the sandalwood seed oil group, which could be related to the presence of XMYA. Since fatty acid has a high turnover rate and can also be oxidised in the kidney, metabolites of XMYA may be found in the renal lipid fraction. As noted in Chapter 4, sandalwood seed oil-fed mice revealed an unusual feature of the kidney

lipids with the presence of a minor unknown peak (0.3%, at retention time 36.17). Careful analysis of its FAME and DMOX suggested that this fatty acid is actually 8,11-diepoxy-8,10-octadecanoic acid ($F_{8,11}$), a previously unidentified oxidation product, which is presumed to originate from XMYA.

6.2.5.7 Liver

The liver serves as an intermediary between the dietary sources of energy and the extrahepatic tissues, which are the main users of energy (217). The liver receives fatty acid transported by the blood after release from adipose tissue. These fatty acids may be esterified to form triacyglycerol to be delivered to the bloodstream in the form of very-low-density lipoproteins for use in peripheral tissues or to be stored in adipose tissue; thus, liver fatty acid compositions may be influenced greatly by plasma fatty acid compositions (185). Clearly, the liver is an organ that is actively concerned with lipid metabolism, and is sensitive to dietary lipid changes (124).

In addition, the liver plays a very important role in lipid metabolism. The enzyme systems are capable of introducing additional double bonds between carboxyl and existing methylene interrupted sequence (37, 39). These processes are known to be controlled by the microsomal chain elongation and desaturation enzyme systems (37). For example, both 18:2(n-6) and 18:3(n-3) are metabolised in the liver to derivatives in which the chain has been elongated and desaturated at the carboxyl end of the molecule.

The fatty acid compositions of the total liver lipid were markedly altered by the dietary fats as shown in Table 6.10. Most notably, the contents of 16:0, 18:0 and 18:1(n-9) from mice on the standard lab diet almost reflected those of the dietary composition. Although the canola oil diet contained the highest 18:1(n-9) (71.1%), mice in this group exhibited the lowest concentration of 18:1(n-9) (26.6%), and the highest 18:0 (13.1%) compared with those of standard lab diet-fed and sandalwood seed oil-fed.

Table 6.10 Relative Percentage Composition of Fatty Acid in Mouse Liver Lipid

Fatty acid	SLD*	CO*	SWO*	SWO (4 wks)
14:0	tr	tr	0.17 ± 0.03^{a}	0.21 ± 0.05^{a}
16:0	31.51 ± 1.33^{b}	27.51 ± 2.77^{ab}	24.61 ± 2.00^{a}	28.86 ± 1.77^{ab}
16:1(n-7)	2.04 ± 0.71^{a}	1.26 ± 0.77^{a}	2.14 ± 0.89^{a}	2.87 ± 0.46^{a}
18:0	8.95 ± 0.83^{b}	13.11 ± 2.02^{c}	5.87 ± 2.60^{a}	3.70 ± 0.41^{a}
18:1(n-9)	34.47 ± 2.87^{b}	26.61 ± 2.67^{a}	54.35 ± 2.12^{c}	53.37 ± 2.42^{c}
18:1(n-7)	1.76 ± 0.44^{a}	1.73 ± 0.26^{a}	2.27 ± 0.60^{a}	2.55 ± 0.57^{a}
18:2(n-6)	12.42 ± 2.13^{b}	10.69 ± 2.58^{b}	3.89 ± 0.52^{a}	3.27 ± 0.77^{a}
18:3(n-3)	0.17 ± 0.06^{a}	0.76 ± 0.15^{b}	0.24 ± 0.2^{a}	0.52 ± 0.31^{ab}
20:1(n-9)	tr	tr	0.24 ± 0.04	0.19 ± 0.03
20:4(n-6)	6.00 ± 1.45^{b}	13.38 ± 1.83^{c}	1.84 ± 0.57^{a}	1.47 ± 0.40^{a}
22:0	tr	tr	tr	tr
22:4(n-6)	tr	tr	tr	tr
22:6(n-3)	2.49 ± 0.72^{a}	6.19 ± 0.87^{b}	3.87 ± 1.64^{a}	2.40 ± 0.75^{a}
XMYA	nd	nd	0.24 ± 0.04	0.41 ± 0.22
$F_{8,11}$	nd	nd	0.27 ± 0.13	0.18 ± 0.06
Subgroups				
Total SFA*	40.46 ± 2.16	40.62 ± 4.79	30.48 ± 4.60	32.56 ± 2.18
Total MUFA	38.27 ± 4.02	29.60 ± 3.70	59.00 ± 3.65	58.98 ± 3.48
Total (n-3)	2.66 ± 0.78	6.95 ± 1.02	4.11 ± 2.21	2.92 ± 1.06
Total (n-6)	18.42 ± 3.58	24.07 ± 4.41	5.73 ± 1.09	4.74 ± 1.17
C22:6(n-3) /C20:4(n-6)	0.42^{a}	0.46^{a}	2.10^{b}	1.63^{b}

In this Table, each value represents the mean ± S.D., n = 5. Same letters (a, b, ab and c) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses. tr = trace (<0.01%); nd = not detected.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

SFA* = saturated fatty acid; MUFA* = monounsaturated fatty acid.

The sandalwood seed oil-fed mice demonstrated the highest 18:1(n-9), together with a marked increase in the proportion of 18:1(11t). The total increase in the proportion of 18:1(n-9) and 18:1(n-7) in sandalwood seed oil-fed mice may suggest that these acids were derived from XMYA.

It has previously been reported by Kawashima and coworkers that animals have the capability to synthesise 18:1 fatty acid by *de novo* synthesis from 16:0 or from dietary 16:0 or 18:0 (218). If essential fatty acid deficiency is occurring, animal liver will increase the synthesis of 18:0 by inducing stearoyl-CoA desaturase and palmitoyl-CoA elongase to rapidly supply 18:1 (218). Recently, Pennacchiotti *et al.* found that treatment of mice with clofibric acid, a pharmacological active derivative of clofibrate, not only increased the proportion of 18:1 in the total lipid of liver, but also altered the proportion of arachidonic and docosahexaenoic acids (219). Similarly, the increased proportion of 16:1(n-7) ($p>0.01$) and 18:1(n-9) and the corresponding decreased proportion of 16:0 and 18:0 in the sandalwood seed oil group animals may indicate that the Δ-9 desaturase system is stimulated by XMYA. These findings are similar to those of adipose tissue, heart, plasma and muscle of mice fed the sandalwood seed oil diet. They suggest that XMYA and sandalwood seed oil may have potential to be used as therapeutical agents in selective fat reduction. However, these aspects of the pharmacological activity of sandalwood seed oil will require further clinical investigation.

The amount of 18:2(n-6) was the lowest in the group C (SWO) possibly because it had the lowest amount of this fatty acid in the diet. The level of 18:3(n-3) tended to directly reflect those in the respective diets, thus lowered levels in a diet were matched by lower levels in the liver lipids.

Dietary manipulation of long-chain polyunsaturated fatty acid in animal liver lipids has been reported by Scott and Bazen who observed that after injection with [1-C^{14}] 18:3(n-3) in mouse pups, loss of labeled docosahexaenoic acid from liver and serum was equivalent to the gain of labeled docosahexaenoic acid in brain and retina within six hours after injection. They suggested that the liver plays a major role in supplying

docosahexaenoic acid to nervous tissue systems (220). In adult rats, it was determined that 18:3(n-3) has to be elongated and desaturated by the liver to provide docosahexaenoic acid for the various nervous tissues; and the minimal amount of dietary 18:3(n-3) requirement is 0.26% of dietary energy, which can maintain normal liver membrane maximal docosahexaenoic acid level (202).

The present study has found that fatty acid elongation and desaturation have occurred in all mice fed various diets. It is possible that 18:2(n-6) was readily available for the synthesis of arachidonic acid, and 18:3(n-3) as the precursor for docosahexaenoic acid. Mice fed the canola oil diet had the highest levels of arachidonic acid suggesting a more active process of elongation and desaturation of 18:2(n-6) in the liver. The elongase and desaturase activity in mice liver was also affected by the sandalwood seed oil diet. The low levels of arachidonic acid in sandalwood seed oil-fed mice may indicate that XMYA is likely to competitively inhibit the desaturation and elongation reactions. This finding is supported by an earlier study in which rats fed with a control diet containing 0.5% of eicosatetraynic acid showed an increase in the proportion of 18:1(n-9) and 18:2(n-6), and a decrease in arachidonic acid percentage (212). Therefore, it has been suggested that eicosatetraynic acid not only affects the Δ9-desaturase system, but also inhibits the elongation of unsaturated fatty acid.

Previous studies have indicated that deficiency of polyunsaturated fatty acids can cause liver disease such as acute hepatitis and cholestasis (37, 39). This is because the most important property of membranes is their fluidity, and the major factor influencing membrane fluidity is the content of polyunsaturated fatty acids. Thus, polyunsaturated fatty acid deficiency may decrease the fluidity of cell membranes and hence impair their biological functions. Some polyunsaturated fatty acids have critical physiological functions such as being the precursors of the eicosanoids *via* the cyclo-oxygenase or lip-oxygenase enzyme system (37, 39, 46, 95). The most abundant eicosanoids are those resulting from the oxidation of arachidonic acid. In contrast, the eicosanoids derived from eicosapentaenoic or docosahexaenoic acids have many more attenuated biological activities than the arachidonic acid-derived corresponding products (112).

It was found that the level of docosahexaenoic acid in the sandalwood seed oil group is

higher than in the standard lab diet fed mice but was not statistically significant. Moreover, the C22:6(n-3)/C20:4(n-6) ratio was considerably higher in mice fed sandalwood seed oil (2.1:1, or 1.6:1) than either standard lab diet (0.42:1) or canola oil (0.45:1) diets. Clearly, the presence of XMYA stimulates the formation of docosahexaenoic acid and suppresses the formation of arachidonic acid, which provide a biochemical basis for the therapeutic use of sandalwood seed oil, as discussed previously in relation to muscle lipid.

Low levels of XMYA were detected in the liver lipids and these results are further discussed in section 6.2.6. The metabolite of XMYA, $F_{8,11}$, was also detected and this has been discussed in detail earlier in section 3.2.4 (page 68).

6.2.6 Ximenynic acid incorporation into tissue lipids

Lipid samples extracted from organs of the mice fed on the sandalwood seed oil diet were carefully examined with FAME analysis by GC/MS. As noted previously, the presence of XMYA methyl ester or DMOX derivatives can be characterised both by retention time and fragmentation pattern with certain signatures. A comparison of results is shown in Table 6.11.

Table 6.11 Relative Percentage of Ximenynic Acid in the Fatty Acids of Tissue of Mice Fed Sandalwood Seed Oil Diet.

Diet	Adipose	Plasma	Brain	Heart	Kidney	Liver	Muscle
SWO*	3.29	tr	nd	0.51	0.59	0.24	2.82
	$\pm$ 0.86[a]			$\pm$ 0.31[a]	$\pm$ 0.30[a]	$\pm$ 0.16[a]	$\pm$ 0.56[a]
SWO	3.07	tr	nd	1.62	1.45	0.41	2.89
(4 wks)	$\pm$ 0.54[a]			$\pm$ 0.42[b]	$\pm$ 0.79[b]	$\pm$ 0.22[a]	$\pm$ 0.35[a]

In this Table, each value represents the mean $\pm$ S.D., n = 5.

Same letters (a, b) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

tr = trace (< 0.01%); nd = not detected; SWO* = sandalwood seed oil.

XMYA is not incorporated into brain lipids and generally only 0.3-3.3% could be detected in lipid fractions of adipose tissue, heart, liver, muscle, kidney and plasma. The incorporation of XMYA (%) is dependent on the particular tissue, e.g., lower in liver (0.2%), heart (0.5%), and kidney (0.6%), but higher in muscle (2.8%) and adipose tissue (3.3%). The level of XMYA in heart, kidney and liver tissue was approximately 2-3 times higher in the four weeks group (D) than the eight weeks group (C).

Inspection of the XMYA in specific tissues of sandalwood seed oil-fed for four weeks (group D) and eight weeks (group C) showed interesting parallels. Similarly, a previous investigation has reported that XMYA can achieve a relatively steady state of incorporation into tissue lipids of rats after 10 days compared with 20 days of feeding (100). It is known that lipids in a living organism is their dynamic state, which is continual being broken down or removed from the tissue and replaced (39). The lower levels of XMYA in plasma may indicate a rapid turnover of XMYA, arising from triximeynin-XMYA cycle which are subject to lipolysis by enzymes to meet energy needs. By contrast, the higher concentration of XMYA in adipose tissue and muscle may reflect the slower turnover of fatty acid/triacylglycerol at these sites, and may also reflect the characteristics of XMYA as *trans* fatty acid.

The time course of this acid incorporation into the eight weeks group (C) of mice was not determined and therefore it cannot be stated whether there was any change of XMYA content in tissues during that period.

6.2.7 Suggested mechanism for tissue metabolism of ximenynic acid

XMYA, as a conjugated acetylenic fatty acid, has unique chemical properties which differentiate it from many other fatty acids. Considering the chemical structure of XMYA, its triple bond is less reactive than the double bond when treated with chemical oxidants, however when treated with a reductant, the triple bond is more reactive than the double bond to the extent that selective hydrogenation is a feasible option, as discussed earlier on page 71 (14, 221). Based on the biochemical analysis data presented in this study, it can be presumed that there are two possible metabolic

pathways for XMYA biotransformation. The first is the possibility of XMYA being partially and/or completely oxidised to CO_2 and H_2O by β-oxidation. The second is the ability of this acid to serve as a substrate for biohydrogenation to produce 18:0, 18:1(9c or 11t) or 18:2(9c, 11t). However, proof of these metabolism pathway will require further study.

Regarding the first pathway, all fatty acids are potential substrates for β-oxidation and subsequent production of CO_2, H_2O and ATP (39). In fact, different types of fatty acid may be oxidised to different extents. It has been reported that fatty acids derived from medium chain triacyglycerol are absorbed partially and largely oxidised by the liver to CO_2, acetate and ketone bodies (95). Moreover, the metabolism of acetylenic fatty acids have been studied . For example, 4-decynedioic acid, a metabolite of crepenynic acid, was isolated from the urine of rats which were fed *Crepis rubra* seeds (98). The metabolism of stearolic acid in rats has also been reported and the main metabolic steps were ω-, β- and presumably α-oxidation, and the major metabolic product is CO_2 together with a series of six different dicarboxylic acids, all retaining the triple bonds (99). These results indicate that α-oxidation involves the methyl end of the compounds together with varying degrees of β-oxidation (98-99).

In the present study, consideration of GC/MS data gathered in the FAME and DMOX analysis of liver and kidney fatty acid samples showed the presence of the unusual compound provisionally identified as 8,11-epoxy-8,10-octadecadienoic acid ($F_{8,11}$), an oxidised metabolite of XMYA.

The three major C18 unsaturated fatty acid, 18:1(n-9), 18:2(n-6) and 18:3(n-3) may be substrates for conversion to the corresponding C20 and C22 derivatives, such as arachidonic and docosahexaenoic acids, by processes of desaturation and elongation (37, 106). However, Dhopeshwarker and Mead found biohydrogenation occurred, an uncommon reaction in guinea pigs, in which the *trans* 18:1(n-7) was considered to be the precursor to form 18:0 (101). The hydrogenation of double bonds in fatty acid has been noted in a few organisms such as rumen micro-organisms and certain bacteria, with the major products being either stearate or an octadecenoate (39, 102).

Such results may indicate that XMYA has mainly been biohydrogenated. It was presumed that XMYA is first hydrogenated to give the conjugated diene, which then would be available as a precursor for further hydrogenation to give *cis* 18:1(n-9) or *trans* 18:1(n-7), as shown in Figure 6.4.

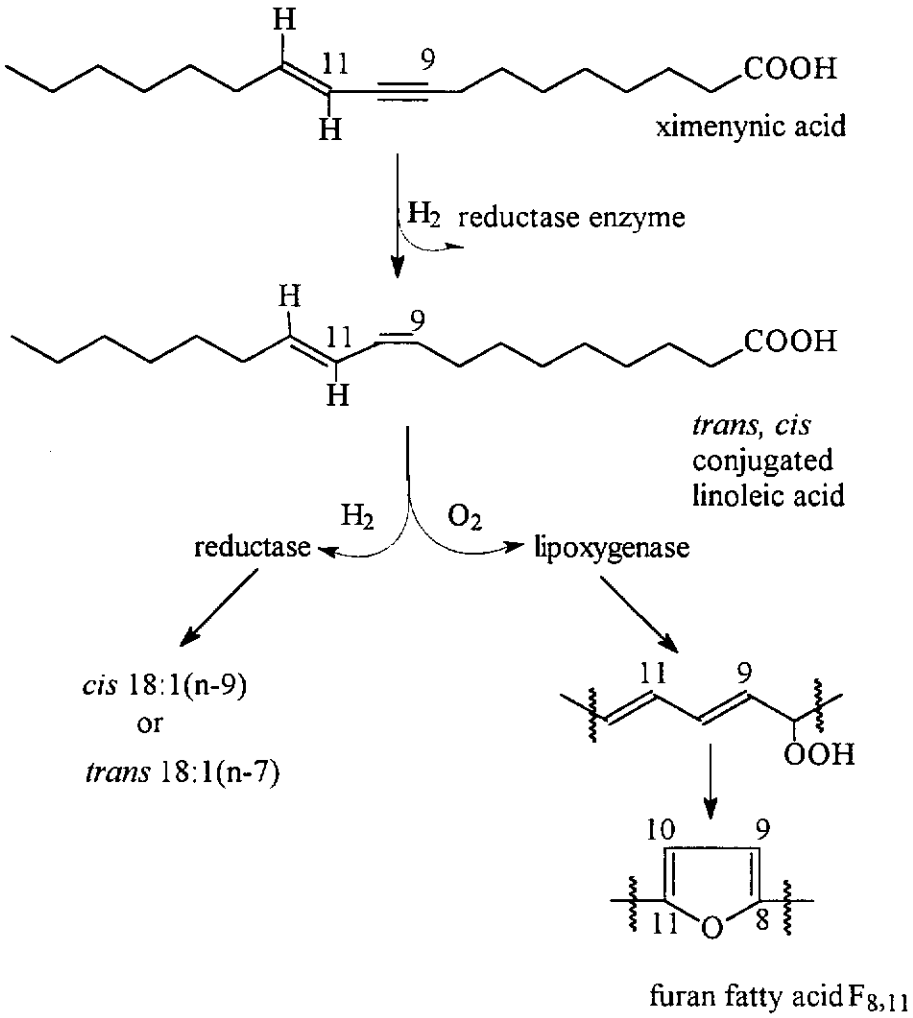


Figure 6.4. A scheme for ximenynic acid metabolism.

A marked increase in the proportion of 18:1(n-9) in adipose tissue, muscle, and especially in liver was shown in Table 6.12.

Examination of the TIC of tissue lipids failed to provide conclusive evidence of the

presence of conjugated linoleic acid, when compared with GC/MS data from authentic conjugated linoleic acid, which was synthesised from XMYA (see Chapter 3).

Table 6.12 Relative Percentage of Oleic Acid in Tissues of Mice Fed Different Diets.

Samples	SLD*	CO*	SWO*	SWO (4 wks)
Diet	30.24	71.05	51.83	51.83
Adipose	53.28 ± 2.69^{a}	65.75 ± 1.29^{b}	70.78 ± 1.03^{c}	68.22 ± 2.82^{bc}
Plasma	21.11 ± 1.16^{a}	26.79 ± 2.44^{b}	35.53 ± 2.13^{c}	34.46 ± 2.26^{c}
Heart	21.65 ± 2.69^{a}	28.84 ± 3.81^{b}	31.65 ± 3.30^{b}	39.99 ± 2.28^{c}
Kidney	16.43 ± 3.77^{a}	25.56 ± 4.36^{b}	24.86 ± 1.18^{b}	35.90 ± 10.98^{c}
Liver	34.64 ± 2.87^{b}	26.61 ± 2.67^{a}	54.35 ± 2.12^{c}	53.37 ± 2.42^{c}
Muscle	47.50 ± 4.49^{a}	44.84 ± 3.38^{a}	60.92 ± 3.66^{b}	64.78 ± 3.59^{b}

In this Table, each value represents the mean ± S.D., n = 5.

Same letters (a, b and c) indicate samples which are not significantly different ($p<0.05$) within a row using the One-Way ANOVA analyses.

SLD* = standard lab diet; CO* = canola oil; SWO* = sandalwood seed oil.

6.2.8 Clinical aspects of *Santalum spicatum* seed oil

The seed of *Santalum spicatum* was recognised to possess medicinal properties by the Australian Aborigines (6, 7). They used the macerated seed kernel as a topical application for skin lesions and the kernels have also been eaten to treat rheumatoid arthritis, a chronic inflammatory disease in joints (222). Current orthodox therapies use various classes of medicines such as nonsteroidal anti-inflammatory drugs (e.g., aspirin and indomethacin), which have historically been essential therapy for this kind of disease. However, nonsteroidal anti-inflammatory drugs may exhibit major adverse

reactions such as gastrointestinal side effects (223). To date, clinical evidence suggest that dietary supplementation with large amounts of fish oil may decrease joint pain,

increase mobility and generally improve both subjective and objective parameters of rheumatoid joint disease (112). Metabolites of arachidonic acid have been demonstrated to be inflammatory mediators (224). Levels of 5(S),12(R)-dihydroxy-6,8,10-(*trans/trans/cis*)-14-*cis*-eicosatetraenoic acid (leukotriene B_4) and 5-hydroxy-6,8,11,14-eicosatetraenoic acid have been shown to be selectively increased in the joints of patients with rheumatoid arthritis. Fish oils containing high concentrations of (n-3) polyunsaturated fatty acid, such as eicosapentaenoic and docosahexaenoic acids, may modulate the production of arachidonic acid-derived mediators and competitively inhibit arachidonic acid utilisation, and as a consequence reducing the production of prostanoids.

A previous study has indicated that rats fed a diet containing fish oil (10% of eicosapentaenoic acid by weight) had significantly increased eicosapentaenoic acid content in cell phospholipids and a 35% reduction of arachidonic acid levels compared to those fed on a mixed coconut/safflower oil diet (225). Similarly, it was also found that supplementation of the diet with olive oil (rich in oleic acid), has some beneficial effects in improving the symptoms of rheumatoid arthritis (226). Olive oil and fish oils might influence expression of rheumatoid arthritis by decreasing arachidonic acid derived pro-inflammatory mediators such as leukotriene and prostaglandins (226, 227).

Data from the present study shows that mice fed the sandalwood seed oil diet had decreased arachidonic acid levels by 12.1%, 38.6%, 60.3% and 86.2% respectively in heart, plasma, muscle and liver total lipids compared with those on the canola oil diet. An important finding is that the markedly low levels of arachidonic acid were compensated by higher levels of docosahexaenoic acid in sandalwood seed oil group. Higher incorporation of oleic acid, 18:1(n-9) in various tissues was also found in animals fed the sandalwood seed oil diet, which were 8.9%, 24.6%, 26.4% and 51.0% higher respectively in the above order of tissues than those of canola oil-fed mice (201).

The physiological significance of the XMYA effect on enzyme activity is to produce an anti-inflammatory activity, as a results of a competitive inhibition of cyclo-oxygenase

and lipoxygenase enzymes in a variety of tissues (118, 130). In this present study, an oxidised metabolite of XMYA, $F_{8,11}$, in relative low concentration resulting possibly by lipoxygenase activity, was also identified. Moreover, large increases in the levels of 18:1(n-9) and docosahexaenoic acid that accompany decreases in arachidonic acid content in body lipids suggest useful biochemical parameters for further clinical investigation of the treatment of inflammatory disease in humans (178).

6.2.9 Histopathology

Samples of liver and skin biopsies from the nose were removed from samples of mice from each group by dissection after killing the mice during the study of fatty acid deposition in tissues. The samples were fixed in buffered formalin at room temperature. Histological sections were prepared by standard procedures as given in Chapter 2 (page 48). Microscopical examination of tissue sections showed absence of unusual structural features or pathological changes. Samples of liver from standard lab diet-, canola oil- and sandalwood seed oil-diet fed mice were essentially similar without any accumulation of excess fatty deposits or fatty liver tissue (Figure 6.5).

It has been noted in section 6.2.3 that group C mice fed the sandalwood seed oil diet for 8 weeks showed a marked and unusual loss of snout whiskers after the third week. These mice, shown in Figure 6.2 in comparison with canola oil-fed mice, displayed no other unusual characteristics other than an apparent reddening of the 'whisker-bed' areas. Histological examination failed to show any marked differences other than mere loss of the whiskers. There were no deformities or malformations to be observed in that tissue region, as shown in Figure 6.6.

The mechanistic basis for whisker loss remains unknown. This phenomenon was not observed in mice fed a sandalwood seed oil-diet after four weeks of the standard lab diet (group D). Perhaps whisker loss was related to mouse age and development of the whisker bed tissue.

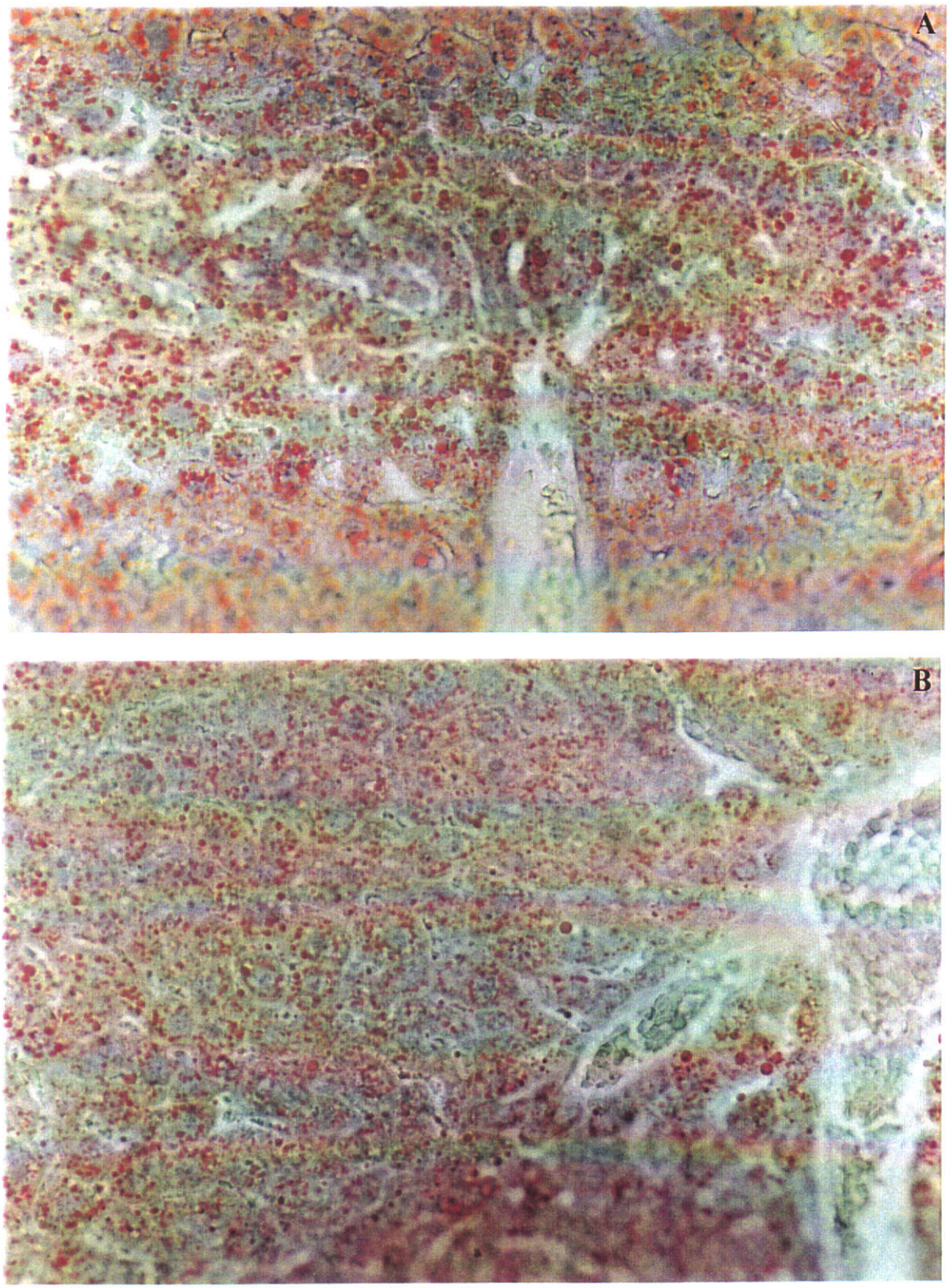


Figure 6.5 Visible light micrograph of a cross section of mice liver tissue, with H&E stained (100 ×). Samples A, B, C and D represent different diet groups as described on page 41.

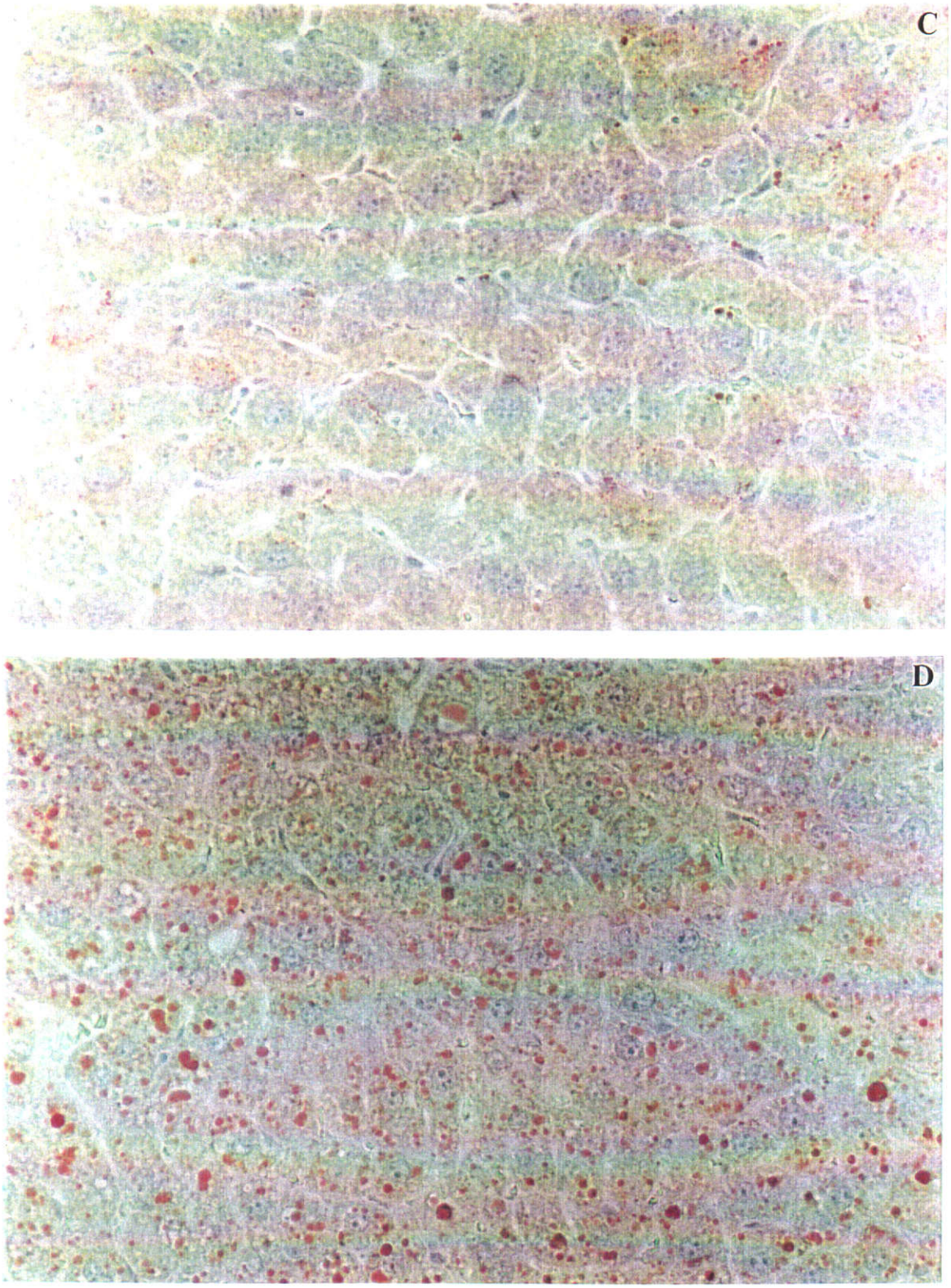


Figure 6.5 (continued)

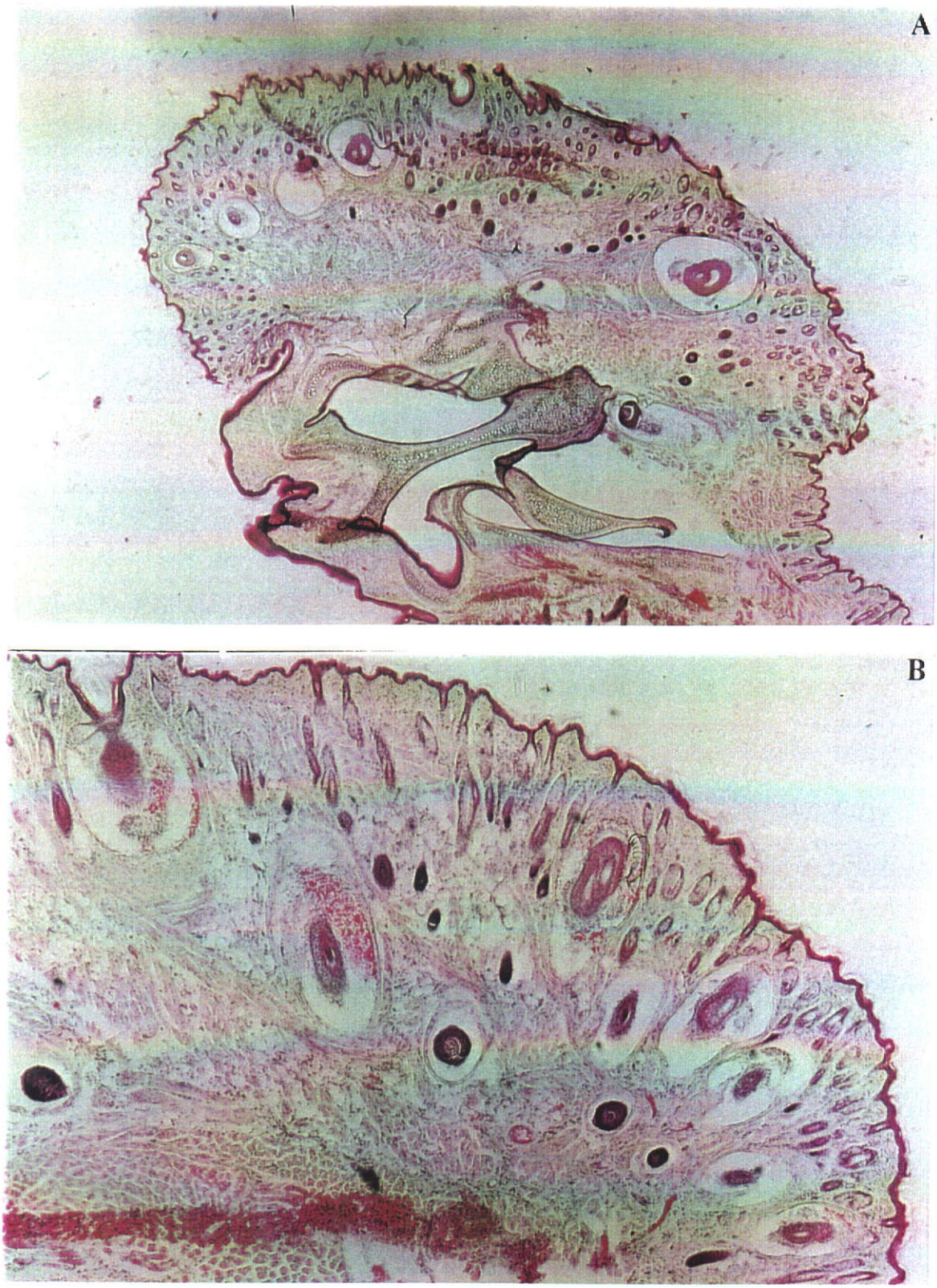


Figure 6.6 Visible light micrography of a cross section of mice snout tissue, H&E stained. A and C (6.6 ×); B and D (13.2 ×). Samples A, B, C and D represent different diet groups as described on page 41.

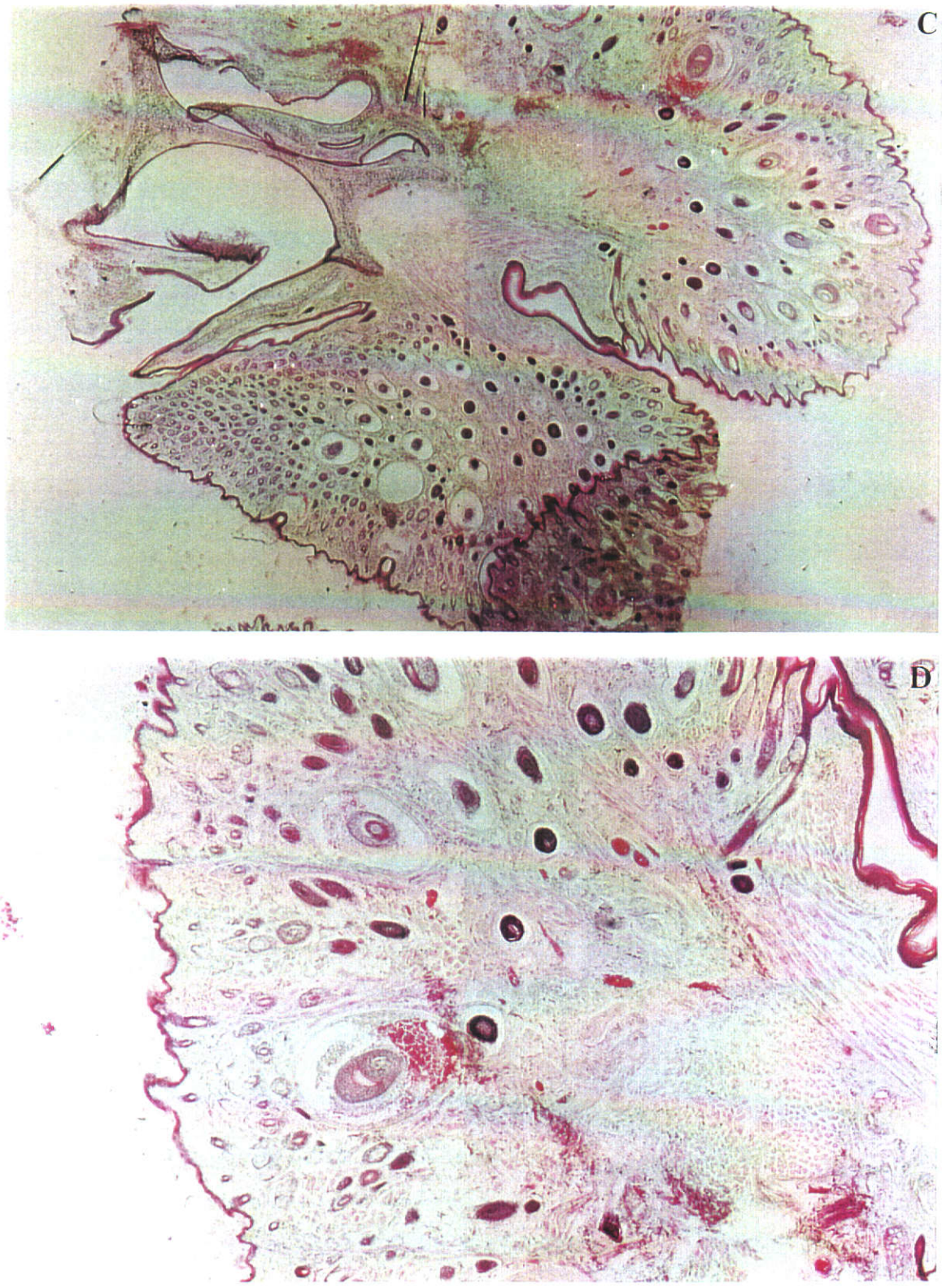


Figure 6.6 (continued).

6.3 CONCLUSION

The results obtained reveal that the inclusion of sandalwood seed oil in a mouse diet affects various biological functions, such as growth, food intake, ASAT activity and fatty acid metabolism.

The weight gain of mice fed sandalwood oil did not differ from the weight gain of mice fed the standard lab diet. Two high fat diets, 15% of canola oil and 15% sandalwood seed oil resulted in different feed intake and weight gain ($p<0.05$). At the end of the experiment the mean weight of sandalwood seed oil-fed mice increased 11.45%, while the respective value of standard lab diet- and canola oil-fed mice were 15.8% and 19.6%. The investigation of blood serum reveals an increase in ASAT activity in mice fed the sandalwood seed oil diet. After switching mice from a control diet (four weeks feeding) to a sandalwood seed oil diet, the activity of ASAT markedly increases after receiving the sandalwood seed oil diet. Increases in ASAT may indicate liver damage with enzyme linkage but this was not substantiated by histological studies.

XMYA is deposited in mouse body fat only to a minor extent, not exceeding 3%, whereas large amounts of oleic acid to a maximum of 70% are found when mice are fed the sandalwood seed oil diet. This study has indicated that there appears to be conversion of dietary XMYA to oleic acid by a biohydrogenation mechanism since the high proportion of XMYA (34%) in the seed oil is not reflected in body tissue composition studies.

No serious nutritional or metabolic disorders were noted in sandalwood seed oil-fed mice, although there were some symptoms of possible essential fatty acid deficiency such as lower growth rates and loss of whiskers. This study has indicated a few health benefits from consumption of large quantities of sandalwood seed oil in the diet. The increased of 16:1/16:0 and 18:1/18:0 ratios, regulation of incorporated oleic, arachidonic and docosahexaenoic acids levels in various tissues could be attributed to the presence of XMYA in the diet. Composition of major fatty acids in mouse tissues are shown in Figure 6.7.

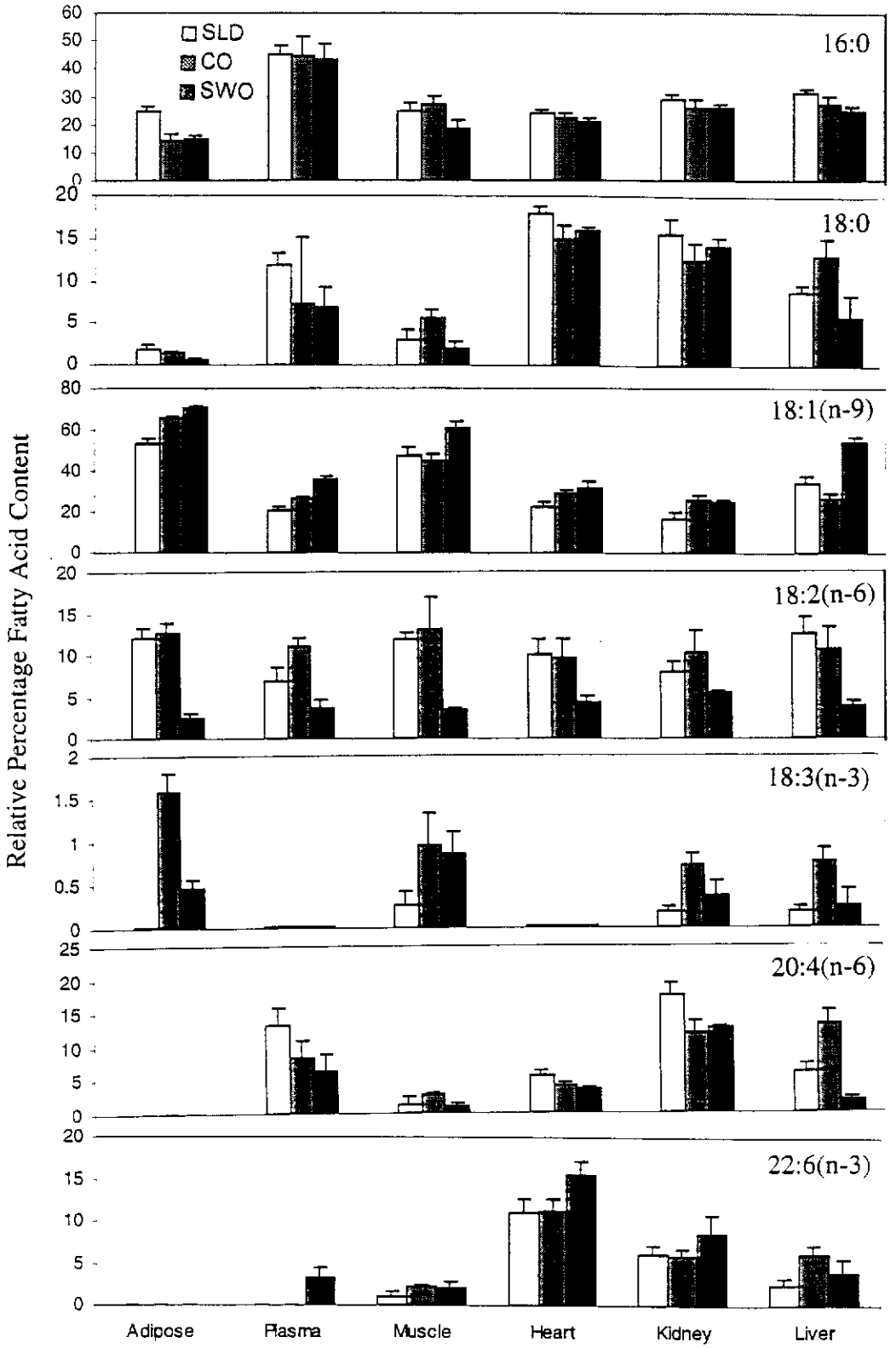


Figure 6.7 Relative percentage content of fatty acids in mouse tissues following feeding with either standard lab, canola oil or sandalwood seed oil diets. Values are means for 5 mice, with standard deviation indicated by bars.

CHAPTER 7: GENERAL CONCLUSIONS AND RECOMMENDATIONS

Conclusions

The triacylglycerols derived from *Santalum spicatum* seed oil contains many different fatty acids. The predominant fatty acids are oleic acid and acetylenic ximenynic acid. Commonly, fatty acids are converted to their methyl esters or 4,4-dimethyloxazoline derivatives for gas chromatography separation. Although fatty acid methyl esters are traditionally used for fatty acid analyses, they are accompanied by some disadvantages, particularly with respect to unsaturated fatty acid or fatty acids with different functional groups. The separation and identification of these fatty acids is possible with 4,4-dimethyloxazoline derivatives. This method-directed fragmentation coupled with good gas chromatographic ability enables the structure elucidation of modified fatty acids in complex mixtures. Fatty acids present in the seed oil of *Santalum spicatum* and mice tissue lipid fraction have been identified by gas chromatography with mass spectrometry of their 4,4-dimethyloxazoline derivatives. The isomers of ximenynic acid and the oxide metabolite of ximenynic acid were identified in mouse liver lipid fraction.

Moisture content decreased significantly, in association with large increases in lipid content during seed development. The accumulation of oil in developing sandalwood seed occurs at markedly different rates, depending on the stage of maturity of the fruit. Oil accumulation can be divided into three stages beginning from flowering. There is little accumulation of carbohydrate, oil and protein in the first period (from seven to 84 days after flowering) of *Santalum spicatum* seed development. During this period the seed has a very high moisture content. A rapid accumulation of oil was found in stage two, which began at about 98 days after flowering at a level of 4 g kg^{-1} and continued to about 396 g kg^{-1} at 147 days after flowering. Finally, there is only minor amounts of oil accumulation and decreasing in seed moisture content. Protein and mineral ash contents displayed similar trends to that of the oil with a corresponding decrease in moisture content. Fatty acid analysis of the seed oil demonstrated marked changes in

composition during seed development. The percentage of oleic and ximenynic acids increased rapidly, while the content of palmitic, linoleic and α-linolenic acids decreased markedly as the seed matures from 91 to 147 days after flowering). Although the biogenetic pathway for ximenynic acid is presently unknown, the parallel increase in the proportions of oleic and ximenynic acids may be related and suggests a precursor effect where oleic acid gives rise to ximenynic acid. It is possible that conjugated linoleic acid, [18:2(9c, 11t)] could be the intermediate for the conversion of oleic acid to ximenynic acid by further dehydrogenation. Fatty acid biosynthesis in *Santalum spicatum* seed is unusual in its production of an acetylenic fatty acid as an end product rather than olefinic linoleic and α-linolenic acids as generally occurring in other plant seed oils.

The total 'oil content' in the seed of *Santalum spicatum* can be readily determined through the use of either the Soxhlet or cold solvent method. The separation and quantitation of lipid constituents by chromatographic techniques are well established and were applied to *Santalum spicatum* seed oil to determine the molecular species of triacylglycerol occurring in *Santalum spicatum* seed oil, both qualitatively and quantitatively by means of gas chromatography on a low polarity capillary (HT-5) column and reverse-phase high performance liquid chromatography. Using a combination of thin layer chromatography, high performance liquid chromatography and gas chromatography techniques, the seed oil was effectively separated into three relatively pure molecular species which were triximenynoyl-glycerol, oleoyl-diximenynoyl-glycerol and dioleoyl-ximenynoyl-glycerol.

Ximenynic acid in the diet may induce physiological effects in mice. It was found that the seed oil moderately suppressed mice body weight increase and food intake. At the end of the experiment, mice fed on *Santalum spicatum* seed oil displayed only a 11.5% weight increase. Those fed on standard lab diet showed an increase of 15.8%, and those in canola oil diet group gained even more weight of 19.6%. The latter is the best 'control' to show low weight gain on sandalwood seed oil diet.

The serum aspartate aminotransferase levels of standard diet-fed mice increased about 8% over the eight weeks test period, remaining within normal range, whereas the canola diet caused a marked increase of 80%. The *Santalum spicatum* seed oil diet only had an increase of 28% over the same period, although greater than the normal range. In the diet group that had been fed seed oil from *Santalum spicatum* for four weeks, mice also showed a marked increase (52%) of serum aspartate aminotransferase levels, which may indicate liver damage with enzyme leakage. Mice fed the *Santalum spicatum* diet lost whiskers after 3 weeks. However, histological examination of snout and liver sections of mice from diet groups showed no pathological differences.

The amounts of total lipid deposited in the mice tissues examined were approximately the same on *Santalum spicatum* seed oil-enriched diet, canola oil diet and standard lab diet. The effects of dietary *Santalum spicatum* seed oil on the fatty acid composition of total lipids of mice various tissues have been evaluated.

An important observation was that ximenynic acid appears to alter the amounts of unsaturated fatty acids such as arachidonic and docosahexaenoic acids in various mice tissues. When the mice were fed *Santalum spicatum* seed oil for four or eight weeks, the lipogenic activity in adipose tissue expressed by the 16:1/16:0 and 18:1/18:0 ratio was significantly higher in the mice than those fed on canola oil or standard lab diets. This suggests that ximenynic acid could stimulate Δ9-desaturase enzyme activity. Ximenynic acid fed to mice as a component of the sandalwood seed oil diet to the extent of 34% by weight of the dietary fatty acids, was deposited in the body fat in low concentration (0.3-3%) in various tissues. In contrast, the brain fatty acid composition was unaffected by this diet. This suggests that mouse control of brain lipid fatty acid composition.

Oleic acid is suggested to be a precursor of ximenynic acid. There appears to be a minor conversion of ximenynic acid to furan fatty acid. It is a possible that conjugated linolenic acid, 18:2(9c, 11t) is the metabolic intermediate. Results suggest that the metabolism of ximenynic acid may involve both biohydrogenation and oxidation reactions in mice.

Recommendations

It will be obvious from this study that although some work has been done on the seed oil of *Santalum spicatum*, several aspects of ximenynic acid biosynthesis in developing seed and its metabolism in the animal body are still unclear. The biosynthesis of ximenynic acid seems to involve oleic acid as the precursor. Perhaps radioactive studies may substantiate this proposed biological pathway. Further work is also required to analyse the details of the pattern of lipid accumulation during seed maturation to give a developmental profile for *Santalum spicatum*.

Although there were no observed adverse health effects or histopathological changes in mice fed *Santalum spicatum* seed oil, a long term toxicity study is required.

Ximenynic acid as a component in the diet has a great influence on the fatty acid composition of various tissues in mice. The metabolism consequences of alteration of tissue fatty acid by ximenynic acid are unknown. More research is needed on the intestinal absorption with some dynamic absorption and metabolic experiments on ximenynic acid. There also needs to be further clinical investigation of response of rheumatoid arthritis and coronary heart disease to using *Santalum spicatum* seed oil. Studies should be performed with pure ximenynic acid or triximenynin, rather than *Santalum spicatum* seed oil. The effect of ximenynic acid could be than be determined, and might give some metabolic information. There is a need to quantify dose-response relationship.

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228. Liu, Y.D. and Longmore, R.B. Separation and identification of triximenynin from *Santalum spicatum* R.Br. *Journal of the American Oil Chemists' Society* 1997; 74: 1269-1272.

APPENDIX 1: PUBLICATION AND PRESENTATION OF WORK FROM THE THESIS

PAPERS PUBLISHED

i) Liu, Y.D., Longmore, R.B., Fox, J.E.D. and Kailis, S.G. A comparison of kernel compositions of sandalwood (*Santalum spicatum*) seeds from different Western Australian locations. *Mulga Research Centre Journal* 1995; 12: 15-21.

ii) Liu, Y.D., Longmore, R.B. and Fox, J.E.D. Separation and identification of ximenynic acid isomers in the seed oil of *Santalum spicatum* R.Br. as their 4,4-dimethyloxazoline derivatives. *Journal of the American Oil Chemists' Society.* 1996; 73(12): 1729-1731.

iii) Liu, Y.D., Longmore, R.B. and Kailis, S.G. Proximate and fatty acid composition change in development sandalwood seeds (*Santalum spicatum*). *Journal of the Science of Food and Agriculture.* 1997; 75 (1): 27-30.

iv) Liu, Y.D. and Longmore, R.B. Dietary sandalwood seed oil modifies fatty acid composition in mouse adipose tissue, brain, and liver. *Lipids* 1997; 32: 965-969.

Liu, Y.D., Longmore, R.B., Boddy, M. and Fox, J.E.D. Separation and identification of trisantalbin from the seed oil of *Santalum spicatum. Journal of the American Oil Chemists' Society.* 1997; 74 (10): 1269-1272.

PAPERS AND POSTERS PRESENTED AT CONFERENCES

i) Kailis, S.G., Liu, Y.D., Longmore, R.B. and Fox, J.E.D. Acetylenic fatty acid development in the fruits and kernels of *Santalum spicatum* (abstract). *Australian Journal of Hospital Pharmacy.* 1994; 24(2): 218. Presented as a paper at the

Australian Pharmaceutical Science Association Scientific Meeting, Brisbane, December 1993.

ii) Liu, Y.D. and Longmore, R.B. A biotransformation product of ximenynic acid and its significance. *Proceedings of Nutrition Society of Australia* 1996; 20: 203.

iii) Liu, Y.D. and Longmore, R.B. Effect of feeding sandalwood seed oil on growth and SGOT activity in mice. *Proceedings of Nutrition Society of Australia* 1996; 20: 182

iv) Longmore, R.B. and Liu, Y.D. A biochemical basis for the traditional use of sandalwood seed oil in the treatment of arthritis. *Australian Journal of Hospital Pharmacy*. 1996; 26(6): S18. Presented as a paper at the Australasian Pharmaceutical Sciences Association Annual Scientific Meeting. Melbourne, December, 1996.

v) Liu, Y.D. and Longmore, R.B. Conjugated linoleic acid - A natural antioxidant from ximenynic acid. *Australian Journal of Hospital Pharmacy*. 1996; 26(6): S48. Presented as a poster at the Australasian Pharmaceutical Sciences Association Annual Scientific Meeting. Melbourne, December, 1996.

vi) Liu, Y.D. Biological and nutritional effects of sandalwood seed oil. In: *Book of Abstracts. The Inaugural Mark Liveris Health Sciences Research Seminar*. pp. 38-44. Curtin University of Technology, Perth, Western Australia, 1996.

vii) Longmore, R.B. and Liu, Y.D. A sandalwood seed oil diet rich in acetylenic acid alters the fatty acid composition of mouse heart and plasma. Presented as a poster at the Australasian Pharmaceutical Sciences Association Annual Scientific Meeting. Sydney, December, 1997.

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APPENDIX 2: APPROVAL FOR ANIMAL EXPERIMENT

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