TREE NUT OILS:
CHEMICAL CHARACTERISTICS, OXIDATION AND ANTIOXIDANTS

HOMAN MIRALIAKBARI

TREE NUT OILS: CHEMICAL CHARACTERISTICS, OXIDATION AND ANTIOXIDANTS

BY

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ABSTRACT

The chemical compositions, antioxidant activities and oxidative stabilities of tree nut oils were examined. The oils of almonds, Brazil nuts, hazelnuts, pecans, pine nuts, pistachios and walnuts were extracted using two solvent extraction systems, namely hexane and chloroform/methanol. The chloroform/methanol system resulted in higher oil yield for each tree nut type examined. Pine nuts had the highest oil content while almonds had the lowest. The lipid class compositions of tree nut oils were analysed using thin layer chromatography-flame ionization detection and showed that triacylglycerols were the predominant lipid class present. Smaller amounts of sterols, sterol esters, phospholipids and sphingolipids were also present. The fatty acid compositions of tree nut oils were analysed using gas chromatography, showing that oleic acid is the predominant fatty acid in all samples except pine nut and walnut oils, which contained high amounts of linoleic acid. The sterol and stanol content and compositions were analysed using gas chromatography; β-sitosterol was the predominant sterol compound present in all samples, with lower amounts of campesterol, stigmasterol, Δ5-avenasterol, 22-nordehydrocholesterol, 24-methylenecholesterol, cholesterol, cholestanol and β-sitostanol also present. The tocopherol compositions were analysed using high performance liquid chromatography, showing that α- and γ-tocopherols are the predominant tocopherol isomers present; however δ- and β-tocopherols were also detected in some samples.

The antioxidative components of tree nut oils were extracted using a solvent stripping process. The minor component stripped oils that remained after the

solvent stripping process were analysed for their chemical compositions as described above, and were also used in the oxidative stability studies. The lipid composition of the tree nut oil extracts showed that they contained phospholipids, sphingolipids, sterols and tocopherols. The total phenolics contents of tree nut oil extracts were analysed and showed that chloroform/methanol extracted oils had higher amounts of phenolic compounds than their hexane extracted counterparts. The antioxidant activity of tree nut oil minor component extracts were assessed using the Trolox equivalent antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl radical scavenging capacity, β-carotene bleaching test, oxygen radical absorbance capacity and photochemiluminescence inhibition assay; results of these studies showed that extracts of chloroform/methanol extracted oils possessed higher antioxidant activities than extracts of their hexane extracted counterparts, while the extract of chloroform/methanol extracted pecan oil possessed the highest antioxidant activity.

The oxidative stability of non-stripped and stripped tree nut oils were examined under two conditions, namely accelerated autoxidation and photooxidation. Progression of oxidation was monitored using tests for conjugated dienes, peroxide value, *para*-anisidine value and headspace volatile analysis. Primary products of oxidation persisted in the earlier stages of oxidation while secondary product levels increased dramatically during the latter stages of oxidation. Hexanal was the major headspace aldehyde formed in all oxidized samples except walnut oil, which contained primarily propanal. Results showed that chloroform/methanol extracted oils were more stable than hexane extracted oils in

both the accelerated autoxidation and photooxidation studies. Oils of pecan and pistachio were the most stable while oils of pine nut and walnut were the least stable.

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LIST OF ABBREVIATIONS

ACL	Antioxidant Capacity of Lipids
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
AUC	Area Under Curve
APCI	Atmospheric Pressure Chemical Ionization
ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)
AAPH	2,2′-azobis(2-amidinopropane) dihydrochloride
β-PE	Beta-phycoerythrin
BL	Blank
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
A-CM	Chloroform/methanol Extracted Almond Oil
BN-CM	Chloroform/methanol Extracted Brazil Nut Oil
HN-CM	Chloroform/methanol Extracted Hazelnut Oil
P-CM	Chloroform/methanol Extracted Pecan Oil
PN-CM	Chloroform/methanol Extracted Pine Nut Oil
PO-CM	Chloroform/methanol Extracted Pistachio Oil
W-CM	Chloroform/methanol Extracted Walnut Oil
CD	Conjugated Dienes
DAD	Diode Array Detector
DPPH	1,1-Diphenyl-2-picrylhydrazyl Radical
N_2O_3	Dinitrogen Trioxide
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
eV	Electron Volts
EDTA	Ethylenediaminetetraacetic Acid
FID	Flame Ionization Detector
FL	Fluorescein
FT-IR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatograph
A-H	Hexane Extracted Almond Oil
BN-H	Hexane Extracted Brazil Nut Oil
HN-H	Hexane Extracted Hazelnut Oil
P-H	Hexane Extracted Pecan Oil
PN-H	Hexane Extracted Pine Nut Oil
PO-H	Hexane Extracted Pistachio Oil
W-H	Hexane Extracted Walnut Oil

HDL	High Density Lipoprotein
HPLC	High Performance Liquid Chromatography
H_2O_2	Hydrogen Peroxide
HpETE	Hydroperoxyeicosatrienoic Acid
OH^-	Hydroxide Ion
HETE	Hydroxyeicosatrienoic Acid
$^{\cdot}OH$	Hydroxyl Radical
HORAC	Hydroxyl Radical Averting Capacity
IU	International Unit
IUPAC	International Union of Pure and Applied Chemistry
kCal	Kilocalorie
LDL	Low Density Lipoprotein
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
MA	Malondialdehyde
MS	Mass Spectrometer
m/z	Mass to Charge Ratio
NCEP	National Cholesterol Education Program
NIST	National Institute of Standards and Technology
$^{\cdot}NO$	Nitric Oxide
$^{\cdot}NO_2$	Nitrogen Dioxide
NMR	Nuclear Magnetic Resonance Spectoscopy
n-3	Omega-3
n-6	Omega-6
n-9	Omega-9
ORAC	Oxygen Radical Absorbance Capacity
OSI	Oxidative Stability Instrument
O_3	Ozone
p-Av	*Para*-Anisidine Value
PV	Peroxide Value
$ROO^{\cdot}$	Peroxyl Radical
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffer Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PCL	Photochemiluminescence
S	Photosensitizer
RDA	Recommended Daily Allowance

$^1O_2$	Singlet Oxygen
$Na_2S_2O_3$	Sodium Thiosulphate
SM	Sphingomyelin
$O_2^{\cdot}$-	Superoxide
TBHQ	Tertiary-butylhydroquinone
TLC	Thin Layer Chromatography
TBA	2-Thiobarbituric Acid
TBARS	2-Thiobarbituric Acid Reactice Substances
TPC	Total Phenolics Contents
TEAC	Trolox Equivalent Antioxidant Capacity
UV	Ultraviolet
VLDL	Very Low Density Lipoprotein
v/v	Volume to Volume
w/w	Weight to Weight

CHAPTER 1

Introduction

Several tree nut varieties serve as valuable oil crops due to their high oil yield, unique flavours and healthful lipid composition. Tree nut oils are primarily composed of triacylglycerols, but also contain diacylglycerols, monoacylglycerols, free fatty acids and other minor components, including natural antioxidants and fat-soluble vitamins. Tree nuts in many cases provide rich sources of food lipids; up to 75% on a wet weight basis [1]. With a few exceptions, tree nut lipids exist as a liquid at room temperature. Generally, tree nut oils are rich in monounsaturated fatty acids (predominantly oleic acid), but contain much lower amounts of polyunsaturated fatty acids (predominantly linoleic acid) and small amounts of saturated lipids [1]. In many parts of the world such as the Middle East and Asia, tree nuts are cultivated for use as oil crops and are important sources of energy and essential dietary nutrients as well as phytochemicals [2]. Tree nut oils are also components of some skin moisturizers and cosmetic products [3].

Tree nuts and their oils are known to contain several bioactive and health promoting components. Tree nuts have long been considered an important component of the Mediterranean diet [4] and in July 2003 the U.S. Food and Drug Administration approved a qualified health claim stating that consumption of 1.5 ounces (42 g) per day of most tree nuts may reduce the risk of heart disease. Epidemiological evidence indicates that the consumption of tree nuts may exert several cardioprotective effects, which are speculated to arise from their lipid component that includes unsaturated fatty acids, phytosterols and tocols [4]. Recent investigations have also shown that dietary consumption of tree nut oils may exert

even more beneficial effects than consumption of whole tree nuts, possibly due to the replacement of dietary carbohydrate with unsaturated lipids and/or other components present in the oil extracts [4].

Much of the existing literature attributes the beneficial health effects of tree nuts and tree nut oils to their high oleic acid content; however, very little research has been conducted on the lipid compositions of tree nut oils and information regarding their minor components are lacking. Therefore, the objectives of this study were to **(1)** analyse the lipid composition of tree nut oils; including analysis of fatty acids, lipid classes, tocopherols, sterols and stanols, **(2)** examine the antioxidant activity of minor components of tree nut oils using a number of novel *in-vitro* assays, and **(3)** assess the oxidative stability of tree nut oils under accelerated autoxidation and photooxidation conditions. A further objective of this work was to **(4)** compare the effects of two oil extraction solvents, namely hexane and chloroform-methanol, on the previously stated components and variables.

CHAPTER 2

Literature Review

2.1 Chemistry and Health Effects of Fats and Oils

Fats and oils are essential dietary nutrients for humans, providing 9 kilocalories per gram (kCal/g) of energy [5]. Fats and oils are the two major types of edible lipids; fats are edible lipids from animal origin that are generally solid at ambient temperature whereas oils from plant sources are generally liquid at ambient temperature. Fats and oils are important dietary carriers of fat-soluble vitamins (vitamins A, D, E, and K) and are sources of essential fatty acids. Food lipids also impart desirable and in some cases characteristic tastes, flavours, and texture/mouth feel to foods [5].

Fats and oils contain predominantly triacylglycerols, which consist of three fatty acid molecules esterified to a molecule of glycerol. The fatty acid molecules of a triacylglycerol can be the same (simple triacylglycerol) or different (mixed triacylglycerol) [6]. The types of fatty acids present in fats and oils are largely responsible for their chemical and physical properties as well as their health effects. Fats and oils also contain a number of other minor components including phospholipids, sphingolipids, sterols and sterol esters, tocols, pigments and waxes, among others [7]. The amounts and types of minor components in fats and oils have long been recognized for their ability to improve the storage and keeping properties of fats and oils [8], however, a number of studies have also shown that some minor

component classes, such as tocols and sterols, exert beneficial health effects including cardioprotective and anticarcinogenic effects [9].

2.1.1 Fatty Acids

Natural fatty acids are alkyl carboxylic acids and generally contain an even number of carbon atoms, they are usually unbranched straight chain molecules, however, odd carbon and branched chain fatty acids do occur in nature [6]. When the carbon atoms of the hydrocarbon chains of fatty acids are bound to their full complement of hydrogen they are classified as saturated. When the hydrocarbon chains of fatty acids contain one or more carbon-carbon double bonds, they are called unsaturated. Fatty acids with one double bond are referred to as monounsaturated while those with two or more double bonds are referred to as polyunsaturated. Generally, animal lipids contain high amounts of saturated fatty acids and tend to remain solid at ambient temperatures whereas most vegetable lipids contain predominantly unsaturated fatty acids and exist as liquids at ambient temperatures [6].

Trivial names have been given to many of the common fatty acids, but systematic names based on the International Union of Pure and Applied Chemistry (IUPAC) system of nomenclature indicate the length of the acid, the position and configuration of double bonds as well as the position of any functional substituents located on the alkyl chain of the acid. The IUPAC system numbers double bonds with relation to their position from the carboxyl group of the acid. However, another naming system accounting for the biological activity of fatty acids numbers their double bonds from the methyl end group; using this latter system it is only necessary to designate the position of the first double bond [10].

2.1.1.1. Polyunsaturated Fatty Acids

The omega-3 (n-3) and omega-6 (n-6) are the two most common groups of polyunsaturated fatty acids [10]. In nature the double bonds of these fatty acids are in the *cis* conformation and are methylene interrupted; this methylene group is called the *bis*-allylic group and is highly prone to hydrogen abstraction, which is known to initiate the development of rancidity [11]. Since the n-3 and n-6 fatty acids can not be synthesised by the body they must be obtained from dietary sources, thus they comprise the two essential fatty acid groups.

Examples of n-3 fatty acids include their parent compound, α-linolenic acid (18:3 n-3), eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexanenoic acid (DHA; 22:6 n-3). Alpha-linolenic acid is the main n-3 fatty acid in plants and is present in the seeds of flax and evening primrose as well as in walnuts, canola and soybeans in significant amounts. EPA and DHA are derived almost exclusively from algal and marine sources. The n-6 fatty acids include their parent compound, linoleic acid (18:2 n-6), γ-linolenic acid (18:3 n-6), and arachidonic acid (20:4 n-6), among others. The main sources of n-6 fatty acids in Western diets are the oils from cottonseed, soybean, peanut, corn, sunflower and canola [12].

The parent compounds of the n-3 and n-6 fatty acid families are used to synthesise the other fatty acid members of these families *in-vivo*. Linoleic acid and α-linolenic acid are metabolised by a series of alternating enzymes known as the desaturases (which introduce another double bond into the fatty acid chain) and elongases (which elongate the fatty acid chain by two carbon atoms) [5]. It is believed that both n-3 and n-6 fatty acids are metabolized by the same group of enzymes and it has been established that the n-3 and n-6 fatty acids compete with

each other for entry into the desaturation and elongation pathways [13]. However, the enzyme systems have been shown to have a higher affinity for the n-3 fatty acids, such that the n-3 fatty acids will be preferentially metabolized [14].

In western diets, n-6 fatty acid intake far exceeds that of n-3 fatty acids which serves to significantly reduce the production of elongated and desaturated n-3 fatty acids by the body, despite the higher affinity of n-3 fatty acids for the enzymes involved with these processes. Because of this fact, nutritionists and dieticians consider the ratio of n-6 to n-3 fatty acid intake to be a better indication of essential fatty acid status rather than just the overall intake of each polyunsaturated fatty acid type. Optimal dietary n-6 to n-3 fatty acid ratios have been estimated to be between 4:1 and 10:1, however, current Western diets have ratios between 10:1 and 20:1 which indicates the need for dietary modifications in these populations [15].

Polyunsaturated fatty acids of the n-6 and n-3 families have several roles in the body including modulation of cell membrane structure, formation of short lived lipid based hormones known as eicosanoids (prostanoids, leukotrienes and hydroxy fatty acids), maintenance of skin integrity, and regulation of cholesterol metabolism [12]. A broad range of beneficial health effects have been associated with n-3 fatty acids including metabolic, genetic, cardiovascular, immunologic and mental health conditions [16].

2.1.1.2. Monounsaturated Fatty Acids

Monounsaturated fatty acids contain only one double bond and are the most widely occurring fatty acids [10]. More than 100 monounsaturated fatty acids have been identified but the most common are the omega-9 (n-9) monounsaturated fatty acids such as palmitoleic acid (16:1, n-9) and oleic acid (18:1, n-9), and the n-6

fatty acid, namely vaccenic acid (18:1, n-6). Since monounsaturated fatty acids do not contain a *bis*-allylic methylene group, they are far more resistant to oxidation compared to polyunsaturated fatty acids [17]. With few exceptions, tree nuts and their oil extracts are rich sources of monounsaturated fatty acids. Many tree nut oils contain oleic acid at levels that exceed those in olive oil. Oleic acid has long been associated with cardiovascular health [18] and more recently with modulation of cellular signalling cascades in cancer cells [19].

2.1.1.3. Health Effects of Fatty Acids

High dietary intake of saturated fats has been linked to arteriosclerosis and coronary disease [20]. Arteriosclerosis is a process in which fatty substances, especially cholesterol and triacylglycerols, are deposited in the walls of medium-sized and large arteries. Cholesterol in the blood is transported in combination with specific aggregates of lipids and proteins called lipoproteins. Normally, most cholesterol is carried in low density lipoprotein (LDL), which is also a significant risk factor for coronary heart disease. Other plasma cholesterol in transported in high-density lipoprotein (HDL) [21]. Research findings indicate that when plasma HDL levels are high, coronary heart disease risk is lowered [22].

Saturated fats, particularly myristic and palmitic acids, have been shown to increase plasma cholesterol and LDL levels, however, stearic acid has not been shown to have atherogenic effects [23]. Consuming mostly polyunsaturated fatty acids tends to reduce the levels of both LDL and HDL, while research indicates that consuming mostly monounsaturated fats tends to reduce only LDL [24]. Grundy [23] has reported measuring plasma LDL and HDL after 4 weeks on a diet containing 40% fat from either palm oil (saturated fat), high oleic safflower oil

(monounsaturated fat), or high linoleic safflower oil (polyunsaturated fat). The poly- and monounsaturated fat diets are known to have equal effects on lowering plasma LDL. However, the polyunsaturated diet lower plasma HDL more frequently than the monounsaturated diets [23].

Studies using porcine endothelial cells show that oleic acid blocks endothelial activation [25]. For example, oleic acid reduces the damage caused by oxidation within endothelial cells [26], and it blocks the production of proinflammatory eicosanoids that cause endothelial inflammation [27]. Oleic acid has also been shown to reduce the passing of white blood cells into blood vessel walls, which is a key step in endothelial activation and atheroma development. In one study, oleic acid decreased the production of vascular cell adhesion molecule-1 that attaches white blood cells to the endothelium by 40% [28]. Oleic acid may achieve this effect by controlling the genes responsible for making the protein [29].

Fatty acids have also been shown to influence the development of cancerous tumours, with monounsaturated and n-3 fatty acids receiving the most attention for the potential beneficial effects. Recently, oleic acid was shown to reduce the activity levels of a gene called Her-2/neu, which occurs at high levels in over a fifth of breast cancer patients and is associated with highly aggressive tumours with a poor prognosis. In addition to suppression of this gene, oleic acid also improved the effectiveness of herceptin, a drug commonly used to fight aggressive breast cancers [30].

2.1.2. Acylglycerols

Acylglycerols are acyl esters of glycerol and include monoacylglycerols, diacylglycerols and triacylglycerols (Figure 2.1). The terminal carbon atoms of the

glycerol backbone of acylglycerols are referred to as the *sn*-1 and *sn*-3 positions, or as the α-positions. The central carbon atom is referred to as the *sn*-2 or β-position. Monoglycerols may be either 1-acyl (α-monoacylglycerol) or 2-acyl (β-monoacylglycerol) isomers [10]. Diacylglycerols may be 1,2- or 2,3-diacylglycerols (α,β) or 1,3-diacylglycerols (α,α). Mono- and diacylglycerols are present in trace amounts in animal and plant lipids; however, significant levels are indicators of rancidity and are usually the products of active lipooxygenase enzymes [31].

Triacylglycerols are the main class of lipids in natural fats and oils. Natural triacylglycerols rarely contain three identical acyl groups; in many cases they contain two or three different acyl groups. The potential number of triacylglycerols present in a fat or oil rises quickly with the number of fatty acid moieties present, such that hundreds of triacylglycerols could be theoretically derived from 10 fatty acids. This, along with the fact that physical properties of triacylglycerols do not change significantly with fatty acid distribution, makes the analysis of triacylglycerols a very tedious and time consuming task [32].

Hydrolysis of acylglycerols by acid or alkali gives glycerol and a mixture of fatty acids. Enzymatic hydrolysis is another effective method for hydrolysis of fatty acids and can be used to perform hydrolysis with higher specificity, for example, pancreatic lipase can used be to hydrolyse the terminal fatty acids while leaving the β-monoacylglycerol intact [10].

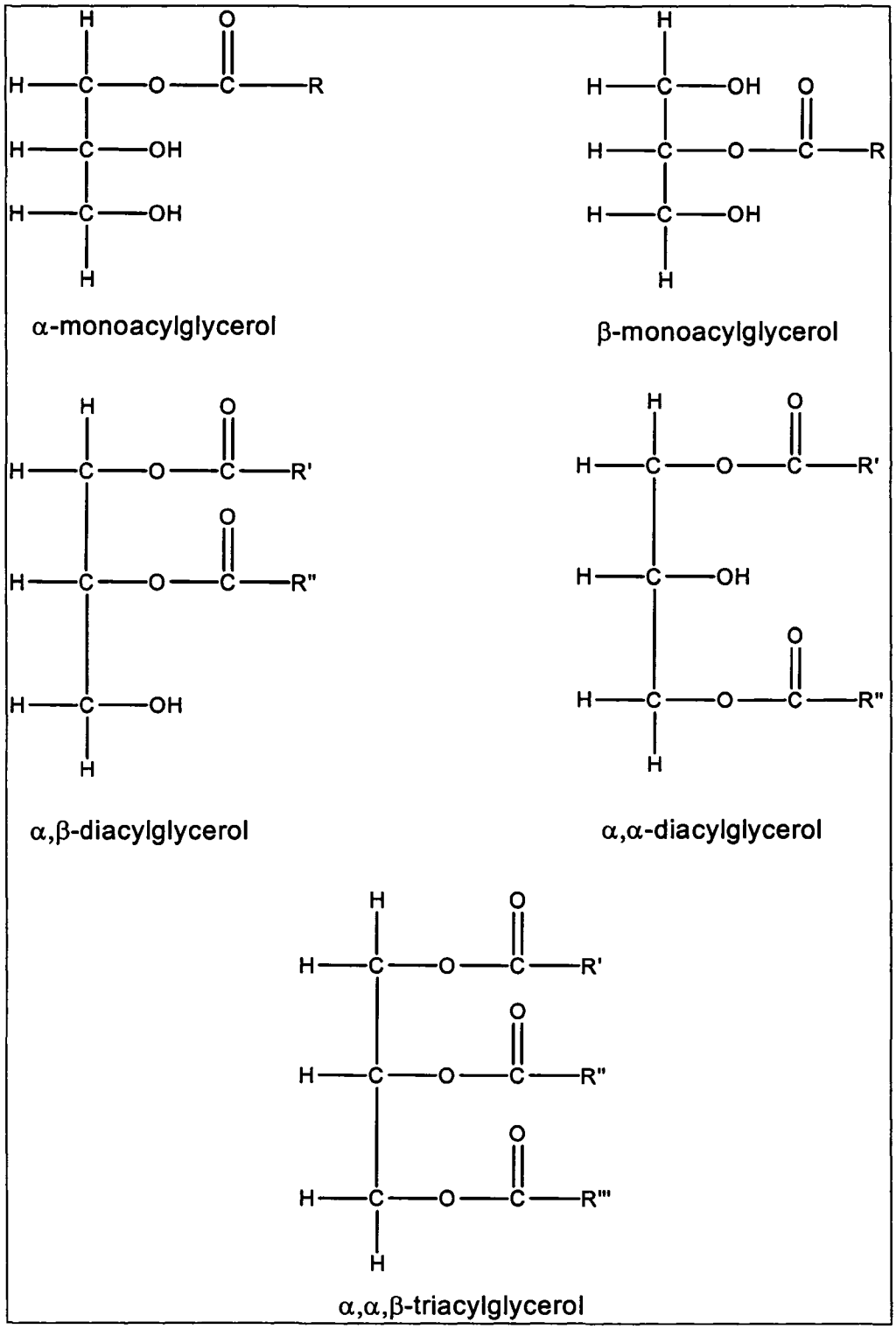


Figure 2.1. Chemical Structures of Acylglycerols

2.1.3. Phospholipids

The term phospholipid denotes any lipid containing a phosphate group as either a mono- or diester. Many phospholipids also contain amino groups. Phospholipids are divided into two subclasses that include the glycerophospholipids and the sphingolipids [33]. All phospholipids contain a negatively charged phosphate group and two acyl chains. The hydrocarbon chains are hydrophobic whereas the phosphate and amino groups with opposite charges are hydrophilic, which makes phospholipids amphipathic molecules.

A variety of groups can be added to the phosphate moiety of phospholipids using a phosphate diester (phosphodiester) linkage, which is referred to as the phospholipid head group. Phospholipids are a major component of biological membranes that separate cells and intercellular organelles from their external environments. Due to their thermodynamic properties phospholipids can spontaneously form a double layer in aqueous environments. In a real cell the membrane phospholipid molecules create a spherical three dimensional lipid bilayer shell around the cell [10].

Glycerophospholipids are the most common and abundant group of membrane phospholipids. They are essentially α,β-diacylglycerols with a head group containing phosphate attached to a terminal position (α-) of the glycerol molecule. With the exception of phosphatidic acid, the phosphate group forms a phosphodiester bond with the primary hydroxyl group of glycerol (*sn*-1 hydroxyl group) and the hydroxyl group of a polar head group substituent. In animal lipids the *sn*-1 position of glycerophospholipids are usually occupied by a saturated fatty acid while the *sn*-1 position almost always contains an unsaturated fatty acids.

There is no general consensus regarding the fatty acid distributions of plant glycerophospholipids [34]. Common glycerophospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidic acid (PA). Phosphatidylcholine (lecithin) is the most abundant glycerophospholipid in animals and higher plants; it contains a phosphorylcholine head group and is present in all cell extracts [35]. Lysophosphatidylcholine (LPC) is similar to PC except that only one of the two available glycerol hydroxyl groups is esterified to a fatty acid. Lysophosphatidylcholine is often found in samples containing PC [34], and in most cases LPC is unesterified at the *sn*-2 position [33]. The second most abundant glycerophospholipid in animals and higher plants is phosphatidylethanolamine which contains a phosphorylethanolamine head group [34]. Phosphatidylserine is a common glycerophospholipid, it is weakly acidic and it is usually associated with potassium, calcium, sodium or magnesium ions which can be removed by washing the compound with water [33]. Its head group consists of phospho-L-serine. Phosphatidylinositol (monophosphoinositide) is a strongly acidic glycerophospholipid and is usually associated with calcium or magnesium [33]. It is widely distributed among the animal and plant kingdoms. The head group of PI is phospho-1-D-myo-inositol, which contains a six carbon cyclic sugar alcohol, inositol. Phosphatidylinositol may be further phosphorylated to give diphosphatidylinositol and triphosphatidylinositol (the polyphosphoinositides) [35]. Phosphatidic acid is a minor but widely distributed glycerophospholipid [35]. It is the precursor of all other glycerophospholipids as well as the triacylglycerols [33].

Unlike the glycerophospholipids which contain esterified acyl chains, the sphingolipids are phospholipids with fatty acids combined as amides of long chain compounds containing an amino and two or more hydroxyl groups [10]. Sphingolipids include long chain amines, cerebrosides, gangliosides and sphingomyleins.

2.1.4. Sterols and Stanols

More than 40 plant sterols (or phytosterols) have been identified, but β-sitosterol, campesterol, and stigmasterol are the most widespread (Figure 2.2). The various sterols differ with respect to the distribution of methyl and ethyl group substitutions on their side chains as well as the position and number of double bonds in their sterol ring structure. Stanols are saturated sterols, containing no double bonds in their sterol ring structure. The major plant stanols are sitostanol and campestanol; in nature they are less abundant than sterols. Sterolins are phytosterols in the glycoside form (attached to a sugar moiety). Sterols and stanols can be esterified with fatty acids to form sterol esters [36].

Plant sterols can be obtained using various methods. Purification of sterols from edible oils such as soybean, rapeseed, and sunflower oils is commonly carried out by different chemical companies to isolate reagent grade phytosterols, which are then further purified to produce pure or analytical grade sterols. Another interesting source material is tall oil, derived from the process of paper production from wood pulp, which contains a high proportion of plant stanols (primarily β-

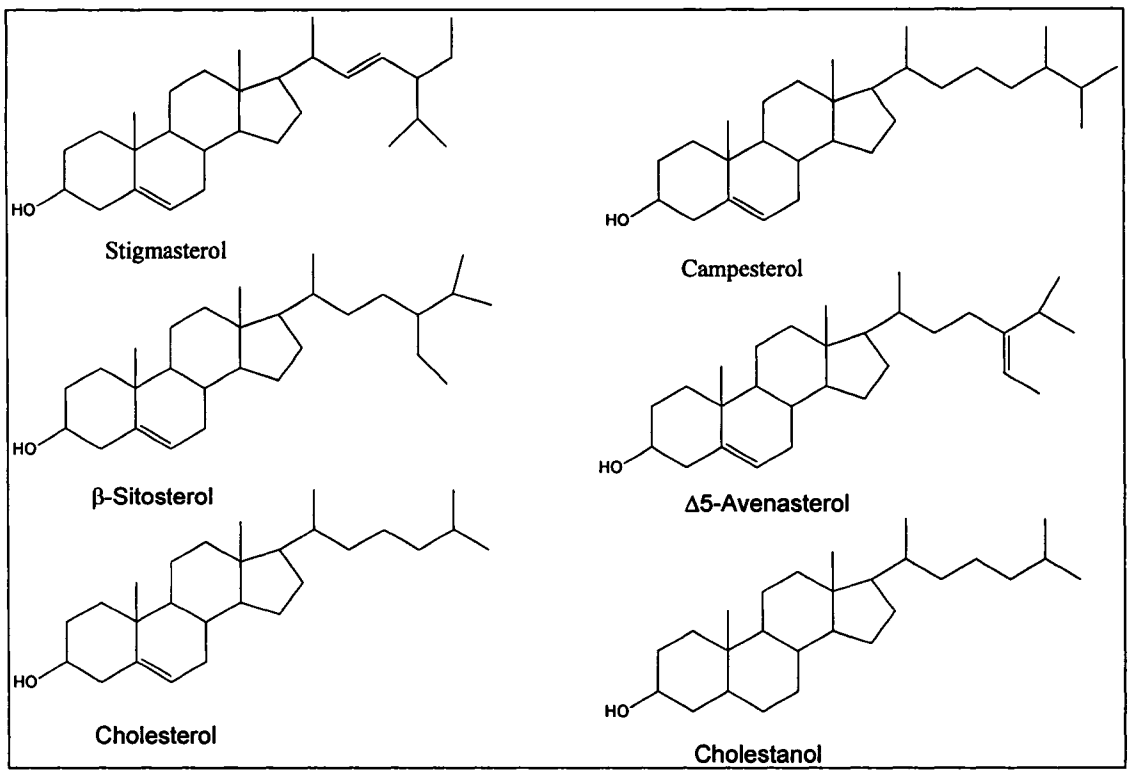


Figure 2.2. Chemical Structures of Sterols and Stanols

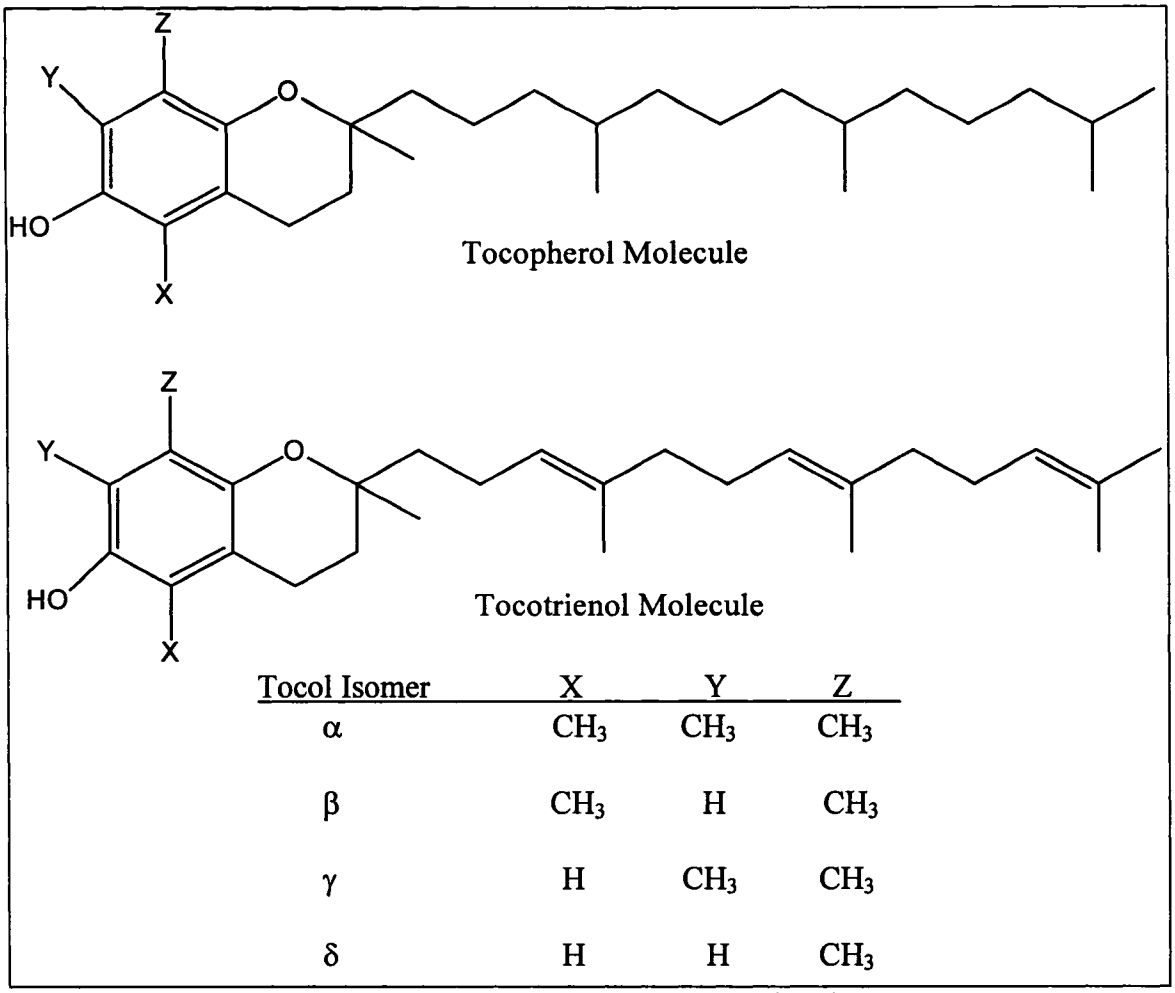
sitostanol) than vegetable oils [37]. Sterol esters are more fat soluble than free sterols. In the intestine, sterol esters are hydrolysed to free sterols as part of the normal digestive process [38]. Approximately 5% of total dietary phytosterols are absorbed in humans, with the actual rate varying for individual sterols. Unabsorbed sterols are metabolised by intestinal bacteria [39].

When ingested, phytosterols are incorporated into micelles within the intestinal lumen and displace dietary cholesterol, thereby reducing cholesterol absorption [37]. Cholesterol absorption from both diet and enterohepatic circulation is strongly reduced in the presence of phytosterols [37]. Unabsorbed dietary cholesterol is removed from the body with the feces. Plant sterols or stanols may also reduce the esterification rate of cholesterol in the enterocyte, and consequently,

the amount of cholesterol secreted as chylomicrons [40]. Unlike cholesterol, phytosterols have a low capacity for intestinal absorption, which together with their high rate of biliary excretion by the liver, results in a low level of phytosterols in the blood [41]. However, when dietary cholesterol is replaced with phytosterols, the concentration of serum phytosterols increases slightly [42]. Controlled trials have shown that daily intake of 2.0 to 2.5g of plant sterols or stanols increases liver LDL receptor expression levels and causes an average reduction of LDL of up to 14% [43]. Phytosterols and phytostanols have also been shown to reduce LDL oxidation and animal studies show that they can reduce atheroma development [44].

2.1.5. Tocols

Tocols occur in nature as eight different isoforms, namely α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols (Figure 2.3). Tocotrienols differ from tocopherols only in their aliphatic tail. Tocopherols have a phytyl side chain attached to their chromanol nucleus, whereas the phytyl chains of tocotrienols contain three double bonds. The various tocol isoforms differ in the number and location of their methyl substituents on the chromanol nucleus. The α- isoforms contain 3 methyl groups, the β- and γ- isoforms have two, and the δ- isoform has only one methyl group [45]. Alpha-tocopherol constitutes about 90% of the tocopherol in animal tissues [45]; it was originally designated d-α-tocopherol on the basis of its optical activity. There are actually three asymmetric carbon atoms in all tocol isomers, one at the 2-position of the chromanol ring, and the other two on the aliphatic chain, at the 4' and 8' positions; all being locations of methyl groups. The IUPAC advocates an R and S system of stereoisomer designation, rather than the "d-" and "l-" prefixes which indicate optical activity. Therefore, the common



Tocol Isomer	X	Y	Z
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

Figure 2.3. Chemical Structures of Tocopherols and Tocotrienols

natural form of α-tocopherol has the IUPAC name 2R,4'R,8'R-α-tocopherol (RRR-α-tocopherol).

Plants can synthesise tocopherols whereas animals can not; animals satisfy their tocopherol requirements by ingestion of preformed tocopherols. The main functions of tocols in both foods and in living organisms are to act as lipid phase antioxidants; however, they also have other important biological functions in animals. In animals, tocotrienols may not be assimilated as well as tocopherols [46, 47]. The first dietary role discovered for the tocopherols was their function as essential nutrients for normal development of rat fetus [48]. Tocopherol fertility

restoration assays provided a basis for the international unit (IU) quantification system. The acetate of RRS-α-tocopherol was arbitrarily assigned the value of one IU per milligram (mg). Natural α-tocopherol (RRR-α-tocopherol) had an activity of 1.49 IU per mg, whereas synthetic α -tocopherol (a racemic mixture of 8 to 23 stereoisomers) had an activity of 1.00 IU per mg. Beta-, γ- and δ-tocopherols had activities of 0.60 IU, 0.30 IU and 0.015 IU per mg, respectively [49]. For α-tocopherol, the asymmetric carbon at the 2-position of the chromanol ring is the major determinant of its biological activity [45]. Enhanced precision of chemical techniques for the analysis of tocopherols and tocotrienols made biological activity assays unnecessary after the 1950's. The IU system is now considered obsolete and the United States Food and Nutrition Board now quotes Recommended Daily Allowances (RDAs) for vitamin E in milligrams rather than in IU [50].

Currently, the biological activity of tocopherols that attracts the most interest is the prevention of lipid peroxidation. Alpha-tocopherol is the most active tocopherol against peroxyl radicals ($ROO^{\cdot}$) and δ-tocopherol is the least active (α>γ> β >δ) [51]. The antioxidant activities of tocopherols are based on the ease with which the hydrogen on the hydroxyl group of their chroman ring can be donated to neutralize a free radical (creating a more stabile tocopheroxyl radical). As with phospholipids, the polar chromanol ring tends to stay near the outer edges of cell membranes, whereas the hydrophobic core will be buried deep into the membrane. When a phospholipid tail becomes peroxidized by a free radical, the tail becomes more polar and migrates to the surface of the membrane where it can react with the tocopherol chromanol ring to be neutralized, concomitantly forming a

tocopheroxyl radical. The tocopheroxyl radical can be regenerated directly by ubiquinol or vitamin C.

Although important as antioxidants, tocopherols have many other biological activities and health functions. The different tocopherol isoforms do not have the same relative activities for each function. For example, γ-tocopherol has been stated to be the most effective isoform for preventing breast cancer [52], whereas α–tocopherol is more effective for reducing LDL oxidation and atheroma development [53]. Although γ-tocopherol is the predominant dietary form of tocopherol in the American diet (mainly from vegetable oil and nuts), the liver preferentially loads LDL with α-tocopherol for delivery to the body; α-tocopherol is at least 5 times more plentiful in the bloodstream than γ-tocopherol. Gamma – tocopherol concentrates in certain tissues, constituting a third of total tocopherol in veins and nearly 40% of total tocopherol in muscle [54].

Tocols have the potential to act as pro-oxidants rather than antioxidants when co-antioxidants such as vitamin C are not available to neutralize the tocopherol radical. This condition is observed in systems under high oxidative stress [55]. There has been concern that α-tocopherol also reduces platelet aggregation through inhibition of protein kinase C [56]. A study of supplementation with 1000 IU daily of RRR-α -tocopherol for 12 weeks in healthy adults indicated that high doses of RRR-α -tocopherol may antagonize vitamin K [57]. However, another study of healthy elderly individuals taking up to 800 IU (727 mg) per day of RRR-α-tocopherol for 4 months showed no increase in bleeding time [58].

2.2. Lipid Oxidation

2.2.1. Introduction to Lipid Oxidation

Lipid oxidation can be defined as the oxygen dependent deterioration of lipids containing any number of carbon-carbon double bonds. Lipid oxidation is initiated by compounds known as sensitizers which include heat, light and metal ions. Lipid oxidation imparts undesirable flavours, aromas and compromises the nutritional quality of fats and oils, and leads to the production of toxic compounds. Lipid oxidation has been noticed since antiquity as a major problem in the storage of fats and oils and was first reported by Swiss chemist Nicolas-Théodore de Saussure (1746-1845) who observed that a layer of walnut oil exposed to air was able to absorb 150 times its own volume of oxygen during one year. He also noted that the oil became more viscous and developed an off-odour [59]. Systematic studies of lipid oxidation have been carried out since the 1940's when it was established that hydroperoxides are the primary products of the oxidation (or peroxidation) of unsaturated lipids [60].

Lipid hydroperoxides are themselves non-radical compounds, but readily decompose into toxic compounds. The formation of hydroperoxides occurs via several possible reactions involving activated species known as reactive oxygen species (ROS), which are also responsible for damage to various tissues in the body. Reactive oxygen species include hydroxyl radicals, lipid oxyl or peroxyl radicals, superoxide, and peroxinitrite formed from nitrogen oxide. Free radicals are defined as species capable of independent existence containing one or more

unpaired electrons. They are formed either by the loss or gain of a single electron from a non-radical.

In fats and oils, the lipids involved in the oxidation process contain unsaturated fatty acids such as oleic, linoleic, linolenic and long chain polyunsaturated fatty acids, however, other unsaturated lipids present in fats and oils such as sterols do become oxidized during this process [60]. The rate at which fatty acids are oxidized increases with the degree of unsaturation. Lipid oxidation can take place via autoxidation, photosensitized oxidation (photooxidation), thermal oxidation, hydrolytic oxidation and enzymatic oxidation.

2.2.1.1. Reactive Oxygen Species

Lipid oxidation can be initiated when certain ROS abstract hydrogen atoms from unsaturated lipids, resulting in stabilization of ROS and the formation of lipid free radicals. Superoxide ($O_2^{\cdot-}$) is one of the most extensively studied ROS and is formed when an electron is added to molecular oxygen. The production of superoxide radicals at the membrane level is initiated in specialized cells with phagocytic functions (macrophages) and contributes to their bactericidal action. The flavin cytosolic enzyme xanthine oxidase found in all tissues and in milk fat globules generates superoxide radicals from hypoxanthine and oxygen. Superoxide is believed to be the initiator of many vascular diseases; most notably in the formation of foam cells by activated macrophages, which is proposed to lead to atherosclerosis [61]. Superoxide is acted upon *in-vivo* by superoxide dismutase to form hydrogen peroxide (H_2O_2), which is then reduced by catalase to form water.

The hydroxyl radical ($^{\cdot}OH$) is a very active ROS which is formed in the presence of Fe^{2+} and H_2O_2 via the Fenton (1894) reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

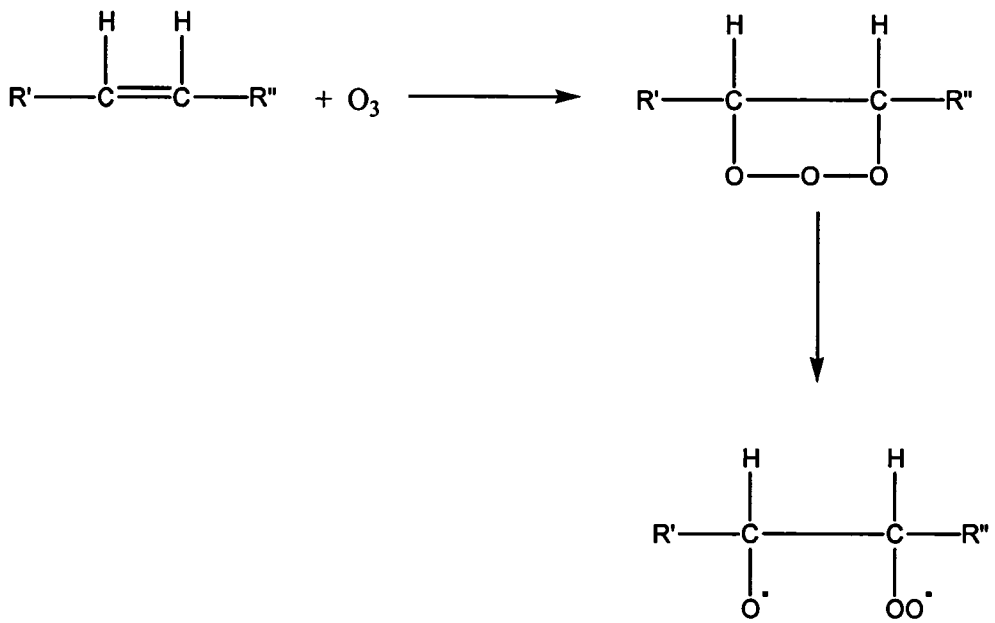
This Fe^{2+} catalysed decomposition of hydrogen peroxide is considered the most prevalent reaction in biological systems and leads to the formation of various deleterious lipid peroxidation products [62].

Nitric oxide ($^{\bullet}NO$) is produced in several biological systems and has been well studied in vascular tissue. While being poorly reactive, it reacts rapidly with oxygen to yield nitrogen dioxide ($^{\bullet}NO_2$) which in turn may react with nitric oxide to yield dinitrogen trioxide (N_2O_3). The rapid reaction of $O_2^{\bullet-}$, produced in different pathological states, with NO gives the extremely reactive peroxynitrite ($ONOO^-$) which mediates oxidation, nitrosation, and nitration reactions. Peroxynitrite decomposition forms $^{\bullet}OH$ and $^{\bullet}NO_2$. The high rate and broad distribution of $^{\bullet}NO$ production *in-vivo*, combined with its high potential to react with oxygen radicals, makes $^{\bullet}NO$ a key interest of researchers studying free radical biology. Multiple mechanisms account for the nitration of lipids by $^{\bullet}NO$-derived species [63]. Nitric oxide is naturally formed in activated macrophages and endothelial cells and is considered as an active agent in several pathologies based on inflammation, organ reperfusion and may also play an important role in atherosclerosis [64].

The singlet oxygen ($^1O_2$) form of oxygen is not a true radical but is important in reactions related to ultraviolet exposition (UV-A, 320-400 nm). Its toxicity is reinforced when appropriate photo-excitable compounds (sensitizers) are present with molecular oxygen. Natural sensitizers known to catalyze lipid oxidation include tetrapyrroles (such as bilirubin), flavins, chlorophylls and related pigments, hemoproteins and reduced pyridine nucleotides (such as nicotinamide adenine dinucleotide). Many of these sensitizers occur in foods and cosmetics, which makes

singlet oxygen a major concern in the long term storage of these products. The presence of metals contributes to the production of singlet oxygen; trace amounts of metal ions significantly accelerate the oxidation of unsaturated lipids and formation of hydroperoxides [65]. Singlet oxygen formation in lipids may account for the chemiluminescence observed during lipid oxidation.

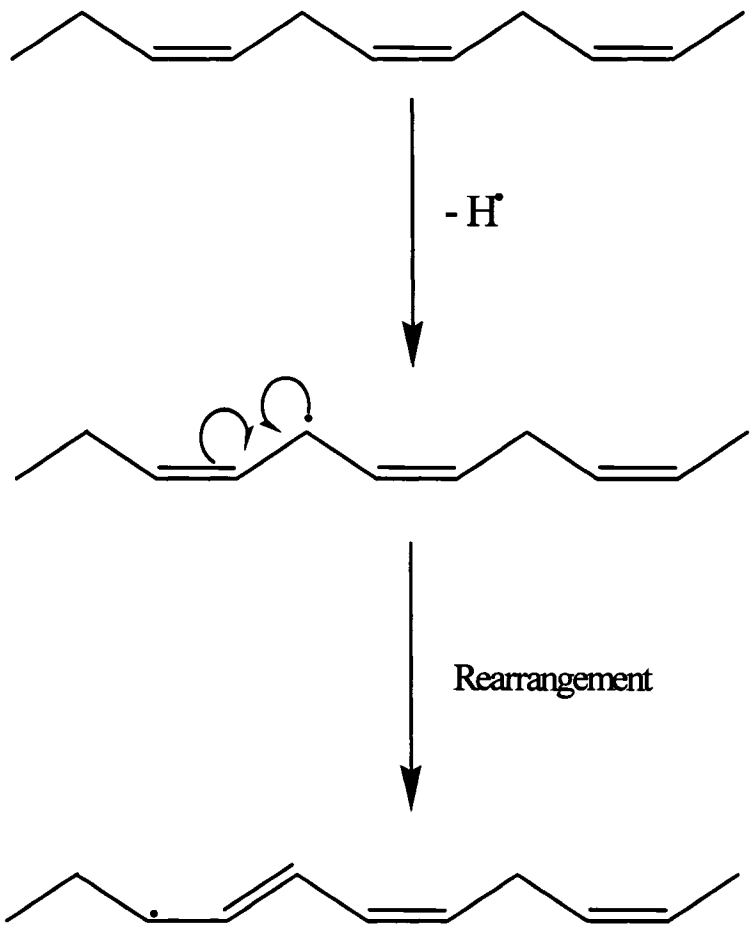
Similar to singlet oxygen ($^1O_2$), ozone (O_3) is not a ROS but can produce them; O_3 has been shown to stimulate lipid peroxidation and induce damage to lipids and proteins *in-vivo*. The exact chemistry of ozone-mediated lipid oxidation is not entirely understood, but it is proposed that O_3 adds on across a double bond and then decomposes to form a free radical. The proposed mechanism is given below.



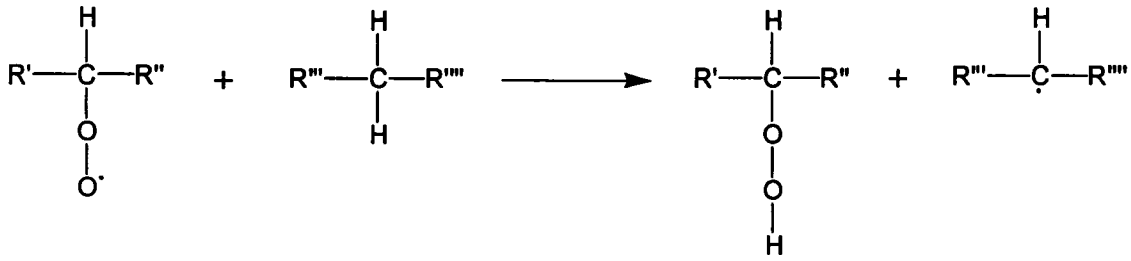
2.2.1.2 Autoxidation

Autoxidation is a chain reaction involving three steps of initiation, propagation and termination [5]. The production of free radicals during the initiation phase requires an activation energy of approximately 35 kCal. For this

reason, initiators such as metal catalysts, light, heat, UV radiation, $^1O_2$, and pigments are required. Once initiated, a hydrogen atom from an allylic or a *bis*-allylic methylene group of a monounsaturated or a polyunsaturated fatty is lost to produce a hydrogen atom and an alkyl radical. The alkyl radical tends to be stabilized by molecular rearrangement to form a conjugated diene.



Under aerobic conditions, conjugated dienes combine with O_2 to give a peroxyl (alkoxy) radical, ROO·. Peroxyl radicals are able to abstract a hydrogen atom from another unsaturated lipid molecule, especially in the presence of metals such as copper or iron, thus causing an autocatalytic chain reaction. When a peroxyl radical combines with a hydrogen atom, a lipid hydroperoxide (or peroxide) is formed.



Alkyl radicals, peroxyl radicals and lipid hydroperoxides are collectively referred to as the primary products of lipid oxidation; these compounds are odourless and tasteless to humans and predominate in the early stages of lipid autoxidation [66]. Lipid hydroperoxides decompose in the presence of catalysts, thereby forming additional radicals; this reaction characterizes the propagation stage. In addition to radical formation, the decomposition of hydroperoxides gives rise to the formation of secondary products of lipid oxidation which include aldehydes, ketones, polymers and hydrocarbons, among others (Figure 2.4). These products are responsible for the characteristic off-odours and off-flavours of oxidized fats and oils.

The chain reactions may be terminated by the formation of non-radical products and the termination steps are favoured when substrates (unsaturated fatty acids) or oxygen have been depleted. Alkyl radicals can react with ROO· to afford non-initiating and non-propagating species such as relatively stable dimers (ROOR). Stable bonds between lipid peroxides and proteins have also been observed. Lipid-phase antioxidants protect unsaturated lipids from oxidation because they act to neutralize free radicals before they can react with lipids.

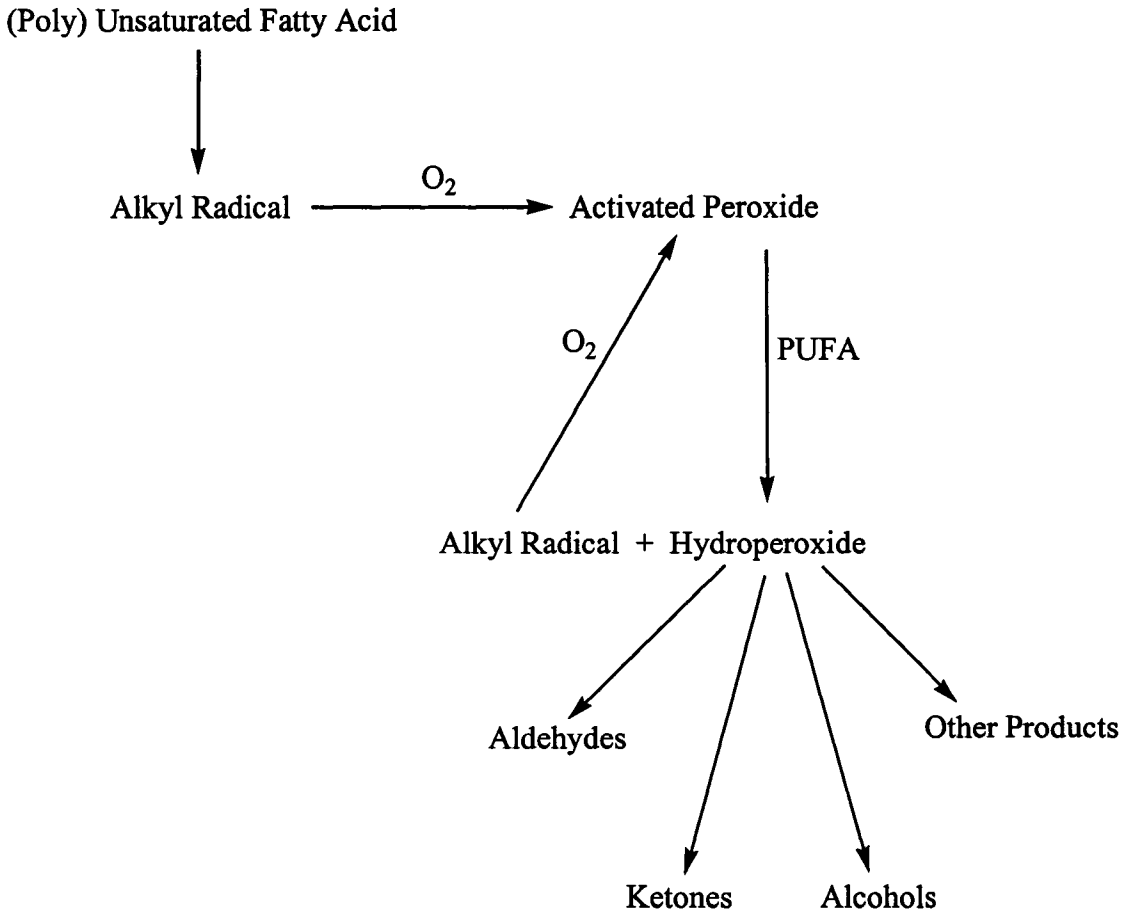


Figure 2.4. Generalized Scheme for Autoxidation of Unsaturated Fatty Acids

2.2.1.3. Photosensitized Oxidation

Photosensitized oxidation is initiated by a sensitizer and light. In fats and oils, singlet oxygen can be generated by the action of photosensitizers such as natural pigments, including chlorophylls, pheophytins and haem-containing compounds [5]. Two pathways have been proposed for photosensitized lipid oxidation [67]. In one pathway (type I), the sensitizer absorbs light and reacts with a lipid substrate to form intermediates that can go on to react with ground state oxygen (triplet oxygen) to yield lipid oxidation products. In another proposed pathway (type II), molecular oxygen reacts with the sensitizer after light absorption and gives rise to oxidation

products. The products of photosensitized oxygen include both non-conjugated and conjugated diene hydroperoxides, whereas free radical mediated lipid oxidation produces only conjugated diene hydroperoxides [68]. Photosensitized oxidation is efficiently inhibited by carotenoids, which is the main protective role played by these compounds in plants. The inhibitory mechanism is thought to be through interference with the formation of singlet oxygen from triplet oxygen, which is achieved by absorbing light energy before it can react with triplet oxygen. In contrast, tocopherols inhibit photosensitized oxidation by quenching the previously formed singlet oxygen before it can react with unsaturated lipids, thus stabilizing the triplet oxygen form. Previously, it was shown that carotenoids are efficient inhibitors of photosensitized oxidation in vegetable oils, but only if tocopherols are also present to protect the carotenoids from oxidation [69].

2.2.1.4. Thermal Oxidation

Thermal oxidation takes place when fats and oils are subjected to high temperatures, such as those encountered during deep fat frying. The breakdown products of fats and oils during thermal oxidation include volatile and non-volatile decomposition products, which influence the flavour of the oil and fried foods [70]. The volatile breakdown products, including aldehydes, lactones and pyrazines, influence the flavour of deep fried foods [71]. The non-volatile products include cleavage products of acylglycerols as well as polymers arising from formation of carbon-carbon bonds or oxygen bridges between radicals [72]. The non-volatile oxidation products contribute to the deterioration of frying fats by reducing their smoke, flash and fire points, and impart off-flavours and potentially toxic compounds to fried foods [73].

2.2.1.5. Hydrolytic Oxidation

Hydrolytic oxidation is due the reaction of lipids and water in the presence of a catalyst or by the action of enzymes. This pathway gives rise to free fatty acids as well as salts of free fatty acids [74]. This type of oxidation occurs most commonly in fats containing short and medium chain fatty acids. However, it also occurs during deep fat frying when the moisture in the material to be fried reacts with the triacylglycerols, hence releasing the free fatty acids.

2.2.1.6. Enzymatic Oxidation

Lipoxygenases (from plants or animals) catalyze reactions between oxygen and polyunsaturated fatty acids containing methylene interrupted double bonds. When arachidonic acid is the substrate, the hydroperoxides formed are the hydroperoxyeicosatrienoic acids (HpETE), which are then transformed into hydroxyeicosatrienoic acid products (HETEs). These HETEs are also formed directly via cytochrome P450 induced reactions (mono-oxygenases) and sometimes also via cyclooxygenase enzymes. Six hydroperoxides (5-, 8-, 9-, 11-, 12-, and 15-HpETE) are known to be formed from arachidonic acid in animal cells. Dihydroperoxy compounds may also be formed via the action of 5- and 15-lipoxygenases. These compounds are important metabolic intermediates but are also bioactive. Cyclooxygenases (in plants and animals) catalyze the addition of molecular oxygen to various polyunsaturated fatty acids and convert them into biologically active molecules called endoperoxides, which are intermediates in the transformation of fatty acids to prostaglandins [75].

2.2.2. Assessment of Lipid Oxidation

Lipid oxidation can be measured by objective or sensory methods. Sensory methods of assessing lipid oxidation in foods are widely used but are time consuming and taste panels are difficult to maintain. Use of trained panelists has also changed the definition and such experiments may now be referred to as objective methods.

Several objective methods have been developed to assess the extent of lipid oxidation in fats and oils and these include measurement of conjugated dienes, peroxide value, thiobarbituric acid reactive substances, *para*-anisidine value, volatile carbonyl compounds and several spectroscopic methods, including nuclear magnetic resonance (NMR) [76] and Fourier transform infrared (FT-IR) [77]. Many of the objective methods used to test lipid oxidation are empirical and their accuracy depends on standardization of the experimental conditions.

The analytical methods used to evaluate the oxidative stability of fats and oils each have limitations and selecting an optimum test is difficult due to the complexity of the chemical processes involved. For this reason it is recommended that the progress of oxidation of edible fats and oils be monitored by more than one method, including at least one test for each of the primary and secondary lipid oxidation products.

2.2.2.1. Conjugated Dienes

Oxidation of polyunsaturated fatty acids is accompanied with an increase in the ultraviolet absorption of the product. Lipids containing methylene interrupted dienes or polyenes exhibit a shift in their double bond position during oxidation, which is due to isomerization and conjugate formation. The resulting conjugated

dienes can by quantified due to their strong absorption maximum at 234 nm. The absorption maximum of conjugated trienes is 268 nm [78]. The conjugated dienes of a sample are quantified by diluting a weighed amount of oil in isooctane and the absorbance is read spectrophotometrically at 234 nm [79].

Carotenoids and other molecules containing double bonds can absorb light at 234 nm and may interfere with conjugated dienes determination, which is a major drawback of this method. However, this method is faster and simpler than other tests for primary products of lipid oxidation, requires a small sample size (>10 mg) and does not depend on chemical or colour reactions [76].

2.2.2.2. Peroxide Value

The peroxide value is a measure of the hydroperoxide content of a fat or oil, and thus is a test for primary lipid oxidation products. The peroxide value is most commonly measured quantitatively using an iodometric titration procedure. The principle of the peroxide value determination is based on the reduction of hydroperoxides with iodide (I^-). The liberated iodine is titrated with a standardized sodium thiosulphate ($Na_2S_2O_3$) solution. Therefore, the amount of released iodine is proportional to that of the peroxides present. The peroxide value is expressed in units of milliequivalents of active oxygen (peroxide) per kg of lipid (meq/kg). The peroxide value is a measure of early stage lipid oxidation due to autoxidation and photosensitized oxidation, however, it is less useful during thermal oxidation because hydroperoxides decompose rapidly under high temperature conditions.

Although popular, iodometric procedures have several potential sources of errors including addition of the liberated iodine to double bonds and also oxidation of iodide by air components. Results are also influenced by the structure and

reactivity of the peroxides as well as the reaction temperature and timing of the experiment. However, most of these problems have been addressed by currently accepted peroxide value procedures [76].

2.2.2.3. Thiobarbituric Acid Test

The 2-thiobarbituric acid (TBA) test is a convenient method for the measurement of secondary products of lipid oxidation, referred to as TBA reactive substances (TBARS). This method tests for carbonyl-containing compounds, and is used primarily to quantify malondialdehyde (MA), an important decomposition product of unsaturated lipids with three or more double bonds, among others. During the TBA test, one molecule of MA reacts with two molecules of TBA to form a pink coloured TBA-MA adduct. The absorption intensity of this chromogen is measured at 532 nm [17].

A major disadvantage of the TBA test is that MA is only formed by fatty acids which contain three or more double bonds, however, other products such alkenals and alkadienals are also favoured, hence these all must be expressed as equivalents of MA. Despite its limitations, the TBA-test is commonly used to test lipid oxidation in fats, oils and foods.

2.2.2.4. *p*-Anisidine Value

The anisidine value is empirically defined as 100 times the absorbance of a solution resulting from the reaction of 1 g of oil or fat and *p*-anisidine in 100 mL of isooctane, measured at 350 nm in a 1 cm cell [76, 80].

The anisidine test involves the condensation reaction between conjugated dienals or 2-alkenals in the sample and the *p*-anisidine reagent in isooctane, followed by absorbance measurement at 350 nm. In the presence of acetic acid, *p*-

anisidine reacts with aldehydes producing a yellowish colour. The molar absorbance at 350 nm increases if the aldehyde contains a double bond. The test is particularly useful with abused oils with low peroxide values such as frying oils. An aniside value of 10 or less is considered acceptable for edible oils.

2.2.2.5. Active Oxygen and Oil Stability Instrument/Rancimat Methods

The active oxygen method, or Swift test, is a common accelerated method for assessing oxidative stability of fats and oils. The method is based on the principle that formation of lipid hydroperoxides is accelerated when lipids are subjected to high temperatures while aerated [81].

Automated versions of the active oxygen method have been developed and include the Oxidative Stability Instrument (OSI), Rancimat and Oxidograph. The OSI and Rancimat tests measure changes in conductivity caused by volatile ionic organic acids, mainly formic acid, automatically and continuously. This differs from the active oxygen method where peroxide value changes are determined. Rancimat and OSI tests proceed slowly at first because little amounts of acids are produced during the induction period. The endpoint of this test is selected as the point at which a rapid rise in conductance begins [82]. The Oxidograph test involves heating an oil sample exposed to air or oxygen which results in pressure decrease inside the reaction vessel. The change in pressure is measured electronically by means of pressure transducers, and the endpoint of the test is reached when pressure inside the vessel exhibits a marked decrease.

2.2.2.6. Headspace Analysis of Volatiles

A common group of secondary oxidation products of unsaturated lipids are volatile aldehydes such as propanal, hexanal and nonanal which arise from the oxidation of n-3, n-6 and n-9 fatty acids. Other aldehyde lipid oxidation products include butanal, pentanal, 2-pentenal and octanal, among others. Malondialdehyde is a common secondary product of polyunsaturated lipid oxidation [83]. Various types of headspace gas chromatographic techniques have been developed to assess the composition of volatiles in oxidized fats and oils [83, 84]. Oxidized lipid samples are placed in closed vials and heated to vaporize the volatile components that result from the decomposition of hydroperoxides. Following the heating period the sample vials are pressurized, and then the volatiles in the headspace above the sample are loaded onto a gas chromatograph column to be analysed. The total peak area of volatiles in this technique increases with the storage period of a sample. This method is particularly suitable for highly volatile compounds because they have a favourable equilibrium between a sample and its headspace. This method is rapid and suitable for routine analysis, but requires a significant investment of money in order to purchase the headspace sampler and a gas chromatograph to which it is dedicated [85].

The main disadvantage of this headspace analysis of volatiles is the difficulty of reaching complete equilibrium with viscous and semi-solid samples. Furthermore, polyunsaturated lipid samples can decompose during the heating period [86].

2.2.2.7. Free Fatty Acids Test

Presence of significant amounts of free fatty acids in a fat or oil is an indication of hydrolytic rancidity, but other lipid oxidation processes can also produce free fatty acids. Free fatty acids in a fat or oil are determined by acid-base titration. The free fatty acids value of a fat or oil is expressed as a percentage of a fatty acid common in the product being tested. Frequently, values are expressed as % oleic acid for tallow or soybean oil. For coconut oil or other oils that contain high levels of shorter chain fatty acids, FFA may be expressed as % lauric acid. It may also be useful to know the composition of the free fatty acids present in a sample to identify their source and understand the cause of their formation. The free fatty acids can be separated from lipid samples, which can then be analysed by gas chromatography for free fatty acid profiles if this information is needed.

2.2.3. Control of Lipid Oxidation

The oxidation of edible fats and oils can be controlled by application of antioxidants, using processing techniques that minimize tocopherol and other natural antioxidant losses, inactivation of prooxidant metals and enzymes, minimizing exposure to oxygen, heat and light, hydrogenation of polyunsaturated fats and the use of an inert gas or vacuum packaging to expel atmospheric oxygen before long term storage. Natural and synthetic antioxidants should meet certain criteria before application in fats and oils including safety, ease of incorporation, effectiveness at low concentrations, absence of undesirable odour, colour and flavour, and availability at low cost [87].

2.2.3.1. Removal of Oxygen

Oxygen is an essential reactant in lipid oxidation reactions. For this reason, control of oxygen availability is a critical factor in minimizing lipid oxidation. The oxygen level can be reduced by vacuum or modified atmosphere packaging and by using oxygen scavengers such as glucose oxidase [77]. These precautions reduce lipid oxidation, especially when combined with antioxidants and low temperature storage in the dark.

2.2.3.2. Addition of Antioxidants

Antioxidants are added to fresh fats and oils to retard oxidation and slow the development of rancidity. Antioxidants can not restore the quality of oxidized oils. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tertiary-butylhydroquinone (TBHQ) are used in fats and oils to inhibit lipid oxidation, however, concern over their use is increasing due to their possible negative health effects, and currently there is considerable interest in natural replacements for these compounds. The effectiveness of natural antioxidants from fruits, vegetables, spices, grains, and herbs to combat lipid oxidation has been investigated [88].

Rosemary and green tea extracts have been shown to have antioxidant activity in lipids and lipid containing foods [89]. The antioxidant activities of rosemary extracts could be due to phenolic compounds such as carnosol, carnosic acid, rosmarinic acid, rosmanol, and rosemaridiphenol. Tocols are effective lipidic antioxidants and are commonly used as replacers of synthetic antioxidants in fats and oils [90].

Antioxidants can be categorized as either chain breaking antioxidants (primary antioxidants) which interfere with the propagation steps of lipid oxidation, or preventative antioxidants (secondary antioxidants or synergists) which reduce the rate of initiation of lipid oxidation. Chain breaking antioxidants retard lipid oxidation by interfering with chain propagation or initiation by readily donating hydrogen atoms to peroxyl radicals. Preventive antioxidants enhance the activity of chain breaking antioxidants through such activities as binding of prooxidant metals, deactivation of singlet oxygen, absorption of ultraviolet radiation and decomposition of hydroperoxides to non-radical products.

Phenolic compounds such as BHA, BHT, TBHQ and tocols are effective chain breaking antioxidants because they produce stable and relatively unreactive antioxidant radicals that do not propagate lipid oxidation reactions [91]. Preventive antioxidants can inhibit or delay lipid oxidation by decreasing ROS levels in the lipid medium. Chelating compounds are an important type of preventive antioxidant; these compounds deactivate metal ions which promote initiation and breakdown of hydroperoxides, and thus reduce the formation of secondary products of lipid oxidation. Chelating compounds include ethylenediaminetetraacetic acid (EDTA), citric acid and phosphoric acid [92]. Phenolic compounds are the most widely occurring natural chain breaking antioxidants. Tocols are important members of this group and serve as both free radical scavengers and singlet oxygen quenchers [93]. Carotenoids are the most common natural singlet oxygen quenchers [94].

2.2.3.3. Packaging

Photooxidation occurs in the presence of light and can be prevented if the fat or oil is stored in the proper packaging material. Containers that are opaque to light and impermeable to air and moisture are ideal for long term storage of fats and oils under ambient conditions. Polyvinyl chloride is a preferred packaging material because of its high impermeability to oxygen. Coloured or opaque containers are also preferred because they retard photooxidation [95].

2.3. Antioxidants and Antioxidant Activity Assays

Antioxidants are defined as substances that when present at low concentrations compared to that of an oxidizable substrate, significantly slow down or delay the oxidation of that substrate [96]. They perform this function through a number of mechanisms, which include decreasing oxygen concentration present in foods, deactivating prooxidant metals and by neutralizing free radicals and ROS. Antioxidants present in foods are known to improve their shelf life by delaying lipid oxidation and development of rancidity. When ingested, antioxidants scavenge free radicals and may effect in the prevention of cancer, atherosclerosis and other chronic diseases [97]. Antioxidative compounds derived from foods are sometimes ingested in capsule form to fight the harmful effects of free radicals in foods and are referred to as nutraceuticals.

Both natural and synthetic antioxidants are commonly added to foods to control lipid oxidation. Synthetic antioxidants approved for food use include phenolic compounds such as BHA, BHT and TBHQ and non-phenolics such as ascorbic acid, ascorbyl palmitate and erythorbic acid [98]. Natural antioxidants include carotenoids, ascorbic acid, amino acids and dipeptides, protein

hydrolysates, phospholipids, tocols and other naturally occurring phenolic compounds [99]. Antioxidants used in foods must be effective at low concentrations, stable through various processing conditions and impart minimal effects on colour, odour and flavour of foods [87].

2.3.1. Testing of Antioxidants and Antioxidant Activity

Foods contain a complex mixture of antioxidants that can be extracted and tested in a variety of ways. Separation and testing of individual antioxidant compounds is impractical, time consuming and would not allow researchers to assess potential synergistic effects of food antioxidants. For this reason, several tests and antioxidant activity assays have been developed to assess the activity of natural mixtures of food antioxidants. The antioxidant activity measured by an individual assay reflects only the chemical reactivity under the specific conditions employed in that assay; for this reason it is appropriate to assess the antioxidant activity of complex antioxidant mixtures using a number of methods.

2.3.1.1. Total Phenolics Content

The total phenolics content (TPC) assay is a colorimetric test for phenolic compounds. This assay is based on the oxidation of phenolic groups with phosphomolybdic and phosphotungstic acids. The main reagent for this assay is the Folin-Ciocalteu reagent. The TPC assay was initially used to determine the phenolics content of beverages such as wine [100], but has also been used for other food types [101]. The exact chemical nature of steps involved in the total phenolics determination assay is unknown but it is believed to be based on one- or two-electron oxidation-reduction reactions between heteropolyphosphotungstates-molybdates and phenolic compounds.

Phenolic compounds react with the Folin-Ciocalteu reagent under basic conditions only. This reaction leads to the formation of a blue-green pigment that has an absorbance maximum at 750 nm. Many nonphenolic compounds such as vitamin C and Cu^{+} react with the Folin-Ciocalteu reagent, making the TPC assay less useful for quantitative determination of phenolic compounds in antioxidant mixtures. However, good correlations have been observed between the TPC assay and antioxidant activity assays making the TPC assay a good measure of antioxidative strength [102]. Despite its non-specific nature, the TPC assay has become a routine test in the study of phenolic antioxidants.

2.3.1.2. Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacity (TEAC) assay was first reported by Miller et al. in 1993 [103] and later revised in 1999 [104]. This assay is used to measure the free radical scavenging activity of antioxidants compared to that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble vitamin E analogue. In the currently accepted method, the free radical ($ABTS^{\bullet-}$) is generated by persulphate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{2-}$) (Figure 2.5). The $ABTS^{\bullet-}$, diluted in ethanol or phosphate buffered saline, is combined with antioxidants and the decrease in absorbance over a 6 min period is followed spectrophotometrically at 734 nm, which corresponds to the absorbance maximum of the $ABTS^{\bullet-}$ radical. A standard curve using Trolox is prepared and used to calculate the TEAC values of antioxidant samples. In a recent modification of the TEAC assay, electrolysis of an $ABTS^{2-}$ solution is done to form the ABTS cation radical ($ABTS^{+\bullet}$), which like $ABTS^{\bullet-}$, takes part in decolourization reactions with antioxidants [105].

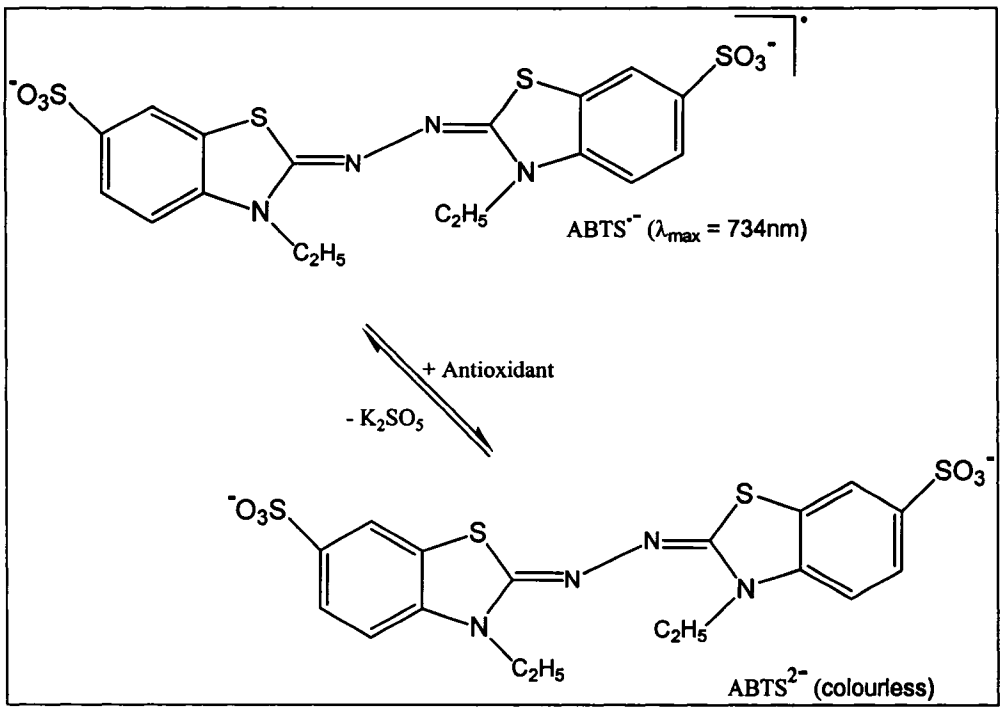


Figure 2.5. Scavenging of $ABTS^{\bullet-}$ by Antioxidants

Due to its simplicity, the TEAC assay is widely used to study the antioxidant capacity of foods, antioxidant extracts and pure antioxidant compounds. The TEAC values of ascorbic acid (1.05), α-tocopherol (0.97), glutathione (1.28), and uric acid (1.01) are almost the same. Ferulic acid (1.90) and *p*-coumaric acid (2.00) have comparable TEAC values. However, caffeic acid has a TEAC value of 1.00 even though its structure is similar to that of ferulic acid. The TEAC value difference between quercetin (3.00) and kaempferol (1.00) is also unexpected as both compounds have similar chemical structures [106].

2.3.1.3. 1,1 Diphenyl-2-picrylhydrazyl Radical Scavenging Capacity Assay

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH, Figure 2.6) is a stable and commercially available organic nitrogen radical and has an absorption maximum at 515 nm. Upon reduction, the DPPH radical mixture fades and its absorbance at 515

nm is lost, making its progress easily monitored by a spectrophotometer. The DPPH radical is diluted in methanol or ethanol, and combined with antioxidants diluted in the same solvent. The absorbance of the mixture is monitored at 515 nm for 30 min or until the absorbance is stable. The percentage of the DPPH remaining at the end-point of the assay is calculated as being proportional to the antioxidant concentration or activity. Another way to describe DPPH radical scavenging capacity is to calculate the concentration of an antioxidant that causes a decrease in the initial DPPH concentration by 50%, defined as the IC_{50} [107].

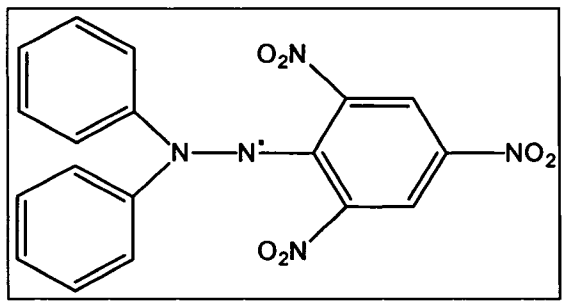


Figure 2.6. Chemical Structure of the DPPH Radical

The DPPH assay is technically simple, but some disadvantages limit its application. The DPPH radical is a relatively long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. Consequently, the antioxidant capacity may not be properly rated [108].

2.3.1.4. Hydroxyl Radical Scavenging Assay

In this assay, hydroxyl radicals ($HO^{\cdot}$) are generated by the Fenton reaction [109], in which Fe^{2+} donates an electron to hydrogen peroxide (H_2O_2) resulting in the formation of Fe^{3+}, hydroxide ions (HO^{-}) and hydroxyl radicals.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^\cdot$$

The production and concentration of hydroxyl radicals can be monitored using electron spin resonance spectrometry with 5,5-dimethyl *N*-oxide pyrroline as the spin trap [110]. However, the Fe^{2+}/H_2O_2 radical generation system has disadvantages when applied to a scavenging assay since many antioxidants are also metal chelators. When the sample is mixed with the radical preparation, it may alter the activity of Fe^{2+} by chelating it, and as a result, it is impossible to distinguish whether an antioxidant is acting as a metal chelator or hydroxyl radical scavenger. Antioxidants in food (such as vitamin C) may act as pro-oxidants by reducing Fe^{3+} to Fe^{2+}, making the generation of hydroxyl radicals catalytic. Ascorbic acid was later used in the Fenton reaction to create a constant flux of hydroxyl radicals [111].

Recently, a fluorometric assay has been developed for screening the metal ion chelating capacity of dietary antioxidants [112] and is named the hydroxyl radical averting capacity (HORAC). The method employs a Co^{2+} complex mediated Fenton-like reaction. Fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1, 9′-xanthen]-3-one; FL) is used as the oxidant probe, and the fluorescence decay curve of FL is monitored in the absence or presence of antioxidants. The area under the fluorescence decay curve (AUC) is then integrated, and the net AUC is calculated by subtracting the AUC of the blank from that of the sample antioxidant. A wide range of phenolic antioxidants have been analyzed using the HORAC assay [112].

2.3.1.5. Oxygen Radical Absorbance Capacity

Originally developed by Cutler and Cao [113], the first version of the ORAC assay employed β-phycoerythrin (β-PE, a fluorescent protein isolated from *Porphyridium cruentum*) as the probe. The fluorescence decay of β-PE is an indication of damage from its reaction with the peroxyl radical. However, later studies found that β-PE suffered from several disadvantages; these include, β-PE, a protein product, has a large lot-to-lot variability, β-PE is extremely sensitive to photobleaching and β-PE interacts with polyphenols due to the nonspecific protein binding and loses fluorescence even without added radical generator.

To solve the problems associated with β-PE, the fluorescent probe was replaced with fluorescein (FL) [114]. Fluorescein (Figure 2.7) is a synthetic non-protein probe that overcomes the limitations of β-PE. The ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals [115]. In general, samples, controls, and standards are mixed with FL solution and incubated at 37 °C before radical solution (2,2′-azobis(2-amidinopropane) dihydrochloride; AAPH) is added to initiate the reaction. The fluorescence intensity (485 nm excitiation/525 nm emission] is measured every min for a predetermined time (usually 30-35 minutes). As the reaction progresses, FL is consumed and its fluorescence intensity decreases. In the presence of antioxidant, the decay of FL fluorescence is retarded.

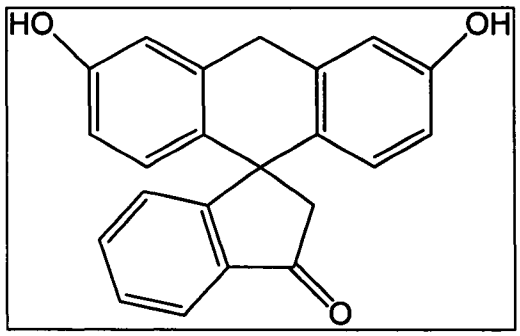


Figure 2.7. Chemical Structure of Fluorescein

The ORAC values of samples are obtained by calculating of the area under the time resolved fluorescence curve (AUC) and net AUC (AUC_{sample} - AUC_{blank}) for each sample, and using a standard curve constructed with a reference antioxidant to relate the net AUC to antioxidant activity. The advantage of the AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phase and those samples that have no lag phase. This approach is particularly useful for food samples and extracts, which are often mixtures of multiple antioxidants and have complex reaction kinetics.

The ORAC assay has been broadly applied in the food and supplement industry as the method of choice to quantify antioxidant activity. When testing lipid soluble antioxidants, compounds that enhance their solubility such as methylated cyclodextrins are added to the assay media; these non-antioxidant compounds are necessary in order to properly assay the ORAC of lipidic antioxidants [115]. Recently, an antioxidant database has been generated archiving ORAC assay and total phenolics content assay results for common foods and supplements [116].

2.3.1.6. Photochemiluminescence Inhibition Assay

In the photochemiluminescence (PCL) inhibition assay, the generation of free radicals is achieved by photochemical excitation of a photosensitizer, which is accompanied by chemiluminescence of a chemiluminescent detection reagent. The chemiluminescence reaction can be accurately measured with fiberoptic based photosensors. The PCL inhibition assay is induced by optical excitation of a photosensitizer (S) causing the generation of the superoxide radical ($O_2^{\cdot-}$).

$$S + O_2 + h\nu \rightarrow S^*O_2 \rightarrow S^* + O_2^{\cdot-}$$

The superoxide radicals generated are visualized through their reaction with chemiluminescent detection reagent, resulting in the emission of light. In the PCL inhibition assay, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is used as both the photosensitizer and the chemiluminescence detection reagent. In the presence of antioxidants that scavenge superoxide, the extent of the chemiluminescent reaction is reduced. The extent of PCL inhibition (in minutes or AUC) is used to calculate antioxidant activity of a sample compared to that of a standard antioxidant. The steps of the reaction as they take place in the Analytik Jena Photochem® (Delaware, OH) are depicted in Figure 2.8. The PCL assay can be performed in either the ACW mode for hydrophilic antioxidants or in the ACL mode for hydrophobic antioxidants. The main difference in the two modes is that the assay mixture in the ACW mode is comprised mainly of carbonate buffer whereas the ACL mode assay mixture contains mostly methanol with small amounts of carbonate buffer. The presence of methanol in the ACL assay mixture allows for complete solubilization of hydrophobic antioxidants when present at low concentrations [117].

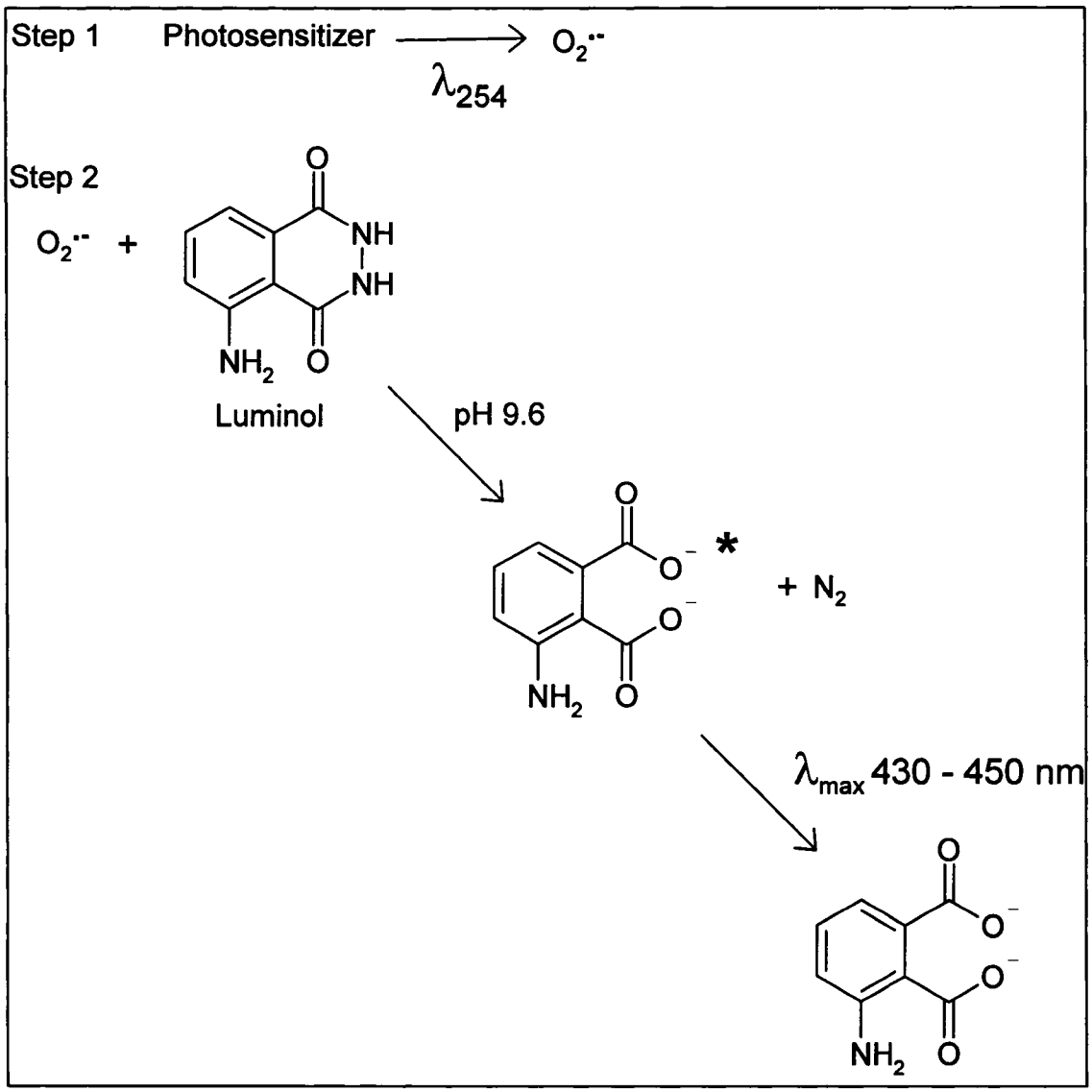


Figure 2.8. Photochemiluminescence of Luminol and Superoxide

2.3.1.7. Low Density Lipoprotein Oxidation Assay

Oxidation of LDL is a known risk factor leading to atherosclerosis, and much interest in the ability of antioxidants to protect LDL from oxidation exists since this may reduce the incidence of cardiovascular disease [118]. Many methods exist that examine the ability of antioxidants and food components to inhibit LDL oxidation. Copper-catalysed and AAPH-mediated oxidation have been widely utilised and the inhibitory activities of antioxidants have been assessed by measuring the production

of hexanal, conjugated dienes and cholesterol ester hydroperoxides [119]. In the copper-catalysed system, antioxidant efficacy can be attributed to both free radical scavenging and metal ion chelation activities of antioxidants. The ability of phenolic compounds to form complexes with proteins may provide an alternate mechanism for their antioxidant activity in the copper-catalysed LDL oxidation assay by blocking copper catalysts from binding to the LDL molecule and thereby protecting LDL lipids from oxidation [120].

2.3.1.8. DNA Scission Assay

During the early twentieth century, radiation biologists discovered that radiolysis of water generates oxygen free radicals (most notably the hydroxyl radical), which are responsible for many of the consequences of irradiation in living organisms. The characterization of radiation-induced oxidative DNA scission, and the connection between radiation and cancer, has led to interest in DNA oxidation. DNA scission is referred to single stranded lesions, or nicks, in the sugar phosphate backbone of DNA and is caused by oxidation of the DNA molecule by free radicals. Living cells do possess DNA repair enzymes, but these systems do not function well during oxidative stress when the concentration of oxidants exceeds the cells ability to quench these reactive compounds and repair the DNA damage they cause [121]. Concerns about artifactual oxidation, combined with the different values that have been generated by alternative methods, have promoted ongoing debate over the most appropriate techniques for studying DNA oxidation. Current methods include GC-MS analysis of oxidatively modified DNA bases, immuno-histochemistry with antibodies reactive towards altered DNA and gel electrophoresis [121]. Each method has advantages and drawbacks. Since DNA

strand scission causes significant changes to the three-dimensional structure of DNA macromolecules, gel electrophoresis followed by treatment with intercalating dyes such as ethidium bromide is the most convenient method for quantitation of DNA scission.

Several model systems have been developed to study the protective effects of antioxidants against free radical induced DNA damage, and one popular system uses a Fe^{2+}-EDTA chelate incubated with DNA and hydrogen peroxide to induce DNA damage by hydroxyl radicals [122].

2.4. Tree Nuts

Tree nuts are widely consumed as snack foods. They are rich sources of protein (up to 30%, w/w) and lipids (up to 75%, w/w). They contain several health promoting components including monounsaturated fatty acids, dietary fiber, phenolics and potentially other healthy components [1, 123]. Currently, there is much interest in the health effects of regular nut consumption, particularly in the long-term prevention of cardiovascular disease [123]

2.4.1. Almond

The almond tree (*Prunus delcis* and *Prunus amara*) and its fruit (containing the almond kernel or 'almond') have long been recognised as being commercially valuable and nutritionally important. California and Italy are the major almond producing regions of the world, however, other parts of Europe, Asia and Australia also contribute to a lower level of production [124]. The only other economically important product of almond trees is the almond hull, which is traditionally used in animal feed preparations. Several studies have reported that almond consumption may improve blood lipid profiles by lowering low density

lipoprotein (LDL) cholesterol and raising plasma high density lipoprotein (HDL) cholesterol levels. Thus, there is much current interest in almond oil as a health promoting edible oil [125]. The proximate composition of almond comprises 50.6% lipid, 21.3% protein, 19.7% carbohydrate, 3.1% ash and 5.3% moisture(w/w) [1].

The defatted meals and hulls of almonds contain several antioxidative compounds as well as other health promoting substances. Senter et al. [126] performed a comparative analysis of phenolic acids in selected tree nut meals including pine nut, almond, hazelnut, chestnut and walnut, among others. The results of this study showed that gallic acid was the predominant phenolic compound in all tree nut meals except pine nut (caffeic acid), almond and hazelnut (protocatechuic acid). Other phenolic compounds identified included *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, vanillic, syringic and ferulic acids [126]. The antiradical activity of ethanolic extracts of almond and almond by-products including brown skins and hulls have been reported [127]. The Trolox equivalent antioxidant activity of brown skins and hulls were 13 and 10 times greater than that of the whole almond extracts [127]. At a concentration of 200 ppm, ethanolic extracts of almond skins and hulls had strong scavenging activities against superoxide radical (95 and 99%, respectively), hydrogen peroxide (91%), hydroxyl radical (100 and 56%, respectively) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (100%) [127]. Sang et al. [128] isolated nine phenolic compounds from almond skins and assessed the DPPH scavenging activity of each compound; catechin and protocatechuic acid had the greatest antioxidant activity, followed by 3′-O-methylquercetin 3-O-β-D-galactopyranoside, 3′-O-methylquercetin 3-O-β-D-glucopyranoside and 3′-O-methylquercetin 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-

glucopyranoside and vanillic and *p*-hydroxybenzoic acid, naringenin 7-O-β-D-glucopyranoside, and finally kaempferol 3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside [128]. Frison-Norrie and Sporns [129] quantitatively determined the flavonol glycoside composition of blanched almond skins using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry, showing the presence of isorhamnetin rutinoside (51μg/g), isorhamnetin glucoside (18μg/g), kaempferol rutinoside (18μg/g) and kaempferol glucoside (6μg/g). More recently, Pinelo et al. [130] reported the total phenolics content and DPPH scavenging activity of almond hull ethanolic extracts at 3.74mg/g and 58%, respectively. Sang et al. [131] also isolated potentially health promoting sterols, nucleotides and one sphingolipid, 1-O-β-D-glucopyranosyl-(2S,3R,4E,8Z)-2-[(2R)-2-hydroxyhexadecanoylamino]-4,8-octadecadiene-1,3-diol, from defatted almond meals. In light of data showing that tree nuts, tree nut oils and tree nut by-products contain heath promoting phytochemicals, Davis and Iwashi [132] examined the effects of dietary consumption of whole almonds, almond oil and almond meal on aberrant crypt foci development in a rat model of colon carcinogenesis. This landmark study showed that both almond oil and almond meal reduced aberrant crypt foci development, but whole almonds showed a significantly stronger anticancer effect in this model, implying a synergistic anticancer activity between the lipidic and non-lipidic constituents of almonds [132].

Shi et al. [133] assessed the fatty acid composition of almond oil; oleic acid was the major fatty acid present (68%), followed by linoleic acid (25%), palmitic acid (4.7%) and small amounts (<2.3%) of palmitoleic, stearic and arachidic acids. Almond oil is also a rich source of α-tocopherol (around 390

mg/kg) and contains smaller amounts of γ-tocopherol (12.5 mg/kg) as well as phylloquinone (70 μg/kg) [1, 134]. Almond oil contains 2.2 to 2.6 g/kg phytosterols, mainly β-sitosterol, with trace amounts of stigmasterol and campesterol [1, 134]. The compositional characteristics of almond oil show that it is rich in several health promoting nutrients. Many of these may be responsible for the observed beneficial effects of dietary almond consumption in cardiovascular diseases [135] and in weight management [136], however, few investigations have explored this topic. Hyson et al. [137] conducted a dietary intervention study to determine whether the consumption of whole almonds or almond oil for 6-weeks would result in similar or different effects on plasma lipids and *ex-vivo* LDL oxidation. Both groups consumed diets with identical almond oil and total fat levels. This study showed that both whole almond and almond oil consumption caused similar reductions in plasma cholesterol and LDL (4 and 6%, respectively) as well as a 14% decrease in fasting plasma triacylglycerols. These findings indicate that the lipid component of almond is responsible for its cardioprotective effects and warrants further investigation [137].

Several lines of evidence suggest that regular consumption of whole almonds as part of a healthy diet can help improve several parameters related to cardiovascular health which include lowering of LDL cholesterol and total plasma lipids [138]. Sabaté et al. [139] compared the effects of two amounts of almond intake with those of a National Cholesterol Education Program (NCEP) Step I diet on serum lipids, lipoproteins, apolipoproteins, and glucose in healthy and mildly hypercholesterolemic adults. The NCEP Step I diet is known to reduce LDL cholesterol by 3–10%. The experimental diets included a Step I diet, a low-almond

diet, and a high-almond diet, in which almonds contributed 0, 10 and 20% of total energy, respectively [139]. Inverse relationships were observed between the percentage of energy in the diet from almonds and the subject's total cholesterol, LDL-cholesterol, and apolipoprotein B concentrations and the ratios of LDL to HDL cholesterol and apolipoprotein B to apolipoprotein A. Compared with the Step I diet, the high-almond diet significantly ($p<0.01$) reduced total cholesterol by 0.24 mmol/L or 4.4%, LDL cholesterol by 0.26 mmol/L or 7.0%, and apolipoprotein B by 6.6 mg/dL or 6.6%, and increased HDL cholesterol by 0.02 mmol/L or 1.7% and decreased the ratio of LDL to HDL cholesterol by 8.8%. Results of this study showed that incorporation of 68 g of almonds (20% of energy) into a 2000 kCal Step I diet markedly improved the serum lipid profile of healthy and mildly hypercholesterolemic adults [139]. Similar findings have been reported by other researchers using roasted almonds [140], and animal model studies have also reported on the cardioprotective effects of almond consumption [141].

2.4.2 Brazil Nut

Brazil nuts (*Bertholletia excelsa*) are widely consumed but are produced mainly in South America, with total world production estimated at about 20,000 metric tonnes. Bolivia, Brazil and Peru are the main Brazil nut producing nations [142]. Brazil nuts are traded mainly in the form of kernels (i.e. shelled) and are used in confectionery, bakery and health foods. Brazil nuts contain 66-69% lipid, 14.3% protein, 12.2% carbohydrate, 3.5% ash and 3.5% moisture (w/w) [1, 143]. Brazil nut oil is used in areas of its production as cooking oil and is being promoted on the export market [142]. Since the export value of shelled Brazil nuts is so high, usually only defective Brazil nuts (cracked and partially oxidized) are extracted for their

oils that can result in oils with acid values and peroxide values as high as 5.9 mg KOH/g oil and 7.6 meq O_2/kg oil, respectively [144]. The fatty acid composition of Brazil nut oil includes 29-48% oleic acid, 30-61% linoleic acid, 14-15% palmitic acid, 6-8% stearic acid and 0.5% myristic acid [143, 145].

2.4.3. Cashew Nut

The cashew (*Anacardium occidentale* L.) is an evergreen species native to tropical America and contains 47% oil (w/w) [1, 143, 146]. Other components of cashew nuts include carbohydrate (27.1%), protein (18.2%), ash (2.5%) and moisture (5.2%). The predominant fatty acid in cashew nut oil is oleic acid (57.3-65.1%), followed by linoleic (15.6-18.6%) and palmitic (9.0-14.2%) acids [146]. Cashew nut oil contains 1.4% unsaponifiable matter (w/w), of which 76.2 to 82.7% is β-sitosterol. Other sterols present in cashew nut oil include Δ^5-avenasterol, campesterol, fucosterol, cholesterol and stigmasterol [146]. Cashew nut oil contains 45.3 to 83.5 mg/100g γ-tocopherol, 2.8 to 8.2 mg/100g α-tocopherol and 2.0 to 5.9 mg/100g δ-tocopherol [146]. The testa of cashew nut serves as a good source of tannins [147], catechin and epicatechin, as well as polymeric proanthocyanidins, leucocyanidins and leucopelargonidins [148].

2.4.4. Hazelnut

Hazelnuts or filberts (Corylus *sp.*) are a rich source of energy with a 61 to 63% lipid content (w/w) [1, 149]. Other components of hazelnuts are protein (13.0%), carbohydrate (15.3%), ash (3.6%) and moisture (5.4%) [1]. Turkey is the world's largest producer of hazelnuts, accounting for approximately 75% of total hazelnut production, followed by Italy which accounts for 10% of total global production. In the US, the state of Oregon is the largest producer and in Canada,

southwestern British Columbia produces a small amount of hazelnuts; North America contributes less than 5% to the total world hazelnut production which is about 850,000 metric tonnes (unshelled basis) [150].

Few researchers have investigated the potential of hazelnuts as a source of natural antioxidants. Yurttas et al. [151] assessed the phenolic composition of methanolic extracts of defatted hazelnuts (hazelnut meal), showing that gallic acid, *p*-hydroxybenzoic acid, caffeic acid, epicatechin, sinapic acid, and quercetin as being the predominant phenolic acids. The composition of phenolic acid constituents in hazelnut meal has also been assessed by Senter et al. [126] who showed that protocatechuic acid was the main phenolic present in hazelnut meal (0.36 μg/g); trace amounts (<0.1 μg/g) of *p*-hydroxybenzoic, vanillic, gallic and caffeic acids were also present. Moure et al. [152] examined the antioxidant activity of ethanolic extracts of hazelnut hulls, showing DPPH bleaching activities ranging from 86.2 to 94.4%. Similar values were reported by Krings and Berger [153] using ethanolic extracts of both roasted and unroasted hazelnut meals. The extracts of roasted and unroasted hazelnut meals exhibited comparable antioxidant activities in both the DPPH bleaching assay and stripped corn oil model system [153]. Wu et al. [116] recently examined the antioxidant capacities of both lipophilic and hydrophilic extracts of hazelnuts using the ORAC assay with fluorescein as the fluorescent probe. Grated hazelnuts were packed into extraction cells with sand and extracted with two solvent systems using an accelerated solvent extractor (Dionex ASE 200). During the first treatment, lipophilic extracts were obtained with hexane/dichloromethane (1:1, v/v), followed by a second treatment with acetone/water/acetic acid (70:29.5:0.5, v/v/v) to obtain the hydrophilic extracts.

Results of this study [116] showed that the lipophilic hazelnut extracts had ORAC values of 3.7 μmol Trolox equivalents/g hazelnut, whereas the hydrophilic extracts had ORAC values of 92.8 μmol Trolox equivalents/g hazelnut.

The fatty acid composition of hazelnut oil is as follows: 78-83% oleic acid, 9-10% linoleic acid, 4-5% palmitic acid and 2-3% stearic acid as well as other minor fatty acids [1]. Parcerisa et al. [154] examined the lipid class composition of hazelnut oil, showing that triacylglycerols constituted 98.4% of total lipids, glycolipids comprised 1.4% of total lipids, while trace amounts (<0.2%) of phosphatidylcholine and phosphatidylinositol were also present. Hazelnut oil contains 1.2 to 2.2 g/kg of phytosterols primarily in the form of β-sitosterol and is a very good source of α-tocopherol (382 to 472 mg/kg) [1, 134]. The main odorant in hazelnut oil responsible for its characteristic flavour is 5-methyl- (E)-2-hepten-4-one or filbertone, which can produce intense hazelnut oil-like aroma at the very low odour threshold value of 5 ng/kg oil [155]. The oil from unroasted hazelnuts typically contains about 6 μg filbertone/ kg oil whereas the oil from roasted hazelnuts contains over 315 μg filbertone/ kg oil [155].

Several reports have shown that hazelnut is a health promoting food and a contributing factor for the beneficial health effects of the Mediterranean style diet [156], however, few studies have investigated the health effects of hazelnut oil. Balkan et al. [157] examined the effects of hazelnut oil administration on plasma peroxide levels, plasma lipid profiles, plasma LDL and VLDL levels, and atherosclerotic plaque development in male New Zealand white rabbits. In this study, animals were divided into four groups receiving normal diets (control), diets rich in cholesterol (0.5% w/w), diets rich in cholesterol (0.5% w/w) with hazelnut

oil supplementation (5% w/w) or a diet with hazelnut oil supplementation (5% w/w) without cholesterol for 14-weeks. The results showed that hazelnut oil supplementation in low cholesterol diets reduced plasma cholesterol and apoB-100 containing lipoprotein levels by an insignificant level ($p > 0.05$). No differences were observed in the high cholesterol diet group supplemented with hazelnut oil which implies that hazelnut oil may be an effective health promoting agent in diets with normal lipid intake, but can not reverse the effects of high cholesterol intake [157].

2.4.5. Macadamia Nut

Macadamia trees (*Macadamia sp.*) were originally cultivated in Australia, but the United States (Hawaii) is currently the world's largest producer of macadamia nuts. Edible macadamia nuts are from two species; *Macadamia integrifolia* (smooth-shell type) and *Macadamia tetraphylla* (rough-shell type). The macadamia nut industry in Hawaii, Australia, and many other producing areas, is based primarily on the smooth-shell type [158]. Oil yields from macadamia nuts range from 59 to 78% (w/w) [1, 159]. Macadamia nuts also contain 7.9% protein, 13.8% carbohydrate, 1.1% ash and 1.4% moisture (w/w) [1]. Compositional studies of macadamia nut oil shows that it is rich in oleic and palmitoleic acids, has 18-54 mg/kg tocol isomers (predominantly α-tocotrienol) and up to 1.5g/kg phytosterols (predominantly campesterol) [160]. Macadamia nut oil has been shown to have a relatively high smoke point of 198°C. The Rancimat method has been used to assess the oxidative stability of several varieties of macadamia nut oil, resulting in induction periods of between 3.6 and 19.8h [160]. Macadamia nut has been shown to contain 2,6-dihydroxybenzoic acid, 2′-hydroxy-4′-methoxyacetophenone, 3′,5′-

dimethoxy-4′-hydroxyacetophenone and 3,5-dimethoxy-4-hydroxycinnamic acid [161].

2.4.6. Pecan

Pecan tree (*Carya illinoinensis*) is native to the United States but has also been naturalized for commercial pecan production throughout the world, including Australia, South Africa, some middle eastern countries and several countries of South America [162]. Fat is the predominant constituent in all pecan varieties ranging between 65 and 75% (w/w) [1, 163]. Other constituents include 9.1% protein, 13.9% carbohydrate, 1.5% ash and 3.5% moisture (w/w) [1]. The predominant fatty acids present in pecan oil are oleic (55%), linoleic (33%), linolenic (2%), palmitic (7%) and stearic (2%) acids [163]. The most predominant tocopherol in pecan oil is γ-tocopherol (176 mg/kg), followed by α-tocopherol (10 mg/kg), and then δ- and β-tocopherols (6.2 mg/kg) [1]. Pecan oil is also estimated to contain 0.73 g/kg phytosterols that exist primarily in the form of β-sitosterol (around 90%) [1].

Early studies have shown that pecan oil is a very stable food oil despite its high content of unsaturated fatty acids, thus making it an excellent dietary oil [164]. Demir and Cetin [165] examined the total yields, compositions and oxidative stability of expeller pressed and hexane extracted pecan oils. Total yields were higher for solvent extracted batches (67-79%, w/w) than pressed batches [165]. The expeller pressed pecan oil had a significantly higher total tocopherol content when compared with hexane extracted oil (260 and 23 mg/kg, respectively). However, the solvent extracted oil exhibited a greater oxidative stability with an induction period of 6h at 100°C, as compared to 5.5h for pressed oil. These findings may imply that

antioxidative constituents besides tocopherols may be contributing to the enhanced oxidative stability of the hexane-extracted oils, however, previous publications [162, 163] using similar solvent extraction methods have shown much higher concentrations of tocopherols in pecan oils and thus contradict the findings of Demir and Cetin [165]. Toro-Vasquez and Perez-Briceno [166] studied the oxidative stabilities of solvent extracted pecan oils from 22 Mexican pecan varieties; all varieties tested had high oxidative stability values (8.5-10.8h at 110 °C).

Epidemiological findings have shown that pecan enriched diets can favourably alter serum lipid profiles in humans and thus reduce cardiovascular disease risk [167]. However, the effects of pecan oil intake on human blood lipid profiles have not been reported.

2.4.7. Pine Nut

Pine nuts (pinon or pignolia) are the edible seeds within the pine cone of several varieties of pine trees (*Pinus sp.*) but most commonly *Pinus pinea* or 'stone pine'. Pine nuts are harvested all over the world, most notably Russia, China, North Korea, Spain, Italy and Turkey, among others. Pine nuts have been shown to yield 48 to 61% lipids by weight [1, 143]. Other constituents of pine nut include protein (11.6%), carbohydrate (19.3%), ash (2.2%) and moisture (5.9%) [1]. Pine nut oil contains predominantly linoleic acid (46.4%) and oleic acid (38.1%). Maritime pine nut (*Pinus pinaster*) oil also contains two fatty acids that are unique among tree nut oils; pinoleic acid and sciadonic acid, both of which exist at 7% each in pine nut oil and may have antiatherogenic effects [168]. Caffeic acid is the predominant phenolic compound in defatted pine nut meal [126].

2.4.8. Pistachio

The pistachio tree (*Pistacia vera*) is native to the Middle Eastern region and has been naturalized in many parts of the world. The world's largest producer of pistachio nuts is Iran (Kerman province) with an annual output of 300,000 tonnes. Other major producers are Turkey, China the United States (California), India, France, Italy, Chile and Syria [168-170]. The proximate composition of pistachio includes 44% lipid, 21% protein, 28% carbohydrate, 3% ash and 4% moisture (w/w) [1], however, some reports have shown that pistachio nuts contain between 45 and 72% oil, depending on the variety and stage of harvest [171]. The main use of pistachio oil is in the cosmetics and condiment industries. The predominant fatty acid of pistachio oil is oleic acid (56-64%), followed by linoleic acid (23-31%), palmitic acid (9-13%) and small amounts of other fatty acids [171]. Pistachio oil contains large amounts of phytosterols (5 g/kg, 85% β-sitosterol) [172], 270 mg/kg of tocopherols [1] and has an acid value higher (2.32 mg KOH/g oil) than other tree nut oils [172]. Evidence from several epidemiologic studies suggests that pistachio consumption can reverse several adverse blood lipid parameters such as hypercholesterolemia [173], however, investigations on the health effects of pistachio oil consumption are not readily available or have not been conducted.

2.4.9. Walnut

The walnut (*nux* juglandes) is harvested from walnut tree (*Juglans regia*) and is the most popular nut ingredient in North American cooking. Over 30 varieties of walnut trees have been developed for various characteristics including pest tolerance, early/late harvest and shell thickness and these are currently harvested.

Walnuts contain about 65% lipids, however, considerable differences exist among varieties with a range of 52-70% lipid (w/w) [1, 174]. Walnuts also contain 15.8% protein, 13.7% carbohydrate, 1.8% ash and 4.1% moisture (w/w) [1]. The defatted meals of walnuts serve as a good source of natural antioxidants, containing predominantly caffeic, vanillic and *p*-hydroxybenzoic acids [126]. Wu et al. [116] showed that lipophilic walnut extracts had ORAC values of 4.8 μmol Trolox equivalents/g walnut and hydrophilic walnut extracts had ORAC values of 130.6 μmol Trolox equivalents/g walnut. Gunduc and El [175] have assessed the total phenolics contents of ethanolic extracts of several Turkish foods including walnuts using the Folin-Ciocalteu colorimetric method, reporting a total phenolics content of 7.1 mg/g (as gallic acid equivalents) for whole walnuts. This group [175] have also compared the ability of food extracts for inhibiting the *in-vitro* oxidation of LDL, showing that both walnut and red wine extracts inhibited LDL oxidation to the greatest degree among food samples tested. Fukuda et al. [176] studied the composition and antioxidant activity of butanolic extracts of walnut polyphenols. Using semi-preparative liquid chromatography and one and two dimensional NMR analyses, Fukuda et al. [176] isolated 14 polyphenolic constituents from walnut extracts including three new hydrolyzable tannins, glansrins A, B, and C (ellagitannins with a tergalloyl or related polyphenolic acyl group), along with pendunculagin, tellimagrandin I and II, casuarinin, rugosin C, casuarictin, and ellagic acid. Adenosine and adenine were also identified in the walnut extracts [176]. The 14 walnut polyphenols had superoxide dismutase-like activities and strong DPPH bleaching activities, indicating that ellagitannin polyphenols act as strong antioxidants [176]. Similar findings were reported by Anderson et al. [177]

who studied the composition of methanolic extracts of walnut and their ability to inhibit both azo-mediated and Cu^{2+}-mediated LDL oxidation in a dose-dependant manner. Anderson et al. [177] reported that walnut extracts contained a total phenolics content of 20 mg/g (as gallic acid equivalents); LC-MS analysis confirmed the presence of ellagic acid and other related ellagitannins, but no tocopherols were detected in the walnut extracts. Sze-Tao et al. [178] reported the hydrolyzable tannin content of several walnut batches was 363 to 1095 mg catechin equivalents/ 100 g of walnuts, using two modified vanillin assays. Differences in total hydrolyzable tannin contents of the various walnut samples were attributed mainly to the different processing and storage conditions employed for each walnut batch [178]. Recently, walnut phenolic extracts have been shown to inhibit fibrillar amyloid beta-protein (A) production which may exert beneficial effects in Alzheimer's disease sufferers since fibrillar amyloid beta-protein (A) is the principal component of amyloid plaques commonly seen in Alzheimer's disease [179]. Fukuda et al. [180] have studied the effects of walnut polyphenols on blood lipid profiles and oxidative stress in type II diabetic mice (nine week old C57/BL/KsJ-db/db male mice). In this study [180] seven mice were supplemented orally with purified ethanolic walnut extracts at a daily level of 200 mg/kg body weight for four weeks, while eight mice were used as controls. Results of this study showed that supplementation of walnut polyphenolics significantly reduced serum triacylglycerols and urinary 8-hydroxy-2'-deoxyguanosine (an *in-vivo* marker of oxidative stress) after four weeks. No significant differences were observed in body weight, blood glucose or total serum cholesterol between the experimental and control groups [180].

The fatty acid composition of walnut oil is unique compared to other tree nut oils for two reasons; walnut oil contains predominantly linoleic acid (49-63%) and also has considerable amounts of alpha-linolenic acid (8-15.5%). Other fatty acids present include oleic acid (13.8-26.1%), palmitic acid (6.7-8.7%) and stearic acid (1.4-2.5%) [174]. The tocopherol content of walnut oil varies among different cultivars and extraction procedures and ranges between 268 and 436 mg/kg. The predominant tocol isomer is γ-tocopherol (>90%), followed by α-tocopherol (6%), and then β- and δ-tocopherols [181]. Non-polar lipids have been shown to constitute 96.9% of total lipids in walnut oil, while polar lipids account for 3.1%. The polar lipid fraction consisted of 73.4% sphingolipids (ceramides and galactosylceramides) and 26.6% phospholipids (predominantly phosphatidylethanolamine) [181]. Walnut oil contains approximately 1.8 g/kg phytosterols [1], primarily β-sitosterol (85%), followed by Δ5-avenasterol (7.3%), campesterol (4.6%), and finally cholesterol (1.1%) [181].

Evidence from epidemiological studies, intervention studies and clinical trials show that walnut consumption has favourable effects on serum lipid levels in humans such as lowering LDL, raising HDL and reducing total serum triacylglycerol levels, all of which reduce the likelihood of suffering from a cardiovascular event [182, 183]. Many of the beneficial findings associated with walnut consumption have previously been attributed to the polyunsaturated fatty acid intake and have prompted health researchers to investigate which of these effects, if any, can be attributed to the lipid components of walnuts. Lavedrine et al. [184] conducted a cross-sectional study to assess the association between whole walnut and walnut oil consumption and blood lipid levels. This study included 933

men and women aged 18-65 years living in Dauphine, France (a major walnut producing area). Factors used to assess cardiovascular disease risk included a one-year dietary recall questionnaire and serum levels of HDL, LDL, total cholesterol and levels of the apolipoproteins apoA1 and apoB. Results from this study showed that higher levels of HDL cholesterol and apoA1 were associated with higher amounts of walnut oil and kernel consumption, with a positive trend existing between the various degrees of walnut oil/kernel consumption in this cohort. Other blood lipids did not show any significant association with walnut consumption; the nature of the cohort group made it impossible to separate the effects of whole walnut and walnut oil consumption [184]. More recently, Zibaeenezhad et al. [185] examined the effects of walnut oil consumption on plasma triacylglycerol levels in hyperlipidemic men and women. In this trial, 29 patients were given 3g/day walnut oil (six 500mg capsules per day) for 45 days; 31 patients were given placebo and were used as controls. Supplementation of walnut oil reduced serum levels of LDL, triacylglycerol and total cholesterol while increasing serum HDL levels, however, only the decrease in serum triacylglycerols reached significance ($p \leq 0.05$) [185]. The fatty acid composition of walnut oil has been suggested as being responsible for its cardioprotective feature, but results from studies such as that of Espin et al. [186] show that the antioxidative components of walnut oil have significant antiradical properties that may exert a protective effect against the oxidation of biomacromolecules such as LDL, a known risk factor for atheroma development and thus heart disease.

CHAPTER 3

Materials and Methods

3.1. Materials

Commercially available shelled and unsalted almonds, Brazil nuts, hazelnuts (filberts), pecans, pine nuts, pistachios and walnuts were purchased fresh from the market or acquired from the International Treenut Council (Reus, Spain) or its affiliates. Samples were stored at -20 °C until use. All chemicals were obtained from Sigma-Aldrich Canada (Oakville, ON) or Fisher Scientific (Ottawa, ON), unless otherwise stated. All solvents were of American Chemical Society grade, or better, unless otherwise specified.

3.2. Fat Extraction

3.2.1. Hexane Extraction

Forty grams of each tree nut sample were first ground into a fine powder and combined with 400 mL of hexanes, followed by homogenization at 8000 rpm using a polytron (Polytron model PT 3000, Kinematica, Littau, Switzerland) for 3 minutes. The resulting mixture was filtered through a Whatman #4 filter paper with suction using a Büchner funnel. The residue was re-extracted twice; the filtrates from the three extractions were combined and a portion of the solvent was removed from the extract using a rotary evaporator (Rotavapor model 461, Büchi, Flawil, Switzerland) at 40 °C. The hexane-oil mixture was then passed through a layer of anhydrous sodium sulphate placed over a filter paper in a funnel and the remaining

solvent was removed using a rotary evaporator at 40 °C. The resulting oil was weighed and transferred into 10 mL sample vials, capped with nitrogen and stored at -80 °C until use.

3.2.2. Chloroform/Methanol Extraction

Forty grams of each tree nut sample were first ground into a fine powder and combined with 400 mL of chloroform, followed by homogenization at 8000 rpm using a polytron (Polytron model PT 3000, Kinematica, Littau, Switzerland) for 3 minutes. The resulting mixture was filtered through a Whatman #4 filter paper with suction using Büchner funnel. The spent residue was re-extracted twice with 400 mL of 1:1 (v/v) chloroform/methanol; the filtrates from the three extractions were combined and solvent was removed using a rotary evaporator (Rotavapor model 461, Büchi, Flawil, Switzerland) at 40 °C. The oil was then redissolved in chloroform and then passed through a layer of anhydrous sodium sulphate placed over a filter paper in a funnel. The solvent was then removed using a rotary evaporator at 40 °C. The resulting oil was weighed and transferred to 10 mL sample vials, capped with nitrogen and stored at -80 °C until use.

3.3. Chemical and Instrumental Analysis

3.3.1. Lipid Class Analysis

The lipid class composition of the tree nut oil samples were analysed using an automated thin layer chromatography-flame ionization detector apparatus [150].

(i) Instrumentation. The crude lipids were chromatographed on silica gel-coated Chromarods S III and then analysed using an Iatroscan MK-5 (Iatroscan

Laboratories Inc.,Tokyo, Japan) analyser equipped with a flame ionization detector (FID) connected to a computer loaded with TSCAN software (Scientific Products and Equipment, Concord, ON) for data handling. A hydrogen flow rate of 160 mL/min and an air flow rate of 2000 mL/min were used in operating the FID. The scanning speed of the rods was 30 s/rod.

(ii) Preparation of Chromarods. The Chromarods were soaked in concentrated nitric acid overnight, followed by thorough washing with distilled water and acetone. The Chromarods were then impregnated by dipping in a 3 % (w/v) boric acid solution for 5 min in order to improve separation. Finally, the Chromarods were scanned twice to burn any remaining impurities.

(iii) Standards and Calibration. A stock solution of each of the nonpolar lipids, namely free fatty acid (oleic acid), cholesterol ester, cholesterol, monoacylglycerol (monoolein), diacylglycerol (diolein), and triacylglycerol (triolein), and the polar lipids, namely phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lysophosphatidyl-ethanolamine (LPE), and sphingomyelin (SM), were prepared by dissolving them in hexane. A range of dilutions of the stock solution from 0.1 to 10 mg/mL was prepared for use as working standards. Each standard was developed individually and run on the Iatroscan FID to determine its R_f value. The peak area was then plotted against a series of known sample concentrations to construct a calibration curve.

(*iv*) *Iatroscan TLC-FID Analysis of Lipids.* The nut oils were dissolved in hexane in order to obtain a concentration of 10 mg lipid per millilitre. A 1μL aliquot of the sample was spotted on silica gel-coated Chromarods S III and conditioned in a constant humidity chamber containing a saturated $CaCl_2$ solution for 20 min. The Chromarods were then developed in two solvent systems. First, hexane/diethyl ether/glacial acetic acid (80:19.8:0.2, v/v/v) was used as the solvent system for nonpolar lipids. Following this, the Chromarods were dried at 110 °C for 3 min and scanned completely to reveal nonpolar lipids. For polar lipids, the Chromarods were first developed in the same solvent system as used for nonpolar lipids and then dried at 110 °C for 3 min to remove the solvents. This was scanned partially to a point just beyond the monoacylglycerol peak to burn the nonpolar lipids. These partially scanned Chromarods were developed in a second solvent system consisting of ethyl acetate/propan-2-ol/chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9, v/v/v/v/v) for the separation of polar lipid classes. After development, the Chromarods were dried at 110 °C for 3 min and scanned completely to reveal polar lipids. The identity of each peak was determined by comparison with a chromatogram of standards acquired concurrently with the samples. The percentages of individual lipid classes (by weight) were determined using the calibration curves procured for each authentic standard.

3.3.2. Fatty Acid Analysis

Fatty acid methyl esters (FAMEs) were prepared for each oil sample and were analysed using gas chromatography [150]. FAMEs were prepared by adding 5 mg

of oil to 2 mL of 3 % sulphuric acid in methanol followed by the addition of 0.5 mg of hepatadecatrienoic acid as an internal standard and then heating for 16 h at 60 °C. Butylated hydroxytoluene was added as an antioxidant (0.1 mg). The FAMEs were extracted with optima grade hexane three times and then analyzed using a Hewlett-Packard 5890 Series II (Palo Alto, CA) gas chromatograph equipped with a FID and an autosampler (Hewlett-Packard model 7673). Samples (1μL) were injected into a Supelcowax 10 column (30 m × 0.25 mm i.d., 0.25 μL film thickness; Supelco, Bellefonte, PA) coated with poly(ethylene glycol). The oven temperature was programmed as follows: 180 °C for 2 min, increased to 200 °C at 2 °C/min, held at 200 °C for a further 10 min, and then raised to 215 °C at 2 °C /min and kept there for 10 min. The injector and detector temperatures were 200 and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. Identification of FAMEs was based on retention times and compared with those of standard FAMEs.

To confirm the identity of each FAME peak, samples were also analysed using GC-MS (Finnigan-Mat Magnum, Thermo-Finnigan, Montreal, QC). The column, column conditions and oven conditions employed were identical to those used in the GC-FID analysis and the detector was operated in EI mode (70 eV) with a 1.5 scan per second interval over a 40-650 *m/z* range.

3.3.3. Sterol Analysis

The sterol composition of tree nut oil samples were analyzed qualitatively using GC-MS and quantitatively using GC-FID.

Oil samples (20mg ± 0.1 mg) were transmethylated as described in Section 3.3.2. Sterols were separated from methylated extracts using column chromatography. Glass wool was placed in the tip of a Pasteur pipette so that it just filled the tip, and pipettes were then burned in a muffle furnace (450 °C) for 12 h. Approximately 0.8 g of activated silica gel (150 °C for 1 h) was packed into each pipette. Columns were washed with 2 mL of diethyl ether followed by 4 mL of hexane. Derivative extracts were applied to the top of the washed column, then FAMEs were eluted with 10 mL of 93:7 (v/v) hexane/diethyl ether, and finally sterols and alcohols were eluted using 10 mL of 1:1 (v/v) diethyl ether/hexane [187]. Solvents in the sterol extracts were evaporated to dryness under nitrogen, and two drops of *N,O*-bis(trimethylsilyl)acetamide and four drops of *N,O*-bis(trimethylsilyl)-trifluoroacetamide were added. Samples were then heated for 15 min at 85 °C in order to afford trimethylsilyl ethers and then were dried under nitrogen and resuspended in 1 mL of a 0.02 mg/mL solution of 5α-cholestane in hexane (internal standard); all chromatographic analyses were performed within 6 h of silyation. Sterols and related compounds were identified using a Varian 3800 GC connected to a Varian 2000 MS (Walnut Creek, CA). The column was a DB-5 (cross-linked 5% phenyl/methyl-siloxane, 30 m length, 0.32 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). Helium was used as the carrier gas, and the pressure was constant at 10 psig. The column temperature profile was as follows: 60 °C for 1.0 min, ramping to 100 °C at a rate of 25 °C/min, ramping to 150 °C at a rate of 15 °C/min, and holding at 315 °C for 2 min after ramping at 3 °C/min. The MS was in the EI mode (70 eV) with a 1.0 scan per second interval over a 40-650

m/z range. Sterols were identified using Varian Saturn GC-MS workstation software version 5.4 with reference to their mass spectra, retention times, known standards, published spectral data and the National Institute of Standards and Technology (NIST) mass spectral library version 2.0 (Gaithersburg, MD) [187].

To more accurately quantify sterols, sterols were also analysed on an Agilent 6890 GC-FID equipped with an Agilent 7683 autosampler (Palo Alto, CA). The column was a DB-5 (30 m length, 0.32 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). The column pressure and temperature profile were the same as those previously described for the GC-MS analyses, whereas the detector and injector temperatures were set to 310 and 315 °C, respectively [187].

3.3.4. Tocopherol Composition

3.3.4.1. Tocopherol Extraction

The tocopherol compositions of tree nut oil samples were analysed as previously described [188], with minor modifications. Three grams of oil were accurately weighed into 50 mL glass tubes, followed by the addition of 5 mL of KOH (60%, w/v), 5 mL ethanol, and 10 mL of ethanolic pyrogallol (6%, w/v). The glass tubes were then sealed and subsequently heated at 70°C for 1 h. The tubes were then cooled and 5 mL of deionized water were added. The mixture was subsequently extracted with 15 mL of hexane/ethyl acetate (9:1, v/v) using a desktop vortex (Fisher Scientific; Ottawa, ON) for 3 minutes. Next the hexane/ethyl acetate fraction was carefully transferred into a 50 mL round bottom flask using a

pipette. The extraction process was repeated three times. The pooled extracts were evaporated to dryness and redissolved in 3 mL of HPLC grade methanol and filtered through a 3 μm pore size syringe filter before HPLC analysis.

The linearity and reproducibility of the extraction process was confirmed for each tocopherol isomer by testing the recovery of pure tocopherols and tocopherol mixtures from stripped corn oil (Acros Organics, Geel, Belgium) with exogenously added tocopherols. The tested ranges of tocopherol recoveries were from 20 to 1000 ppm for each pure tocopherol and for 1:1:1:1 (w/w/w/w) mixtures of the four tocopherol isomers.

3.3.4.2. Tocopherol Analysis

The tocopherols in tree nut oil samples were analysed using an Agilent model 1100 HPLC/UV-DAD/MS system (Agilent Technologies, Palo Alto, CA) as previously described [189], with minor modifications. Separation of tocopherol isomers was achieved using a Phenomenex Luna C18 column (150 mm x 4.6 mm; Phenomenex, Torrance, CA) packed with 5 μm particles. Forty microlitres of the tocopherol extract were loaded onto the column and then eluted isocratically using 98% methanol (v/v) with a flow rate of 1 mL/min at ambient temperature. Tocopherol isomers were quantified using an ultraviolet-diode array detector (UV-DAD) at a wavelength of 290 nm. Quantification of tocopherol isomers was achieved by comparison of each sample peak response to that of the corresponding authentic standard. In order to confirm the identity of each tocopherol peak, the HPLC/UV-DAD effluent was channelled into an Agilent 1100 APCI-MS (Palo

Alto, CA) operating in the negative mode. The MS conditions were as follows: auxiliary gas flow, 10 units; sheath gas pressure, 70 psig; capillary temperature, 150 °C; vaporizer temperature, 400 °C; corona current, 5 μamps; scan time, 1s; scan range, 40-600 *m/z*. Analysis of chromatographic and spectral data was performed using Agilent Chemstation software (Palo Alto, CA).

3.3.5 Iodine Values of Tree Nut Oils

The iodine values of tree nut oils were determined using the Wijs method [80]. Briefly, 0.100 ± 0.001 g of oil samples were accurately weighed into 250 mL Erlenmeyer flasks wrapped with aluminium foil. Twenty-five millilitres of Wijs iodine solution (0.3 N) were then accurately pipetted into the flask which was then allowed to stand at room temperature for 30 min in the dark. Subsequently, 10 mL of 15% KI solution were added to the solution followed by 100 mL of deionized water. The mixture was then titrated with 0.1 N sodium thiosulphate ($Na_2S_2O_3$) until the sample mixture became a light yellow colour. Starch indicator was then added to the sample and the titration was continued until the blue colour of the starch-iodine complex disappeared. The iodine value (IV) was calculated using the following formula:

$$IV = \frac{\text{mL of } Na_2S_2O_3\,(B) - \text{mL of } Na_2S_2O_3\,(S) - \text{N of } Na_2S_2O_3 \times 12.692}{\text{Weight of sample taken (g)}}$$

3.4. Antioxidant Activity of Tree Nut Oil Extracts

3.4.1. Extraction of Minor Components and Stripping of Tree Nut Oils

Minor components were extracted from tree nut oils as previously described [190], with minor modifications. Twenty grams of oil were combined with 200 mL of hexane in a lightly tarred 500 mL separatory funnel. To this 100mL of methanol were added, and the separatory funnel was sealed and agitated for 5 min with periodic venting. The separatory funnel was then sealed with nitrogen and stored at 4 °C for 1 h. The methanol fraction was decanted into a 1L round bottom flask. The methanol extraction was repeated 4 times and the pooled extracts were evaporated down to 20 mL, resulting in an extract with a final concentration of 1 g oil equivalent per mL. The extracts were stored in methanol at -80 °C until use within a maximum of one week.

After the methanol extraction process the hexane fraction was washed once with cold (4 °C) deionized water, and then the hexane fraction was passed through sodium sulphate. Hexane was removed from the elutate using a rotary evaporator at 40 °C. The resulting stripped oil was weighed to assess oil recovery and to calculate the weight of the minor component extract by difference. The stripped oil was then transferred to 10 mL sample vials and stored under nitrogen at -80 °C until use.

3.4.2. Total Phenolics Content (TPC) of Tree Nut Oil Extracts

The total phenolics content of oil extracts was assessed using a modified version of the method described by Singleton and Rossi [191]. Folin and Ciocalteu's reagent (1 mL) was added to centrifuge tubes containing 1 mL of oil extract, followed by 6 mL of deionized water and finally, 2 mL of sodium carbonate solution (75 g/L). The mixture was then vortexed and allowed to stand for 60 min with subsequent centrifugation at 4250g for 5 min. The absorbance of supernatants was read at 725 nm. A blank containing 1 mL of methanol rather than extract was used for background subtraction while standard curves, using gallic acid and α-tocopherol, were constructed. The total phenolics content of the oil extracts represents the total extracted phenolics expressed as milligrams of gallic acid or α-tocopherol equivalents per g oil.

3.4.3. Trolox Equivalent Antioxidant Capacity (TEAC)

The free radical scavenging capacity of extracts was determined as previously described [192]. All solutions were made in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl (phosphate buffer saline, PBS). Equal volumes of 2.0 mM 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonate) solution (ABTS) and 2.5 mM 2,2'-azo-bis (2-methylpropionamidine) dihydrochloride (AAPH) were mixed for production of the ABTS radical ($ABTS^{\bullet-}$). This solution was then heated at 65 °C in the dark for 12 min. Blank measurements (decrease in absorbance of the $ABTS^{\bullet-}$ solution without any sample added) were made for each radical preparation. A standard curve was prepared by measuring the decrease in the absorbance at 734 nm

over a 6 min period with increasing concentrations of Trolox, a vitamin E analog. Trolox equivalent antioxidant capacity values for the tree nut oil extracts were determined in the same manner by mixing 0.1 mL of the oil extract with 2.9 mL of radical solution, then measuring the absorbance of this solution every minute for 6 min. The TEAC value of an extract represents the concentration of a Trolox solution that has the same antioxidant capacity as the extract. The TEAC values were determined as follows:

$$\Delta A_{Trolox} = (A_{t=0\ Trolox} - A_{t=6min\ Trolox}) - \Delta A_{solvent\ (0\text{-}6\ min)}$$
$$\Delta A_{Trolox} = m^* [Trolox]$$
$$TEAC_{extract} = (\Delta A_{extract} / m)^* d$$

Where; ΔA is the reduction in absorbance; A, absorbance at a particular time; m, slope of the standard curve; [Trolox], molar Trolox concentration; and d, the dilution factor.

3.4.4. DPPH Scavenging Capacity of Tree Nut Oil Extracts

Scavenging activities of tree nut oil extracts towards the DPPH radical was assessed as previously described [193], with some modifications. The DPPH radical was dissolved in methanol and a standard curve of DPPH concentration versus absorbance at 540 nm was prepared to establish the linear absorbance range for DPPH concentration, which was found to be between 0.01 and 0.3 mM DPPH. Based on these studies, an initial concentration of 0.125 mM DPPH was selected for use in the scavenging assay; at this concentration, the absorbance of the DPPH mixture remained stable over a 2 h period. The reaction mixture contained 2 mL of 0.25 mM DPPH and 2 mL of tree nut oil extracts or methanol as a blank reaction. The absorbance of the reaction mixture at 540 nm was measured after 20 min (A_{540} t_{20}) and the percent DPPH scavenged was calculated using the following formula.

$$\% \text{ DPPH Scavenged} = ((A_{540}\ t_0 - A_{540}\ t_{20}) / A_{540}\ t_0) * 100$$

Tree nut oil extracts were assayed undiluted (1g oil equivalent / mL extract) and diluted 2, 4, 8 and 10 times; the data were used to construct a % DPPH scavenged versus extract concentration expression. This expression was then used to calculate the extract concentration required to scavenge 50% of the DPPH in the assay media, referred to as the IC_{50}. The DPPH scavenging capacity of α-tocopherol was also assayed at concentrations of 6.25, 12.5, 25.0, 50.0 and 100 μM to develop a DPPH scavenging capacity standard curve for α-tocopherol.

3.4.5. β-Carotene Bleaching Test with Tree Nut Oil Extracts

The β-carotene bleaching test was performed as previously described [194], with some modifications. A solution of 5 mg / 10 mL of β-carotene was prepared in chloroform and 3 mL of this solution were pipetted into a 100 mL round bottom flask. Chloroform was removed under vacuum using a rotary evaporator at 40 °C, then 40 mg linoleic acid, 400 mg Tween 40 emulsifier and 100 mL of aerated distilled water were added to the flask with vigorous shaking. Aliquots of 4.8 mL of this emulsion were transferred into a series of test tubes containing 200 μL of the tree nut oil extracts or methanol (control). Alpha-tocopherol was used as the reference antioxidant (10 μM). Immediately after the addition of the emulsion to each tube, the zero time absorbance was measured at 470 nm. Subsequent absorbance readings were recorded over a two-hour period at 20 min intervals by keeping the reaction tubes in a water bath set to 50 °C. Blank samples devoid of β-

carotene were prepared for background subtraction. The capacity of the extracts to protect against oxidation of β-carotene was determined as follows.

$$[(A_{t=0\ Sample}-A_{t=0\ Blank})-(A_{t=120\ min\ Sample}-A_{t=120\ min\ Blank})] / [(A_{t=0\ Control})-(A_{t=120\ min\ Control})] = \mathbf{C}$$

$$\beta\text{- Carotene retention (\%)} = 100\% - (\mathbf{C})*100\%$$

Where; A is the absorbance at a particular time; and **C**, carotene depletion factor.

3.4.6. Photochemiluminescence Inhibition Capacity of Tree Nut Oil Extracts

The photochemiluminescence (PCL) inhibition capacity of tree nut oil extracts was assessed as previously described [195] with some modifications. The principle of the PCL inhibition capacity is based on measurement of inhibition of the superoxide mediated oxidation of a chemiluminescent compound, luminol, by antioxidants. In this system the reaction is initiated by optical excitation of a suitable photosensitizer, which exclusively results in the generation of the superoxide radical. The radicals are then visualized with a chemiluminescent detection reagent. Luminol plays a dual role, acting as both the photosensitizer and the radical detection reagent. An automated PCL inhibition capacity analyser system (Analytik Jena Photochem, Analytik Jena USA, The Woodlands, TX) was used operating in the antioxidative capacity of lipids (ACL) mode. For lipid-soluble substances, the assay mixture contained 1 mL of methanol, 1.5 mL of 0.1M carbonate buffer with 0.1mM Na_2-EDTA (pH 10.5), 25 µL of 1 µM luminol, and 10 µL of α-tocopherol (0.05-0.25 mM) or 10 µL of tree nut oil extracts (1g oil equivalent/mL) dissolved in methanol. When necessary, extracts were diluted with methanol so that the PCL inhibition time fell within the linear range of the α-

tocopherol calibration curve. The chemiluminescence lag time of the tree nut oil extracts was used to calculate the PCL inhibition capacity, expressed as α-tocopherol equivalents.

3.4.7. Oxygen Radical Absorbance Capacity (ORAC) of Tree Nut Oil Extracts

The ORAC of tree nut oil extracts was studied as previously described [196], with modifications using a FLOUstar Optima fluorescence microplate reader equipped with two reagent injectors (BMG Labtechnologies, Durham, NC). Non-transparent 96-well microplates (Costar model 3095, Corning Life Sciences, Corning, NY) were used in the ORAC assay. Fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used; these conditions correspond to the fluorescence properties of fluorescein. All solutions were prepared with 75 mM phosphate buffer (pH 7.4). Samples were diluted by a factor of 30 with 5% randomly methylated cyclodextrin solution (Cyclodextrin Technologies Inc., High Springs, FL) before being assayed. Twenty microlitres of diluted tree nut oil extracts were manually pipetted into sample wells of the microplate which was then placed in the microplate reader and incubated for 15 min at 37 °C. During the first cycle 120 μL of fluorescein solution (disodium salt, 20 μM) were injected into each well using the first automated reagent injector, each injection was followed by a 1 s mixing cycle. During the second cycle 60 μL of AAPH (45 mM) were injected into each well using the second automated reagent injector, followed by a 1 s mixing cycle. Following the mixing cycle, the initial fluorescence measurement of the assay mixture was read. Fluorescence readings

were then taken every 30 s, with a total assay time of 30 min. A standard curve was prepared with α-tocopherol using a concentration range of 12.5 to 150 μM. The relative fluorescence versus time graph of each sample was recorded by the FLOUstar Optima computer software (BMG Labtechnologies, Durham, NC), from which the area under curve (AUC) of each sample was calculated. The AUC of each sample was used along with the standard curve to calculate the ORAC of each extract, expressed as nmol α-tocopherol equivalents / g oil equivalent.

3.4.8. Lipid Composition of Tree Nut Oil Extracts

The lipid class compositions of the tree nut oil extracts were analysed as described in Section 3.3.1. The tocopherol compositions were analysed as described in Section 3.3.4.2.

3.5. Oxidative Stability of Tree Nut Oils

3.5.1. Accelerated Oxidation Studies

The oxidative stability of stripped and non-stripped tree nut oils were studied using two accelerated oxidation conditions, namely accelerated autoxidation and accelerated photooxidation.

3.5.1.1. Accelerated Autoxidation Conditions

Six grams (± 0.01 g) of stripped or non-stripped tree nut oil samples were accurately weighed into 10 mL clear glass sample vials and loosely capped before being placed in a forced air oven (Precision Scientific Company, Chicago, IL) in the

dark and heated to 60 °C. For each sample, six vials were loaded into the oven and one was removed after 12 h, 1, 3, 6, 9 and 12 days, and stored at -80 °C until used for testing. Each sample was analysed in triplicate [197].

3.5.1.2. Accelerated Photooxidation Conditions

Stripped or non-stripped tree nut oils (2.0 g ± 0.01 g) were accurately weighed into 4 mL clear glass sample vials and placed in stainless steel transmethylation blocks, such that light was able to come into contact with the oils from the top of the vials only. The transmethylation blocks containing samples were then placed in a mirrored box (70 cm (l) x 35 cm (w) x 25 cm (h)) equipped with 2 cool white fluorescent lights suspended 10 cm above the sample vials. The fluorescent radiation was at 2650 Lux and the temperature within the photooxidation chamber was maintained at 27 °C (± 1 °C). For each sample, six vials were loaded into the photooxidation chamber and one was removed after 4 , 8, 12 and 24 hours, and 2 and 3 days and then stored at -80 °C until tested. Each sample was analysed in triplicate [198].

3.5.2. Quality Indicator Tests

The quality indicator tests used to assess the oxidative deterioration of stripped and non-stripped tree nut oils were conjugated dienes and peroxide values and these were used for assessing primary oxidation products. The *p*-anisidine values and headspace chromatographic analysis of volatile aldehydes were used for monitoring secondary oxidation products.

3.5.2.1. Conjugated Dienes

Conjugated diene values of oil samples were measured using the IUPAC method [79]. Oil samples (0.2-0.4 g ± 0.01g) were accurately weighed into 25 mL volumetric flasks, dissolved in isooctane (2,2,4- trimethylpentane) and made to the mark with the same solvent. The mixture was then thoroughly mixed and the absorbance was read at 234 nm; isooctane was used as the reference. Conjugated diene values were calculated as follows:

$$CD = A_{234} / (c * d)$$

Where; A_{234} is the absorbance of the mixture at 234 nm; c, concentration of the sample mixture (g / 100 mL); and d, length of the spectrophotometer cell (cm).

3.5.2.2. Peroxide Value

The peroxide value was measured using the official method of the American Oil Chemists' Society [80]. Oil samples (1.0-2.0 g ± 0.01 g) were weighed into 250 mL glass stoppered Erlenmeyer flasks and dissolved in 30 mL of glacial acetic acid/ chloroform (3:2, v/v) while stirring, followed by the addition of 0.5 mL of saturated potassium iodide (KI). The stoppered flasks were placed in the dark and allowed to stand for exactly 1 min and then mixed with 30 mL of deionized water. The liberated iodine was titrated against a standardized solution of 0.01 N sodium

thiosulphate ($Na_2S_2O_3$) while shaking constantly until the yellow colour had almost disappeared, at which point 0.5 mL of a starch indicator solution (1 % w/v) was added to the Erlenmeyer flask and the titration was continued until the blue colour of the mixture disappeared. Blank titrations were made before each sample titration. Peroxide value was expressed as the uptake of milliequivalents of oxygen per kg of oil using the following formula.

$$PV = (V_{sample} - V_{blank}) * N\ Na_2S_2O_3 * 1000 / \text{sample mass (g)}$$

Where; V is the volume of titrant used (mL); and N, is the normaility of the thiosulphate solution.

3.5.2.3. Anisidine Value

The anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1.0 cm cell of a solution containing 1.0 g of oil in 100 mL of isooctane and *p*-anisidine reagent according to the method described herein (AOCS method CD 18-90) [80]. Oil samples (0.5-1.0 g ± 0.01g) were accurately weighed into 25 mL volumetric flasks and brought to mark with isooctane, absorbance of this solution was measured at 350 nm. Five millilitres of this solution were transferred into a test tube and 1.0 mL of *p*-anisidine reagent (0.25 g *p*-anisidine / 100 mL glacial acetic acid) was then added to it. The absorbance of this solution was read after 10 min at 350 nm. A solution containing 5 mL of isooctane and 1 mL *p*-anisidine reagent was used as blank. The anisidine value (AnV) was calculated using the following formula.

$AnV = [25 * (1.2\ A_s - A_b)] / m$

Where; A_s is the absorbance of the sample containing reaction mixture after 10 min; A_b, absorbance of the blank reaction mixture after 10 min; and m, mass of the sample used (g).

3.5.2.4. Static Headspace Gas Chromatographic Analysis

Quantitative analysis of volatile aldehydes during the oxidation of oil samples was carried out using static headspace gas chromatographic method [199]. A Perkin-Elmer 8500 gas chromatograph equipped with a HS-6 headspace sampler (Perkin-Elmer Corp., Montreal, QC) was used for headspace analysis. Volatile aldehydes were separated using a Supelcowax-10 fused silica capillary column (30 m length, 0.32 mm i.d., 0.10μm film thickness; Supelco, Oakville, ON). Ultra high purity (UHP) helium was used as the carrier gas, employed at 17.5 psig and a split ratio of 7:1. The oven temperature was maintained at 40 °C for 5 min and then ramped to 200 °C at 20 °C/min and held there for 5 min. The injector and FID temperatures were set at 280 °C during the analysis.

Prior to headspace sampling, oils were melted and 0.1 to 0.2 g (± 0.01 g) accurately weighed into 6 mL headspace vials (Supelco Canada Ltd., Oakville, ON) followed by addition of 14 mg of 1% 2-heptanone (internal standard; diluted with stripped corn oil containing 250 ppm α-tocopherol). Headspace vials were capped with Teflon-lined septa, crimped and then loaded into the HS-6 headspace sampler assembly. While in the assembly, the samples were incubated at 90 °C for 30 min, followed by a 6 s pressurization phase, then a vapour sampling phase was carried out to load the headspace volatiles onto the gas chromatograph column. After the

sampling phase the oven temperature program was initiated. Volatile aldehydes were identified by comparison of their retention times with those of the authentic standards.

3.6. Statistical Analysis

All experiments were performed in triplicate; mean values and standard deviations were calculated for each case. Analysis of variance (ANOVA) followed by Tukey's studentized range test were performed at the $p \leq 0.05$ level using Minitab statistical software version 14 (Minitab Inc., State College, PA) to evaluate the significance of differences among different mean values [200].

CHAPTER 4

Results and Discussion

4.1. Oil Yield, Chemical Characteristics and Stripping of Tree Nut Oils

The oils used in this study were extracted from fresh raw tree nuts. Comparison of the two fat extraction processes, namely hexane and chloroform/methanol, show that the latter solvent system resulted in a higher oil yield for all tree nut varieties studied, whereas the hexane solvent system afforded oil with higher clarity; implying that the hexane solvent system provides a more refined oil extract compared to the chloroform/methanol solvent system. Pine nuts yielded the highest amount of oil; 73.9 ± 0.5 % with hexane and 75.4 ± 0.2 % with chloroform/methanol, whereas almonds had the lowest oil yield; 51.2 ± 0.5 % with hexane and 53.5 ± 0.2 % with chloroform/methanol (Table 4.1). The oil yields of several tree nut varieties have previously been reported and show that the oil contents being 50.6% (w/w) for almond, 66.4% (w/w) for Brazil nut, 72.0% (w/w) for pecan, 68.4% (w/w) for pine nut, 46.4% (w/w) for pistachio and 65.2% (w/w) for walnut [1, 123]. Results from this study are therefore in good agreement with those reported previously.

The oxidative qualities of the extracted oils were examined using the conjugated dienes, peroxide value and anisidine value tests, all of which were well below the recommended values for oil acceptability (Table 4.1) [201]. No headspace aldehydes were detected in fresh tree nut oils (data not shown). These

Table 4.1. Oil Yields and Chemical Characteristics of Hexane Extracted and Chloroform/Methanol Extracted Tree Nut Oils[1,2]

Sample	Oil Yield (% ww)	CD	PV (meq/kg oil)	*p*-Av
Hexane-Extracted				
Almond	51.2 ± 0.5^{h}	1.67± 0.01^{h}	0.040 ± 0.002^{a}	0.120 ± 0.003^{m}
Brazil Nut	67.4 ± 0.2^{c}	1.77 ± 0.01^{g}	0.047 ± 0.003^{a}	0.189 ± 0.006^{l}
Hazelnut	60.4 ± 0.4^{e}	2.28 ± 0.01^{e}	0.031 ± 0.001^{a}	0.591 ± 0.008^{c}
Pecan	71.5 ± 0.4^{a}	1.50 ± 0.01^{j}	0.030 ± 0.003^{a}	0.433 ± 0.007^{h}
Pine Nut	73.9 ± 0.5^{a}	1.54 ± 0.01^{i}	0.030 ± 0.002^{a}	0.267 ± 0.002^{j}
Pistachio	52.3 ± 0.2^{g}	1.36 ± 0.01^{k}	0.023 ± 0.002^{a}	0.545 ± 0.006^{e}
Walnut	70.6 ± 0.4^{a}	0.99 ± 0.01^{l}	0.030 ± 0.004^{a}	0.230 ±0.005^{k}
Chloroform/Methanol -Extracted				
Almond	53.5 ± 0.2^{f}	2.74 ± 0.01^{c}	0.030 ± 0.002^{a}	0.561 ± 0.004^{d}
Brazil Nut	68.9 ± 0.3^{b}	2.42 ± 0.01^{d}	0.030 ± 0.003^{a}	0.821 ± 0.004^{a}
Hazelnut	61.9 ± 0.2^{d}	3.80 ± 0.01^{b}	0.058 ± 0.005^{a}	0.288 ± 0.003^{i}
Pecan	73.4 ± 0.3^{a}	1.87 ± 0.01^{f}	0.023 ± 0.002^{a}	0.294 ± 0.005^{i}
Pine Nut	75.1 ± 0.2^{a}	4.07 ± 0.01^{a}	0.015 ± 0.002^{a}	0.735 ± 0.002^{b}
Pistachio	54.1 ± 0.4^{f}	1.64 ± 0.01^{h}	0.015 ± 0.002^{a}	0.735 ± 0.002^{b}
Walnut	72.5 ± 0.3^{a}	1.52 ± 0.01^{j}	0.015 ± 0.002^{a}	0.462 ±0.005^{g}

[1]Values in the same column bearing different superscripts are significantly ($p \leq 0.05$) different.
[2] Iodine values (g iodine / 100g oil) of hexane extracts were 102.3 ± 0.8^{d} for almond oil; 98.6 ± 0.9^{e} for Brazil nut oil; 87.0 ± 0.9^{f} for hazelnut oil; 107.1 ± 0.8^{c} for pecan oil; 145.8 ± 0.4^{b} for pine nut oil; 97.9 ± 0.8^{e} for pistachio oil; and 154.0 ± 0.4^{a} for walnut oil.

findings show that the fat extraction processes employed here were gentle enough to preserve the oxidative integrity of the oils, as expected for fresh products.

A liquid-liquid phase partitioning system was used to strip minor components from tree nut oil samples (solvent stripping process). This method was chosen over solid phase stripping processes [198] because of the relative ease of the solvent stripping process, and to reduce oxidative deterioration of the minor components; thereby preserving their antioxidant activity. The recovery of stripped oil was

between 95.2% and 97.8%, with hexane extracted almond oil affording the highest stripped oil recovery and chloroform/methanol extracted Brazil nut oil affording the lowest stripped oil recovery (Table 4.2). A similar solvent stripping process was employed by Ramadan et al. [190] to extract antioxidative components from black cumin, coriander and Niger oils, reporting DPPH radical scavenging activity for oil extracts examined. However, the nature of the active compounds involved was not investigated [190].

4.2. Fatty Acid Composition of Tree Nut Oils

The fatty acid compositions of tree nut oils used in this study are reported in Table 4.3. Oleic acid was the predominant fatty acid in all samples examined, except for pine nut oil and walnut oil which contained predominantly linoleic acid. The extraction solvent did not significantly ($p>0.05$) influence the fatty acid composition of the oils (Table 4.3). Fatty acid compositions of the stripped tree nut oils were not significantly different from their parent oils ($p>0.05$).

Among samples tested, hazelnut oil had the highest oleic acid content at 83.4 and 83.0% for hexane and chloroform/methanol extracted oils, respectively. Almond oil contained the second highest oleic acid level at 69.9 to 70.0%. Walnut oil contained the lowest amount of oleic acid of around 12.1%. Among the tree nut oils studied, walnut oil was the only significant source of both myristic acid (14.4%) and α-linolenic acid (12.9%). Brazil nut oil was the richest source of palmitic acid (15.0 to 15.7%), stearic acid (10.0%) and total saturated fatty acids (25.0 to 25.7%). The fatty acid compositions of tree nut oils have been previously

Table 4.2. Recovery of Stripped Oils Using the Solvent Stripping Process from Hexane and Chloroform-Methanol Extracted Oils[1]

Nut	Oil Yield (%)
Hexane-Extracted	
Almond	97.8±0.1[a]
Brazil Nut	96.0±0.1[d]
Hazelnut	96.9±0.1[c]
Pecan	96.2±0.1[d]
Pine Nut	97.7±0.1[a]
Pistachio	96.7±0.1[c]
Walnut	96.6±0.1[c]
Chloroform/Methanol Extracted	
Almond	97.3±0.1[b]
Brazil Nut	95.2±0.1[f]
Hazelnut	96.1±0.1[d]
Pecan	95.5±0.1[e]
Pine Nut	97.1±0.1[b]
Pistachio	96.2±0.1[d]
Walnut	96.2±0.1[d]

[1] Values bearing different superscripts are significantly ($p \leq 0.05$) different for each type of solvent extracted oil.

Table 4.3. Fatty Acid Compositions (weight %) of Tree Nut Oils[1,2,3,4]

Fatty Acid	A-H	A-CM	BN-H	BN-CM	HN-H	HN-CM	P-H	P-CM	PN-H	PN-CM	PO-H	PO-CM	W-H	W-CM
14:0	0.00^{d}	0.00^{d}	0.07^{c}	0.08^{c}	0.00^{d}	0.00^{d}	0.09^{c}	0.11^{c}	0.00^{d}	0.00^{d}	2.55^{b}	2.54^{b}	14.44^{a}	14.42^{a}
14:1 n-9	0.00^{b}	0.00^{b}	0.05^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.08^{a}	0.07^{a}	0.00^{b}	0.00^{b}
15:0	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.09^{a}	0.07^{a}	0.00^{b}	0.00^{b}	0.05^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}
16:0	7.28^{c}	7.43^{c}	15.04^{a}	15.71^{a}	5.32^{e}	5.35^{e}	7.64^{c}	7.70^{c}	5.22^{e}	5.25^{e}	8.49^{b}	8.53^{b}	6.52^{d}	6.57^{d}
16:1 n-9	0.77^{a}	0.78^{a}	0.40^{b}	0.40^{b}	0.19^{c}	0.19^{c}	0.11^{c}	0.14^{c}	0.09^{c}	0.00^{d}	0.77^{a}	0.76^{a}	0.00^{d}	0.00^{d}
18:0	1.89^{d}	1.86^{d}	9.97^{a}	10.02^{a}	2.86^{c}	2.81^{c}	2.52^{c}	2.56^{c}	2.19^{d}	2.15^{d}	1.39^{e}	1.38^{e}	3.54^{b}	3.55^{b}
18:1 n-9	69.89^{b}	70.01^{b}	37.80^{e}	37.49^{e}	83.43^{a}	83.31^{a}	49.60^{d}	49.72^{d}	28.90^{f}	29.43^{f}	58.43^{c}	58.41^{c}	12.14^{g}	12.11^{g}
18:1 n-6	0.52^{b}	0.32^{c}	0.02^{d}	0.03^{d}	0.00^{e}	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}	1.63^{a}	1.65^{a}	0.57^{b}	0.58^{b}
18:2 n-6	19.57^{f}	19.49^{f}	35.92^{d}	35.59^{d}	8.22^{g}	8.35^{g}	37.71^{c}	37.34^{c}	59.60^{a}	59.29^{a}	25.15^{e}	25.11^{e}	49.56^{b}	49.55^{b}
18:3 n-3	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	1.47^{b}	1.55^{b}	0.17^{d}	0.15^{d}	0.37^{c}	0.43^{c}	12.86^{a}	12.85^{a}
20:0	0.00^{d}	0.00^{d}	0.38^{b}	0.40^{b}	0.00^{d}	0.00^{d}	0.34^{b}	0.34^{b}	1.33^{a}	1.33^{a}	0.14^{c}	0.11^{c}	0.00^{d}	0.00^{d}
20:1 n-9	0.00^{d}	0.00^{d}	0.28^{c}	0.28^{c}	0.00^{d}	0.00^{d}	0.52^{b}	0.53^{b}	1.34^{a}	1.38^{a}	0.62^{b}	0.64^{b}	0.37^{c}	0.38^{c}
20:1 n-6	0.07^{a}	0.11^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.30^{a}	0.30^{a}	0.00^{b}	0.00^{b}
Total Fatty Acids (g/100g oil)	94.61^{b}	94.47^{b}	94.98^{a}	94.64^{b}	94.85^{a}	94.70^{a}	94.92^{a}	94.80^{a}	93.99^{c}	93.79^{c}	94.12^{c}	94.94^{a}	95.17^{a}	95.03^{a}

[1] Abbreviations used: A-H, almond oil – hexane extracted; A-CM, almond oil – chloroform/methanol extracted; BN-H, Brazil nut oil – hexane extracted; BN-CM, Brazil nut oil – chloroform/methanol extracted; HN-H, hazelnut oil – hexane extracted; HN-CM, hazelnut oil – chloroform/methanol extracted; P-H, pecan oil – hexane extracted; P-CM, pecan oil – chloroform/methanol extracted; PN-H, pine nut oil – hexane extracted; PN-CM, pine nut oil – chloroform/methanol extracted; PO-H, pistachio oil – hexane extracted; PO-CM, pistachio oil – chloroform/methanol extracted; W-H, walnut oil – hexane extracted; W-CM, walnut oil – chloroform/methanol extracted; UI, undifferentiated isomers.

[2] Standard deviations of sample fatty acids from triplicate injections did not exceed 0.05% (data not shown).

[3] Values in the same row with different superscripts are significantly ($p \leq 0.05$) different.

[4] Heptahecanoic acid (17:0) was detected in BN-H (0.08%) and BN-BD (0.07%), heptadecenoic acid (17:1) was detected in PO-H (0.08%) and PO-BD (0.08). Eicosadienoic acid (20:2 n-6) was detected in PN-H (1.12%) and PN-BD (1.02%).

reported, showing that in most cases oleic acid is the predominant fatty acid in most tree nut oils, excluding pine nut and walnut oils which contain predominantly linoleic acid [1, 123]. The fatty acid compositions reported in this study are in good agreement with those reported previously by others [1, 123].

4.3. Lipid Class Compositions of Tree Nut Oils

The lipid class composition of stripped and non-stripped tree nut oils were analysed using an Iatroscan TLC-FID system. Lipid classes detected in tree nut oils included triacylglycerols, sterols and sterol esters, phospholipids and sphingolipids; no hydrocarbons or free fatty acids were detected in oil samples examined. Stripped oils contained higher amounts of triacylglycerols than their non-stripped counterparts, and lower amounts of sterols, phospholipids and sphingolipids (Table 4.4). Stripped oils contained between 98.9 and 99.4 % triacylglycerols and no significant differences ($p>0.05$) were detected among stripped oil samples. Among stripped oil samples, walnut and almond oils contained the highest triacylglycerol content while pistachio and pecan oils had the lowest levels. Similarly, among the non-stripped oil samples, almond oil contained the highest triacylglycerol content while pistachio and pecan oils contained the lowest amount. No significant differences in the sterol ester contents of stripped and non-stripped tree nut oils were observed in this work; however, sterol esters were the smallest detectable lipid class present in tree nut oils (0.03 to 0.09% of total lipid).

The stripping process employed in this study was intended to recover representative samples of minor components of the oils responsible for their antioxidant activity. This process effectively reduced the levels of sterols and polar

lipid classes in tree nut oils ($p \leq 0.05$) (Table 4.4). Sterol levels in stripped oils were reduced by 24 to 53% while phospholipids and sphingolipids were reduced by 25 to 50%. Among individual phospholipids classes, phosphatidylcholine was most effectively removed by the solvent stripping process (Table 4.4). Phosphatidic acid was present in hazelnut oils at trace amounts (0.02 to 0.05%) and was not detected in stripped hazelnut oil.

Few reports on the lipid class composition of tree nut oils exist [123]. Using column chromatography, hazelnut oil lipids have been separated and quantified; showing 98.4% triacylglycerols and less than 0.2% of phospholipids (phosphatidylcholine and phosphatidylinositol) [154]. The lipid class composition of hazelnut oil has also been studied using Iatroscan; revealing 98.8% triacylglycerols and 1.2% polar lipids. Among polar lipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were present at 56.4, 30.8 and 11.7%, respectively [150]. The lipid class composition of walnut oil has been reported to contain 96.9 % non-polar lipids (acylglycerols, fatty acids, sterols and sterol esters) and 3.1% polar lipids. Polar lipids included sphingolipids (73.4%) and 26.6% phospholipids (primarily phosphatidylethanolamine) [181].

4.4. Sterol and Stanol Contents and Compositions of Tree Nut Oils

Sterol and stanol contents of stripped and non-stripped tree nut oils were analysed using gas chromatography. Qualitative analysis of trimethylsilyl ethers of sterols and stanols were carried out using GC-MS with reference to published spectral data and peak retention times. Quantitative analyses were then performed using GC-FID,

Table 4.4. Lipid Class Compositions of Non-stripped (a) and Stripped (b) Tree Nut Oils[1,2]

Lipid Class (g/100g oil)	A-H	A-BD	BN-H	BN-BD	HN-H	HN-BD	P-H
Non-Stripped Oils							
Triacylglycerols	98.2 ± 0.1^{b}	98.0 ± 0.1^{b}	96.7 ± 0.1^{e}	96.6 ± 0.1^{e}	98.0 ± 0.1^{b}	97.6 ± 0.1^{c}	96.4 ± 0.1^{e}
Sterols	0.22 ± 0.01^{a}	0.25 ± 0.03^{a}	0.18 ± 0.02^{b}	0.19 ± 0.02^{b}	0.21 ± 0.03^{b}	0.22 ± 0.02^{b}	0.26 ± 0.02^{a}
Sterol Esters	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.07 ± 0.01^{b}
Phosphatidylserine	0.21 ± 0.04^{e}	0.32 ± 0.03^{d}	0.26± 0.02^{e}	0.32 ± 0.02^{d}	0.27 ± 0.01^{e}	0.36 ± 0.01^{c}	0.39 ± 0.03^{c}
Phosphatidylinositol	0.11 ± 0.05^{c}	0.17 ± 0.03^{b}	0.09 ± 0.03^{c}	0.10 ± 0.02^{c}	0.06 ± 0.02^{c}	0.08 ± 0.02^{c}	0.15 ± 0.02^{b}
Phosphatidylcholine	0.21 ± 0.03^{d}	0.56 ± 0.01^{b}	0.34 ± 0.04^{c}	0.78 ± 0.06^{a}	0.24 ± 0.03^{d}	0.48 ± 0.03^{b}	0.21 ± 0.06^{d}
Phosphatidic Acid	ND	ND	ND	ND	0.02 ± 0.01^{a}	0.05 ± 0.01^{a}	ND
Sphingholipids	0.53 ± 0.02^{e}	0.63 ± 0.05^{d}	0.83 ± 0.03^{b}	0.91 ± 0.02^{a}	0.26 ± 0.04^{f}	0.32 ± 0.03^{f}	0.48 ± 0.02^{e}
Lipid Class (g/100g oil)	P-BD	PN-H	PN-BD	PO-H	PO-BD	W-H	W-BD
Triacylglycerols	96.3 ± 0.1^{e}	97.6^{c}± 0.1	97.1 ± 0.1^{d}	96.2 ± 0.1^{e}	95.8 ± 0.1^{f}	97.2 ± 0.1^{d}	97.1 ± 0.1^{d}
Sterols	0.28 ± 0.03^{a}	0.13 ± 0.03^{b}	0.16 ± 0.01^{b}	0.19 ± 0.02^{b}	0.21 ± 0.02^{b}	0.26 ± 0.03^{a}	0.28 ± 0.02^{a}
Sterol Esters	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	0.05 ± 0.01^{b}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}
Phosphatidylserine	0.47 ± 0.01^{b}	0.23 ± 0.02^{e}	0.33 ± 0.03^{d}	0.47 ± 0.04^{b}	0.59 ± 0.04^{a}	0.37 ± 0.02^{c}	0.46 ± 0.03^{b}
Phosphatidylinositol	0.18 ± 0.03^{b}	0.14 ± 0.01^{b}	0.19 ± 0.02^{b}	0.21 ± 0.01^{b}	0.28 ± 0.03^{a}	0.25 ± 0.04^{a}	0.31 ± 0.02^{a}
Phosphatidylcholine	0.52 ± 0.04^{b}	0.19 ± 0.02^{d}	0.37 ± 0.05^{c}	0.52 ± 0.06^{b}	0.68 ± 0.04^{a}	0.34 ± 0.05^{c}	0.52 ± 0.04^{b}
Phosphatidic Acid	ND	ND	ND	ND	ND	ND	ND
Sphingholipids	0.55 ± 0.04^{e}	0.45 ± 0.02^{e}	0.57 ± 0.03^{e}	0.73 ± 0.03^{c}	0.82 ± 0.01^{b}	0.54 ± 0.02^{e}	0.68 ± 0.02^{d}
Lipid Class (g/100g oil)	A-H	A-BD	BN-H	BN-BD	HN-H	HN-BD	P-H
Stripped Oils							
Triacylglycerols	99.3 ± 0.1^{a}	99.2 ± 0.1^{a}	98.9 ± 0.1^{a}	99.0 ± 0.1^{a}	99.3 ± 0.1^{a}	99.1 ± 0.1^{a}	98.9 ± 0.1^{a}
Sterols	0.14 ± 0.01^{b}	0.16 ± 0.02^{b}	0.09 ± 0.01^{c}	0.11 ± 0.02^{c}	0.09 ± 0.01^{c}	0.11 ± 0.03^{c}	0.14 ± 0.03^{b}
Sterol Esters	0.04 ± 0.01^{b}	0.05 ± 0.02^{b}	0.04 ± 0.01^{b}	0.05 ± 0.02^{b}	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.06 ± 0.02^{b}
Phosphatidylserine	0.16 ± 0.03^{f}	0.21 ± 0.03^{e}	0.14 ± 0.03^{f}	0.24 ± 0.02^{e}	0.16 ± 0.02^{f}	0.23 ± 0.03^{e}	0.24 ± 0.01^{e}
Phosphatidylinositol	0.04 ± 0.02^{c}	0.09 ± 0.02^{c}	0.08 ± 0.02^{c}	0.08 ± 0.01^{c}	0.03 ± 0.01^{c}	0.05 ± 0.03^{c}	0.08 ± 0.03^{c}
Phosphatidylcholine	0.13 ± 0.03^{e}	0.36 ± 0.04^{c}	0.23 ± 0.03^{d}	0.39 ± 0.04^{c}	0.13 ± 0.02^{e}	0.27 ± 0.01^{d}	0.11 ± 0.03^{e}
Phosphatidic Acid	ND	ND	ND	ND	ND	ND	ND
Sphingholipids	0.32 ± 0.02^{f}	0.39 ± 0.05^{e}	0.51 ± 0.03^{e}	0.62 ± 0.02^{d}	0.27 ± 0.04^{f}	0.30 ± 0.03^{f}	0.27 ± 0.02^{f}
Lipid Class (g/100g oil)	P-BD	PN-H	PN-BD	PO-H	PO-BD	W-H	W-BD
Triacylglycerols	98.9 ± 0.1^{a}	99.1± 0.1^{a}	99.2 ± 0.1^{a}	98.9 ± 0.1^{a}	98.8 ± 0.1^{a}	99.4 ± 0.1^{a}	99.3 ± 0.1^{a}
Sterols	0.13 ± 0.02^{b}	0.08 ± 0.01^{c}	0.09 ± 0.02^{c}	0.08 ± 0.01^{c}	0.09 ± 0.01^{c}	0.15 ± 0.01^{b}	0.14 ± 0.03^{b}
Sterol Esters	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	0.04 ± 0.02^{b}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}
Phosphatidylserine	0.23 ± 0.02^{e}	0.13 ± 0.03^{f}	0.18 ± 0.02^{f}	0.26 ± 0.02^{e}	0.31 ± 0.02^{d}	0.20 ± 0.01^{f}	0.29 ± 0.01^{e}
Phosphatidylinositol	0.11 ± 0.02^{c}	0.08 ± 0.03^{c}	0.11 ± 0.01^{c}	0.12 ± 0.02^{c}	0.16 ± 0.01^{b}	0.13 ± 0.01^{c}	0.18 ± 0.04^{b}
Phosphatidylcholine	0.29 ± 0.02^{d}	0.09 ± 0.03^{e}	0.23 ± 0.04^{d}	0.28 ± 0.03^{d}	0.38 ± 0.02^{c}	0.22 ± 0.02^{d}	0.28 ± 0.02^{d}
Phosphatidic Acid	ND	ND	ND	ND	ND	ND	ND
Sphingholipids	0.22 ± 0.04^{f}	0.18 ± 0.02^{f}	0.29 ± 0.03^{f}	0.44 ± 0.03^{e}	0.49 ± 0.03^{e}	0.29 ± 0.02^{f}	0.39 ± 0.04^{e}

[1] Abbreviations used: A-H, almond oil – hexane extracted; A-CM, almond oil – chloroform/methanol extracted; BN-H, Brazil nut oil – hexane extracted; BN-CM, Brazil nut oil – chloroform/methanol extracted; HN-H, hazelnut oil – hexane extracted; HN-CM, hazelnut oil – chloroform/methanol extracted; P-H, pecan oil – hexane extracted; P-CM, pecan oil –chloroform/ methanol extracted; PN-H, pine nut oil – hexane extracted; PN-CM, pine nut oil – chloroform/methanol extracted; PO-H, pistachio oil – hexane extracted; PO-CM, pistachio oil – chloroform/methanol extracted; W-H, walnut oil – hexane extracted; W-CM, walnut oil – chloroform/methanol extracted; ND, not detected.

[2] Values for each lipid class with different superscripts are significantly ($p \leq 0.05$) different.

using 5α-cholestane as internal standard. Results showed that β-sitosterol was the predominant sterol in tree nut oils, with stigmasterol, campesterol and Δ5-avenosterol also being widely distributed among samples, but at lower levels (Table 4.5). Small amounts of stanols (≤ 0.1 mg/g oil) were present in Brazil nut, hazelnut, pine nut and pistachio oils. Walnut oil contained the highest amount of total sterols (2.9 mg/g oil), followed by almond oil (2.7 to 2.8 mg/g oil) and pecan oil (2.6 to 2.7 mg/g oil). Among non-stripped oil samples, hexane extracted pine nut oil contained the lowest amount of total sterols, with 1.3 mg total sterols per gram of oil. The solvent stripping process reduced the total sterol content of tree nut oils by 24 to 53%. Stripping of sterols was most effective with Brazil nut oil and least effective with pine nut oil. Poor results for the stripping of total sterols from pine nut oil may be due to its low total sterol content in combination with a high degree of unsaturation, which may have negatively influenced the partitioning of sterols and similar compounds into the methanol phase during the stripping process. Minor sterols (≤ 0.05 mg/g oil) present in non-stripped samples were completely removed during the solvent stripping process.

Results for total sterols from gas chromatography and TLC-FID analyses of tree nut oils were similar, with oils from walnuts, almonds and pecans having the highest sterol contents and pine nut oil having the lowest. The TLC-FID analyses performed in this work allowed for quantification of both free and unesterified sterols, whereas the gas chromatographic protocol used in this study was not suitable for quantitative determination of free sterol and sterol ester compositions. However, since sterol esters are present in tree nut oils at much lower amounts than free sterols, the difference in results for total sterols from the two analytical

Table 4.5. Sterol and Stanol Contents (mg/g) and Compositions of Non-stripped and Stripped Tree Nut Oils[1,2]

Compound	A-H	A-CM	BN-H	BN-CM	HN-H	HN-CM	P-H
Non-Stripped Tree Nut Oil							
22-Nordehydrocholesterol	ND	ND	0.11±0.01^{a}	0.14±0.02^{a}	ND	ND	ND
Cholesterol	0.02±0.01^{c}	0.03±0.01^{c}	0.12±0.03^{a}	0.18±0.03^{a}	0.13±0.02^{a}	0.13±0.02^{a}	0.07±0.02^{b}
Cholestanol	ND	ND	0.02±0.01^{b}	0.02±0.01^{b}	0.08±0.01^{a}	0.09±0.001^{a}	ND
Campesterol	0.09±0.02^{b}	0.09±0.01^{b}	0.12±0.01^{b}	0.15±0.01^{a}	0.17±0.02^{a}	0.17±0.02^{a}	0.22±0.03^{a}
Stigmasterol	0.19±0.05^{b}	0.19±0.07^{b}	0.22±0.05^{b}	0.23±0.04^{b}	0.32±0.08^{b}	0.39±0.07^{b}	0.44±0.03^{b}
24-Methylenecholesterol	0.00	0.00	0.12±0.01^{a}	0.13±0.01^{a}	0.01±0.01^{c}	0.01±0.001^{c}	0.05±0.01^{b}
β-Sitosterol	2.30±0.09^{a}	2.29±0.06^{a}	1.11±0.02^{c}	1.12±0.07^{c}	1.07±0.06^{c}	1.10±0.09^{c}	1.67±0.08^{b}
β-Sitostanol	ND	ND	ND	ND	ND	ND	ND
Δ5-Avenasterol	0.10±0.01^{b}	0.11±0.01^{b}	0.10±0.001^{b}	0.11±0.02^{b}	0.07±0.01^{b}	0.09±0.01^{b}	0.10±0.02^{b}
Total	2.68±0.07^{a}	2.75±0.06^{a}	1.92±0.08^{b}	2.06±0.07^{b}	1.85±0.06^{b}	1.99±0.09^{b}	2.62±0.07^{a}
Compound	P-CM	PN-H	PN-CM	PO-H	PO-CM	W-H	W-CM
22-Nordehydrocholesterol	ND	0.04±0.01^{b}	0.05±0.01^{b}	0.04±0.02^{b}	0.04±0.01^{b}	ND	ND
Cholesterol	0.08±0.02^{b}	0.02±0.01^{c}	0.02±0.01^{c}	0.03±0.01^{c}	0.04±0.02^{c}	0.08±0.01^{b}	0.10±0.01^{a}
Cholestanol	ND	0.01±0.01^{b}	0.01±0.01^{b}	ND	ND	ND	ND
Campesterol	0.24±0.03^{a}	0.19±0.02^{a}	0.22±0.02^{a}	0.20±0.02^{a}	0.21±0.02^{a}	0.18±0.02^{a}	0.19±0.02^{a}
Stigmasterol	0.60±0.07^{a}	0.13±0.08^{b}	0.15±0.09^{b}	0.10±0.05^{b}	0.11±0.06^{b}	0.33±0.09^{b}	0.35±0.08^{b}
24-Methylenecholesterol	0.05±0.01^{b}	ND	ND	ND	ND	ND	ND
β-Sitosterol	1.75±0.06^{b}	1.20±0.08^{c}	1.12±0.07^{c}	1.14±0.08^{c}	1.19±0.09^{c}	2.16±0.09^{a}	2.25±0.07^{a}
β-Sitostanol	ND	ND	ND	0.08±0.01^{a}	0.10±0.02^{a}	ND	ND
Δ5-Avenasterol	0.11±0.01^{b}	0.06±0.01^{b}	0.07±0.01^{b}	0.13±0.01^{a}	0.15±0.01^{a}	0.17±0.02^{a}	0.18±0.02^{a}
Total	2.76±0.08^{a}	1.29±0.07^{c}	1.60±0.07^{b}	1.52±0.08^{b}	1.69±0.07^{b}	2.92±0.09^{a}	2.99±0.08^{a}
Compound	A-H	A-CM	BN-H	BN-CM	HN-H	HN-CM	P-H
Stripped Tree Nut Oils							
22-Nordehydrocholesterol	ND	ND	0.05±0.01^{b}	0.06±0.01^{b}	ND	ND	ND
Cholesterol	ND	ND	0.07±0.01^{b}	0.08±0.01^{b}	0.07±0.01^{b}	0.07±0.01^{b}	ND
Cholestanol	ND	ND	0.001±0.01^{b}	0.01±0.01^{b}	0.04±0.01^{b}	0.04±0.01^{b}	ND
Campesterol	0.06±0.01^{b}	0.06±0.01^{b}	0.05±0.01^{b}	0.07±0.01^{b}	0.10±0.01^{b}	0.11±0.01^{b}	0.13±0.01^{b}
Stigmasterol	0.07±0.02^{b}	0.02±0.04^{b}	0.06±0.03^{b}	0.08±0.03^{b}	0.17±0.06^{b}	0.20±0.04^{b}	0.16±0.07^{b}
24-Methylenecholesterol	ND	ND	0.05±0.01^{b}	0.06±0.01^{b}	ND	ND	ND
β-Sitosterol	1.56±0.08^{b}	1.65±0.08^{b}	0.58±0.08^{d}	0.59±0.07^{d}	0.61±0.09^{d}	0.64±0.08^{d}	1.14±0.05^{c}
β-Sitostanol	ND	ND	ND	ND	ND	ND	ND
Δ5-Avenasterol	0.05±0.01^{b}	0.06±0.01^{b}	0.04±0.01^{b}	0.05±0.01^{b}	0.04±0.01^{b}	0.05±0.01^{b}	0.05±0.01^{b}
Total	1.68±0.08^{b}	1.78±0.08^{b}	0.90±0.07^{d}	0.99±0.06^{d}	1.03±0.08^{d}	1.11±0.06^{d}	1.47±0.07^{b}
Compound	P-CM	PN-H	PN-CM	PO-H	PO-CM	W-H	W-CM
22-Nordehydrocholesterol	ND	ND	ND	ND	ND	ND	ND
Cholesterol	ND	0.01±0.01^{c}	0.01±0.01^{c}	0.01±0.01^{c}	0.02±0.01^{c}	0.03±0.01^{c}	0.04±0.01^{c}
Cholestanol	ND	ND	0.01±0.01^{b}	ND	ND	ND	ND
Campesterol	0.14±0.01^{b}	0.10±0.02^{b}	0.14±0.01^{b}	0.11±0.01^{b}	0.12±0.01^{b}	0.09±0.01^{b}	0.10±0.01^{b}
Stigmasterol	0.09±0.03^{b}	0.04±0.02^{b}	0.06±0.002^{b}	0.10±0.02^{b}	0.13±0.07^{b}	0.12±0.07^{b}	0.13±0.06^{b}
24-Methylenecholesterol	ND	ND	ND	ND	ND	ND	ND
β-Sitosterol	1.08±0.08^{c}	0.93±0.06^{c}	0.96±0.05^{c}	0.49±0.05^{d}	0.51±0.07^{d}	1.18±0.04^{c}	1.22±0.08^{c}
β-Sitostanol	ND	ND	ND	0.05±0.001^{b}	0.06±0.01^{b}	ND	ND
Δ5-Avenasterol	0.07±0.01^{b}	0.04±0.01^{b}	0.04±0.01^{b}	0.07±0.02^{b}	0.08±0.01^{b}	0.08±0.02^{b}	0.09±0.01^{b}
Total	1.39±0.06^{c}	1.11±0.07^{d}	1.21±0.07^{d}	0.83±0.07^{d}	0.90±0.08^{d}	1.51±0.07^{b}	1.59±0.07^{b}

[1] Abbreviations used: A-H, almond oil – hexane extracted; A-CM, almond oil – chloroform/methanol extracted; BN-H, Brazil nut oil – hexane extracted; BN-CM, Brazil nut oil – chloroform/methanol extracted; HN-H, hazelnut Oil – hexane extracted; HN-CM, hazelnut oil – chloroform/methanol extracted; P-H, pecan oil – Hexane extracted; P-CM, pecan oil – chloroform/methanol extracted; PN-H, pine nut oil – hexane extracted; PN-CM, pine nut oil – chloroform/methanol extracted; PO-H, pistachio oil – hexane extracted; PO-CM, pistachio oil – chloroform/ methanol extracted; W-H, walnut oil – hexane extracted; W-CM, walnut oil – chloroform/methanol extracted; ND, not detected.

[2] Values for each compound with different superscripts are significantly ($p \leq 0.05$) different.

methods was negligible. Comparison of the two solvent extraction systems showed that the chloroform/methanol extracted oils had total sterols contents that were between 6 and 9% higher than those of their hexane extracted counterparts, but the difference reached significance ($p \leq 0.05$) only in pine nut oil.

The sterol compositions reported in this work are in good agreement with those reported previously in the literature [123]; however, little information exists regarding the minor sterol and stanol compositions of tree nut oils [202]. Silicic acid column chromatography was used to purify sterols and related compounds from oil samples before gas chromatographic analysis. This preparative step allowed for injection of sample sterols at higher concentrations and purity than could be achieved with direct injection of transmethylated or saponified samples. Thus, the preparative step allowed for quantification of minor sterol and stanol components, including 22-nordehydrocholesterol in Brazil nut, pine nut and pistachio oils as well as 24-methylenecholesterol in Brazil nut oil, hazelnut oil and pecan oils, both of which have not been reported previously in tree nut oils.

The sterol compositions of almond, hazelnut, pecan, pistachio and walnut oils have been previously reported by others. Almond oil has been reported to contain between 2.2 to 2.6 g/kg phytosterols, mainly as β-sitosterol, with trace amounts of stigmasterol and campesterol [1, 134]. Hazelnut oil contains 1.2 to 2.2 g/kg of phytosterols primarily in the form of β-sitosterol [1, 134]. Pecan oil has been reported to contain 0.73 g/kg phytosterols, primarily β-sitosterol (around 90%) [1]. Pistachio oil has been reported to contain 5 g/kg phytosterols, of which 85% is β-sitosterol [172]. Walnut oil has been shown to contain 1.8 g/kg phytosterols [1], with β-sitosterol comprising 85% of total sterols, followed by Δ5-avenasterol

(7.3%), campesterol (4.6%), and finally cholesterol (1.1%) [181]. These values are in good agreement with those reported in our study.

4.5. Tocopherol Contents and Compositions of Tree Nut Oils

The tocopherol compositions of tree nut oils were analysed in reversed-phase using HPLC; detection was achieved using both ultraviolet detection at 290 nm for quantitative analysis, and negative mode mass spectrometric detection for qualitative analysis of tocopherol isomers. No tocotrienols were detected in tree nut oil samples. Comparison of the hexane and chloroform/methanol extracted oils for total tocopherol contents shows that the chloroform/methanol extraction system afforded oils with higher tocopherol contents, with differences reaching significance ($p \leq 0.05$) in all nut oil samples studied (Table 4.6). Among non-stripped samples, walnut oil contained the highest tocopherol content (519 to 584 mg/kg oil), followed by hazelnut oil (494 to 525 mg/kg oil) and then pecan oil (479 to 509 mg/kg). Hexane-extracted Brazil nut oil contained the lowest amount of tocopherols (192 mg/kg oil). Interestingly, chloroform/methanol extracted Brazil nut oil contained more than twice the amount of total tocopherols than its hexane extracted counterpart, and the difference was due primarily to increased inclusion of δ-tocopherol (201 mg/kg oil) into chloroform/methanol extracted Brazil nut oil. The difference may be attributable to the enhanced ability of the chloroform/methanol extraction solvent to remove lipidic components from the skins of Brazil nuts, which are known to contain high amounts antioxidative components such as tocopherols but are not extracted well with non-polar solvents alone [128].

Table 4.6. Tocopherol Content (mg/kg) and Compositions of Non-stripped and Stripped Tree Nut Oils

Compound	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	β-Tocopherol	Total
Non-Stripped Oils					
A-H	203.4 ± 0.9^{f}	20.9 ± 0.4^{t}	ND	ND	224.3 ± 1.7^{l}
A-CM	311.6 ± 1.2^{d}	21.0 ± 0.6^{t}	ND	14.2 ± 0.2^{d}	346.8 ± 2.2^{k}
BN-H	14.1 ± 0.8^{t}	138.7 ± 1.3^{k}	38.9 ± 0.3^{c}	ND	191.7 ± 2.4^{m}
BN-CM	16.0 ± 1.3^{s}	175.3 ± 1.0^{f}	222.4 ± 0.4^{a}	ND	413.7 ± 2.5^{i}
HN-H	388.5 ± 0.9^{a}	105.2 ± 1.2^{l}	ND	ND	493.7 ± 1.7^{e}
HN-CM	377.5 ± 1.4^{b}	147.6 ± 1.1^{i}	ND	ND	525.1 ± 1.4^{b}
P-H	14.8 ± 0.9^{t}	464.6 ± 1.8^{c}	ND	ND	479.4 ± 2.3^{g}
P-CM	19.9 ± 0.6^{r}	488.8 ± 1.6^{b}	ND	ND	508.7 ± 1.8^{d}
PN-H	123.9 ± 0.7^{h}	240.0 ± 1.2^{e}	26.4 ± 0.5^{d}	32.5 ± 0.5^{a}	422.8 ± 1.6^{h}
PN-CM	178.4 ± 1.0^{g}	265.1 ± 0.7^{d}	23.7 ± 0.1^{e}	20.7 ± 0.2^{c}	487.9 ± 1.2^{f}
PO-H	296.7 ± 0.6^{e}	32.6 ± 1.0^{s}	18.3 ± 0.2^{g}	ND	347.6 ± 2.4^{k}
PO-CM	332.1 ± 0.8^{c}	49.7 ± 1.7^{p}	23.5 ± 0.6^{e}	ND	405.3 ± 1.8^{j}
W-H	34.1 ± 1.1^{q}	463.7 ± 1.1^{c}	20.7 ± 0.4^{f}	ND	518.5 ± 1.7^{c}
W-CM	38.0 ± 1.7^{p}	522.8 ± 1.9^{a}	23.4 ± 0.3^{e}	ND	584.2 ± 2.3^{a}
Stripped Oils					
A-H	63.1 ± 0.4^{n}	6.5 ± 0.3^{w}	ND	ND	69.5 ± 0.8^{v}
A-CM	96.6 ± 0.3^{l}	6.5 ± 0.4^{w}	ND	ND	103.1 ± 0.7^{u}
BN-H	4.4 ± 0.2^{y}	43.0 ± 0.6^{r}	5.0 ± 0.2^{l}	ND	56.8 ± 1.1^{w}
BN-CM	5.0 ± 0.3^{x}	54.3 ± 0.3^{o}	71.0 ± 0.2^{b}	ND	130.3 ± 1.3^{s}
HN-H	120.4 ± 0.8^{i}	32.6 ± 0.3^{s}	ND	ND	153.0 ± 1.1^{q}
HN-CM	117.0 ± 0.7^{j}	45.8 ± 0.5^{q}	ND	ND	162.8 ± 1.4^{o}
P-H	4.6 ± 0.2^{y}	144.0 ± 0.8^{j}	ND	ND	148.6 ± 1.2^{r}
P-CM	6.2 ± 0.2^{w}	151.5 ± 0.9^{h}	ND	ND	157.7 ± 1.1^{p}
PN-H	38.4 ± 0.7^{p}	74.4 ± 0.6^{n}	8.2 ± 0.3^{h}	10.1 ± 0.2^{e}	131.1 ± 1.8^{s}
PN-CM	55.3 ± 0.5^{o}	82.2 ± 0.5^{m}	7.3 ± 0.3^{i}	6.4 ± 0.3^{e}	151.2 ± 0.8^{q}
PO-H	92.0 ± 0.9^{m}	10.1 ± 0.3^{v}	5.7 ± 0.2^{k}	ND	107.8 ± 1.1^{u}
PO-CM	103.0 ± 0.9^{k}	15.4 ± 0.3^{u}	7.3 ± 0.3^{h}	ND	125.6 ± 1.5^{t}
W-H	10.6 ± 0.2^{v}	143.7 ± 0.7^{j}	6.4 ± 0.2^{j}	ND	160.7 ± 1.2^{o}
W-CM	11.8 ± 0.2^{u}	162.1 ± 0.9^{g}	7.3 ± 0.3^{i}	ND	181.1 ± 1.7^{n}

[1] Abbreviations used: A-H, almond oil – hexane extracted; A-CM, almond oil - chloroform/methanol extracted; BN-H, Brazil nut oil – hexane extracted; BN-CM, Brazil nut oil – chloroform/methanol extracted; HN-H, hazelnut oil – hexane extract; HN-CM, hazelnut oil – chloroform/methanol extracted; P-H, pecan oil – hexane extracted; P-CM, pecan oil – chloroform/methanol extracted; PN-H, pine nut oil – hexane extracted; PN-CM, pine nut oil – chloroform/methanol extracted; PO-H, pistachio oil – hexane extracted; PO-CM, pistachio oil – chloroform/methanol extracted; W-H, walnut oil – hexane extracted; W-CM, walnut oil – chloroform/methanol extracted; ND, not detected

[2] Values in the same column with different superscripts are significantly ($p \leq 0.05$) different.

The solvent stripping process employed in this work was able to reduce the amount of total tocopherols in tree nut oils by approximately 70%; however, no individual tocopherol isomer could be completely stripped from oil samples. Alpha-tocopherol and γ-tocopherol were present in all tree nut oils, with α-tocopherol predominating in almond, hazelnut and pistachio, while γ-tocopherol predominating in pecan, pine nut and walnut oils as well as hexane extracted Brazil nut oil. Delta-tocopherol was the predominant tocol isomer in chloroform/methanol extracted Brazil nut oil, and was also present in pine nut, pistachio and walnut oils at much lower amounts (5 to 26 mg/kg oil). Beta-tocopherol was detected only in chloroform/methanol extracted almond and pine nut oils at trace amounts (< 33 mg/kg oil). Alpha-tocopherol is the predominant tocol in almond oil (390-439 mg/kg) and hazelnut oil (382 to 472mg/kg); both are reported to contain smaller amounts of γ-tocopherol (12.5 and 61.2 mg/kg, respectively) [1, 134]. The most predominant tocopherol in pecan oil is γ-tocopherol (176 mg/kg), followed by α-tocopherol (10 mg/kg), and then δ- and β-tocopherols (6.2 mg/kg) [1]. Pistachio oil has been reported to contain 270 mg/kg of tocopherols (primarily α-tocopherol) [1]. Walnut oil has been reported to contain between 268 and 436 mg/kg of tocopherols. The predominant tocol isomer is γ-tocopherol (>90%), followed by α-tocopherol (6%), and then β- and δ-tocopherols [181]. The tocopherol compositions reported here are in agreement with those reported previously [123].

4.6. Lipid Compositions of Tree Nut Oil Extracts

The lipid class compositions of tree nut oil minor component extracts, analysed using TLC-FID, revealed the presence of sterols, phosphatidylserine, phosphatidylinositol, phosphatidylcholine and sphingolipids. Tocopherol compositions were analysed using reversed-phase HPLC. Lipid classes and tocopherols were expressed as parts per million of the final methanolic extract described in section 3.4.1 (ppm; mg/kg oil equivalent). The minor component extract of chloroform/methanol extracted walnut oil contained the highest total tocopherol content (403 mg/kg oil equivalent), followed by chloroform/methanol extracted hazelnut oil (362 mg/kg oil equivalent) (Table 4.7). The minor component extract of hexane extracted Brazil nut oil contained the lowest total tocopherol content (135 mg/kg oil equivalent). The relative percentages of individual tocopherols in tree nut oil extracts did not significantly differ ($p > 0.05$) from the relative percentages in their non-stripped counterparts.

The minor component extracts of chloroform/methanol extracted tree nut oils contained higher amounts of phospholipids and sphingolipids than their hexane extracted counterparts. The minor component extract of chloroform/methanol extracted pistachio oil possessed the highest amount of phospholipids and sphingolipids among all samples tested (10.3 g/kg oil equivalent), followed by chloroform/methanol extracted walnut oil (9.3 g/kg oil equivalent) and then chloroform/methanol extracted pecan oil (8.6 g/kg oil equivalent). The minor component extract of hexane extracted hazelnut oil possessed the lowest amount of phospholipids and sphingolipids (2.6 g/kg oil equivalent). Phosphatidic acid was only detected in the extracts of hazelnut oil.

Table 4.7. Lipid Class Contents (mg/kg oil equivalent) and Compositions of Tree Nut Oil Extracts[1,2]

Lipid Class	Sterols[3]	Phosphatidylserine[3]	Phosphatidylinositol[3]	Phosphatidylcholine[3]	Phosphatidic Acid[3]	Sphingolipids[3]	Tocopherols[4]
A-H	800 ± 152^{a}	500 ± 99^{c}	700 ± 73^{c}	800 ± 92^{d}	ND	2100 ± 142^{a}	154.6± 0.4^{k}
A-BD	900 ± 108^{a}	1100 ± 137^{b}	600 ± 52^{c}	2000 ± 149^{c}	ND	2400 ± 166^{a}	240.4± 0.3^{j}
BN-H	900 ± 99^{a}	1200 ± 186^{b}	200 ± 35^{f}	1000 ± 107^{d}	ND	3200 ± 185^{a}	135.1± 0.8^{l}
BN-BD	800 ± 125^{a}	1200 ± 205^{b}	200 ± 48^{f}	3900 ± 237^{a}	ND	2900 ± 182^{a}	283.4± 0.2^{h}
HN-H	1100 ± 184^{a}	1100 ± 163^{b}	300 ± 37^{e}	1100 ± 175^{d}	200 ± 39^{b}	100 ± 26^{b}	340.7± 0.7^{d}
HN-BD	1100 ± 193^{a}	1300 ± 138^{b}	300 ± 28^{e}	2100 ± 226^{c}	500 ± 63^{a}	200 ± 46^{b}	362.3± 0.4^{b}
P-H	1200 ± 157^{a}	1500 ± 263^{b}	400 ± 34^{d}	1000 ± 78^{d}	ND	2100 ± 238^{a}	330.8± 0.6^{f}
P-BD	1600 ± 179^{a}	2400 ± 206^{a}	700 ± 75^{c}	2300 ± 287^{c}	ND	3200 ± 280^{a}	351.0± 0.3^{d}
PN-H	500 ± 68^{a}	1000 ± 149^{b}	600 ± 47^{c}	1000 ± 95^{d}	ND	2700 ± 261^{a}	291.7± 1.2^{g}
PN-BD	700 ± 128^{a}	1500 ± 242^{b}	700 ± 19^{c}	1400 ± 125^{d}	ND	2800 ± 235^{a}	336.7± 0.6^{e}
PO-H	1100 ± 142^{a}	2100 ± 172^{a}	900 ± 64^{b}	2400 ± 277^{c}	ND	2900 ± 263^{a}	239.8± 0.8^{j}
PO-BD	1100 ± 123^{a}	2800 ± 238^{a}	1200 ± 36^{a}	3000 ± 284^{b}	ND	3300 ± 213^{a}	279.7± 0.7^{i}
W-H	1100 ± 118^{a}	1700 ± 127^{b}	1200 ± 149^{a}	1200 ± 115^{d}	ND	2500 ± 173^{a}	357.8± 0.4^{c}
W-BD	1500 ± 134^{a}	2700 ± 236^{a}	1300 ± 233^{a}	2400 ± 203^{c}	ND	2900 ± 218^{a}	403.1± 0.7^{a}

[1] Abbreviations used: A-H, almond oil – hexane extract; A-CM, almond oil - chloroform-methanol extract; BN-H, Brazil nut oil – hexane extract; BN-CM, Brazil nut oil – chloroform-methanol extract; HN-H, hazelnut oil – hexane extract; HN-CM, hazelnut oil – chloroform-methanol extract; P-H, pecan oil – Hexane extract; P-CM, pecan oil – chloroform-methanol extract; PN-H, pine nut oil – hexane extract; PN-CM, pine nut oil – chloroform-methanol extract; PO-H, pistachio oil – hexane extract; PO-CM, pistachio oil – chloroform-methanol extract; W-H, walnut oil – hexane extract; W-CM, walnut oil - chloroform-methanol extract; ND, not detected

[2] Values in the same column with different superscripts are significantly ($p \leq 0.05$) different. Experiments performed in triplicate.

[3] Analysed using TLC-FID.

[4] Analysed using HPLC.

4.7. Total Phenolics Contents of Tree Nut Oil Extracts

The TPC of tree nut oil extracts, determined by the method of Singleton and Rossi [191], were expressed as equivalents of gallic acid / kg oil equivalent (Figure 4.1, Table 4.8) and as equivalents of α-tocopherol / kg oil equivalent (Figure 4.2, Table 4.8). These two reference standards were because of their different solubility characteristics; gallic acid is a water soluble phenolic while α-tocopherol is a lipid soluble phenolic. Minor component extracts of chloroform/methanol extracted oils had higher TPC than their hexane extracted counterparts, expressed as gallic acid equivalents or α-tocopherol equivalents. This strongly suggests that chloroform/methanol was more effective than hexane for extraction of phenolic compounds. Since several antioxidative phenolic compounds occur in tree nuts [123], inclusion of these compounds in tree nut oils is expected to enhance their antioxidant activity as well as their content of minor components.

Among the oil extracts studied, chloroform/methanol extracted pecan oil had the highest TPC (711 mg/kg gallic acid equivalents or 783 mg/kg α-tocopherol equivalents) followed by chloroform/methanol extracted walnut oil (689 mg/kg gallic acid equivalents or 759 mg/kg α-tocopherol equivalents) and then chloroform/methanol extracted Brazil nut oil (381 mg/kg gallic acid equivalents or 429 mg/kg α-tocopherol equivalents). Hexane extracted almond oil extracts had the lowest TPC (40 mg/kg gallic acid equivalents or 124 mg/kg α-tocopherol equivalents). The α-tocopherol equivalence values of chloroform/methanol extracted pecan and walnut oil extracts were almost two-fold greater than their total tocopherols contents as determined using HPLC, which implies that phenolic compounds other than tocopherols that are present in these extracts enhance their

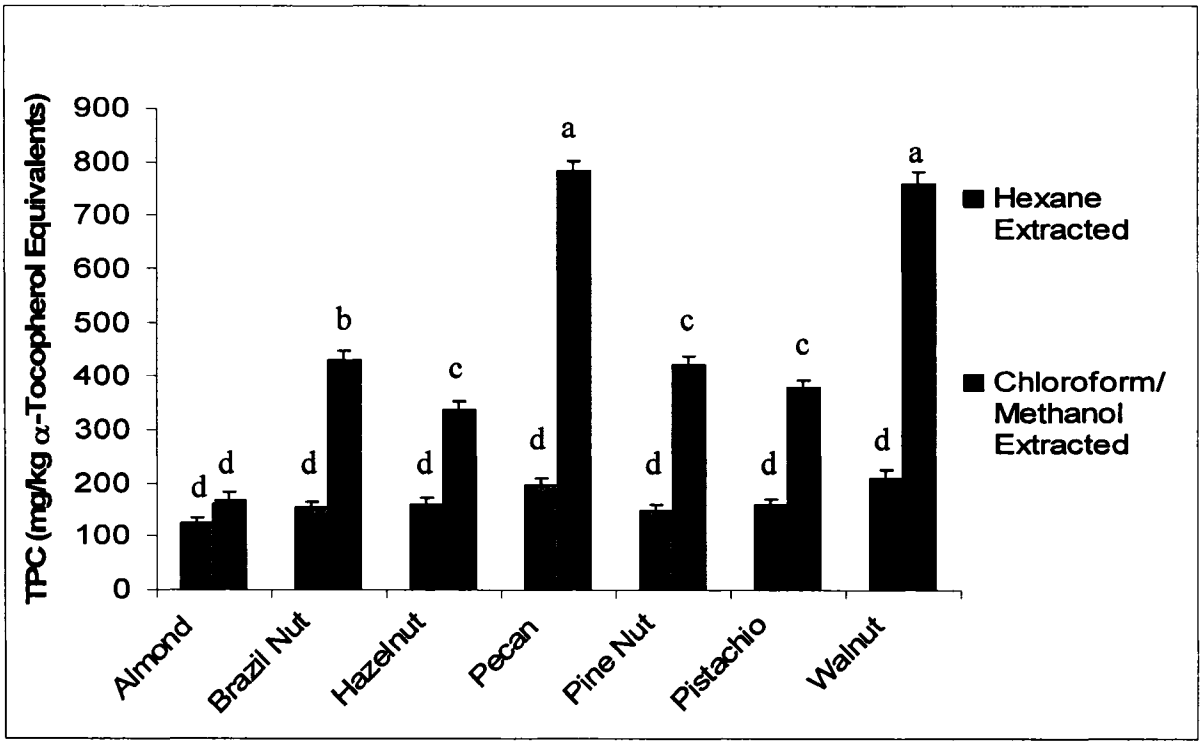


Figure 4.1. Total Phenolics Contents (TPC) of Tree Nut Oil Extracts as Gallic Acid Equivalents[1]

[1] Values with different alphabetical identifiers are significantly ($p \leq 0.05$) different.

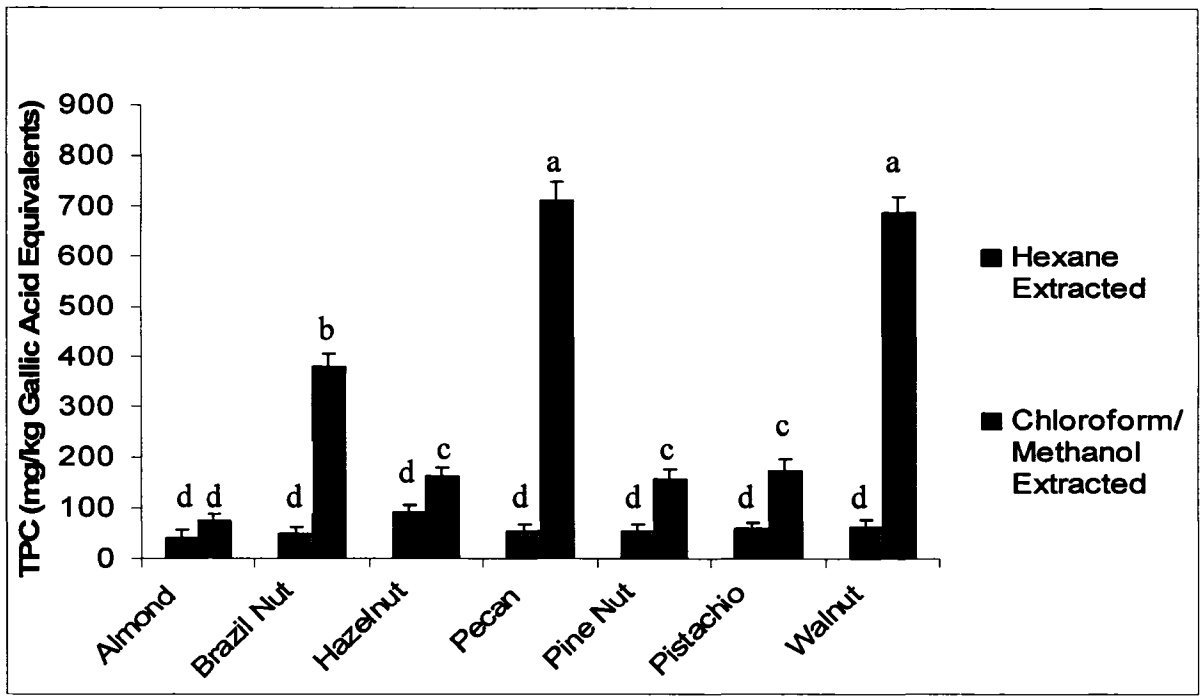


Figure 4.2. Total Phenolics Contents (TPC) of Tree Nut Oil Extracts as α-Tocopherol Equivalents[1]

[1] Values with different alphabetical identifiers are significantly ($p \leq 0.05$) different.

Table 4.8. Total Phenolics Contents (mg/kg) of Extracts from Tree Nut Oils

Nut	Hexane Extracted	Chloroform/Methanol Extracted
Gallic Acid Equivalents[1]		
Almond	124 ± 11^{d}	168 ± 15^{d}
Brazil Nut	153 ± 12^{d}	429 ± 19^{b}
Hazelnut	159 ± 13^{d}	338 ± 14^{c}
Pecan	196 ± 15^{d}	783 ± 18^{a}
Pine Nut	148 ± 12^{d}	423 ± 16^{c}
Pistachio	158 ± 12^{d}	379 ± 15^{c}
Walnut	210 ± 16^{d}	759 ± 22^{a}
α-Tocopherol Equivalents[1]		
Almond	40 ± 5^{d}	73 ± 7^{d}
Brazil Nut	48 ± 4^{d}	381 ± 11^{b}
Hazelnut	91 ± 12^{d}	163 ± 17^{c}
Pecan	54 ± 8^{d}	711 ± 32^{a}
Pine Nut	53 ± 11^{d}	157 ± 15^{c}
Pistachio	59 ± 8^{d}	173 ± 18^{c}
Walnut	63 ± 7^{d}	689 ± 23^{a}

[1] Values with different superscripts are significantly ($p \leq 0.05$) different.

antioxidant activities. No obvious correlations could be observed between α-tocopherol equivalence values (TPC) and total tocopherol contents (HPLC), with 8 out of 14 extracts having lower α-tocopherol equivalence values than total tocopherol contents (HPLC). The difference between α-tocopherol equivalence values and total tocopherol contents did not exceed two fold for any sample. These differences could be due to the presence of non-tocopherol phenolics or other compounds in some samples that reacted with the Folin and Ciocalteu's reagent, and thus increased their TPC beyond what would be expected from their total tocopherol contents alone.

There are no previous reports on TPC of tree nut oils. The total phenolics contents of minor component extracts of black cumin (Nigella *sativa* L.) oil, coriander (*Coriandrum sativum* L.) oil, and Niger (*Guizotia abyssinica* Cass.) oil have been reported as 24, 11 and 5 mg caffeic acid equivalents per kg oil equivalent [190]. The TPC of olive oil has been studied by a number of research groups who have reported values ranging from 11 to 76 mg syringic acid per kg oil equivalents [203]. However, in both of these reports the TPC were considerably lower than the total tocopherol contents of the oils studied, implying that the TPC assay employed in these studies did not adequately assess the total amounts of phenolic compounds present in the oil samples.

4.8. Trolox Equivalent Antioxidant Capacity of Tree Nut Oil Extracts

The Trolox equivalent antioxidant capacity measures the ability of antioxidants to scavenge the 2.5 mM 2,2'-azo-bis (2-methylpropionamidine) dihydrochloride (AAPH$^{\cdot}$) / 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonate) (ABTS$^{\cdot}$) radical pair; the antioxidant activities are expressed as equivalents of

Trolox, a water soluble vitamin E analogue. The TEAC value is defined as the molar concentration of Trolox solution having the antioxidant capacity equivalent to the sample solution being tested. Trolox equivalent antioxidant capacity values of tree nut oil extracts were calculated using a Trolox standard curve and were expressed as μM Trolox equivalents / g oil equivalent (μM Trolox / g oil). Results showed that minor component extracts of chloroform/methanol extracted nut oils possessed greater TEAC values compared to their hexane extracted counterparts (Figure 4.3, Table 4.9). All minor component extracts exhibited antioxidant activity; chloroform/methanol extracted pecan oil had the greatest TEAC value (2047 μM Trolox / g oil), followed by chloroform/methanol extracted Brazil nut oil (1217 μM Trolox / g oil), and then chloroform/methanol extracted walnut oil (959 μM Trolox / g oil) ($p \leq 0.05$). The minor component extracts of hexane extracted almond oil and hexane extracted Brazil nut oil had the lowest TEAC values (68 and 82 μM Trolox / g oil, respectively). The high TEAC value of chloroform/methanol extracted pecan oil may stem from its tocopherol composition that is very rich in γ-tocopherol. However, the presence of other antioxidative components acting alone or synergistically with γ-tocopherol is likely involved since the TEAC value of chloroform-methanol extracted pecan oil greatly exceeds that of its hexane extracted counterpart.

These results indicate that the chloroform/methanol extraction system affords oils with higher amounts of antioxidative components, which are expected to improve oil stability and potentially exert beneficial health effects.

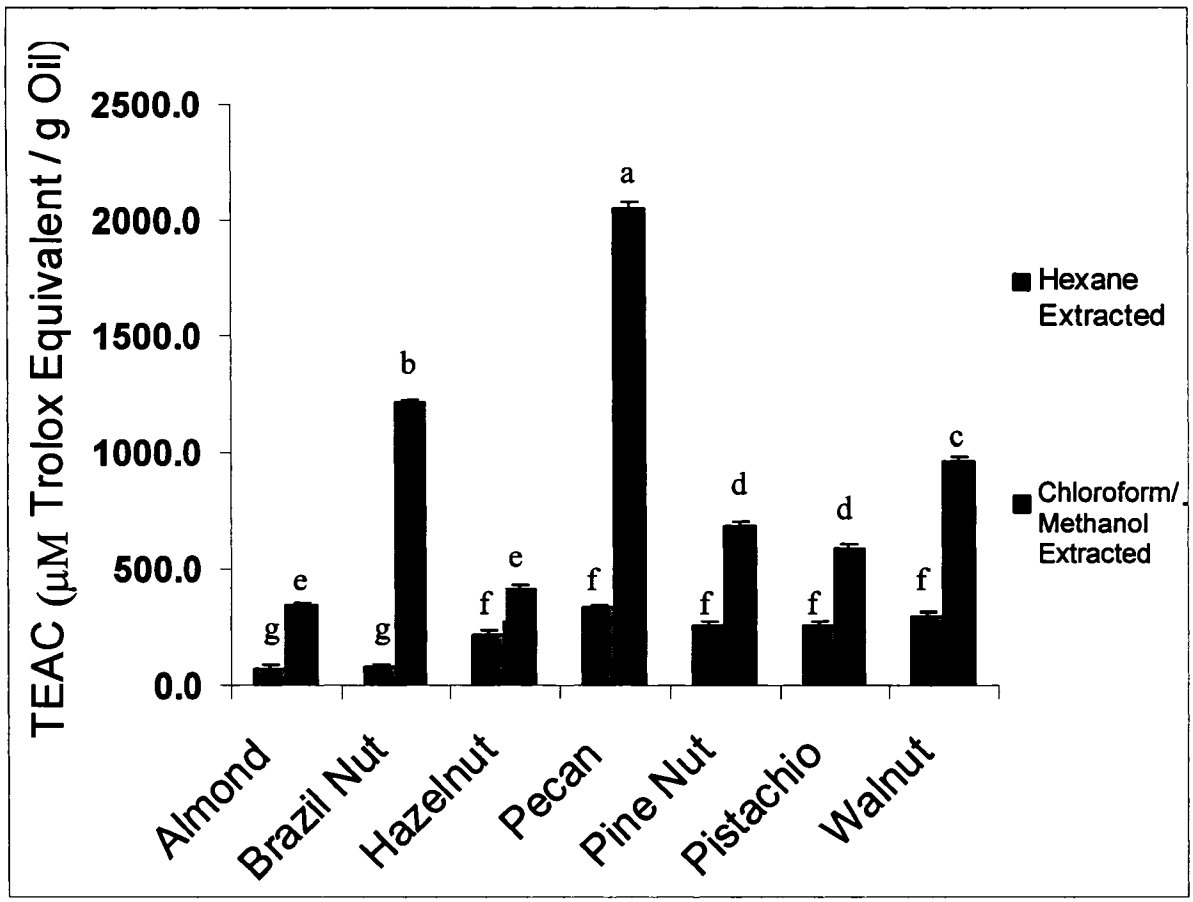


Figure 4.3. Trolox Equivalent Antioxidant Capacity (TEAC) of Tree Nut Oil Extracts[1]

[1] Values with different alphabetical identifiers are significantly ($p \leq 0.05$) different.

Table 4.9. Trolox Equivalent Antioxidant Capacity (μM Trolox Equivalents / g oil) of Tree Nut Oil Extracts[1]

Nut	Hexane Extracted	Chloroform-Methanol Extracted
Almond	67.6 ± 19.4^{g}	345.3 ± 11.6^{e}
Brazil Nut	81.8 ± 9.8^{g}	1216.9 ± 12.6^{b}
Hazelnut	216.9 ± 22.6^{f}	412.8 ± 15.4^{e}
Pecan	329.1 ± 12.5^{f}	2047.3 ± 27.6^{a}
Pine Nut	254.7 ± 18.6^{f}	689.9 ± 17.5^{d}
Pistachio	258.7 ± 13.7^{f}	585.9 ± 18.7^{d}
Walnut	298.0 ± 16.2^{f}	958.9 ± 20.7^{c}

[1] Values with different superscripts are significantly ($p \leq 0.05$) different.

4.9. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Capacity of Tree Nut Oil Extracts

The DPPH radical scavenging capacity assay was used to examine the antioxidant activity of tree nut oil extracts. Tree nut oil extracts were assayed over a range of dilutions to establish the concentration of each extract required to scavenge 50% of the DPPH radical present in the assay medium, referred to as the IC_{50}. Under the assay conditions employed here, the IC_{50} of pure α-tocopherol was 23.6 μg. The minor component extracts of chloroform/methanol extracted oils possessed greater DPPH radical scavenging activity than their hexane extracted counterparts. Among the chloroform/methanol extracted oils, pecan oil had the greatest DPPH radical scavenging capacity with an IC_{50} of 0.03 g oil equivalent which equates to 787μg α-tocopherol equivalents per gram oil equivalent, followed by walnut and Brazil nut oils (337 and 295 α-tocopherol equivalents per gram oil equivalent, respectively), then pistachio and pine nut oils (215 and 182 α-tocopherol equivalents per gram oil equivalent, respectively), followed by hazelnut oil (112 α-tocopherol equivalents per gram oil equivalent) and finally, almond oil (62 α-tocopherol equivalents per gram oil equivalent) ($p \leq 0.05$) (Figure 4.4, Table 4.10). Among the hexane extracted oils, pecan and walnut oils exhibited the greatest DPPH scavenging activity (107 and 98 α-tocopherol equivalents per gram oil equivalent, respectively), followed by pistachio, hazelnut and pine nut oils (87, 84 and 76 α-tocopherol equivalents per gram oil equivalent, respectively), then Brazil nut oil (66 α-tocopherol equivalents per gram oil equivalent), and finally almond oil (46 α-tocopherol equivalents per gram oil equivalent) ($p \leq 0.05$).

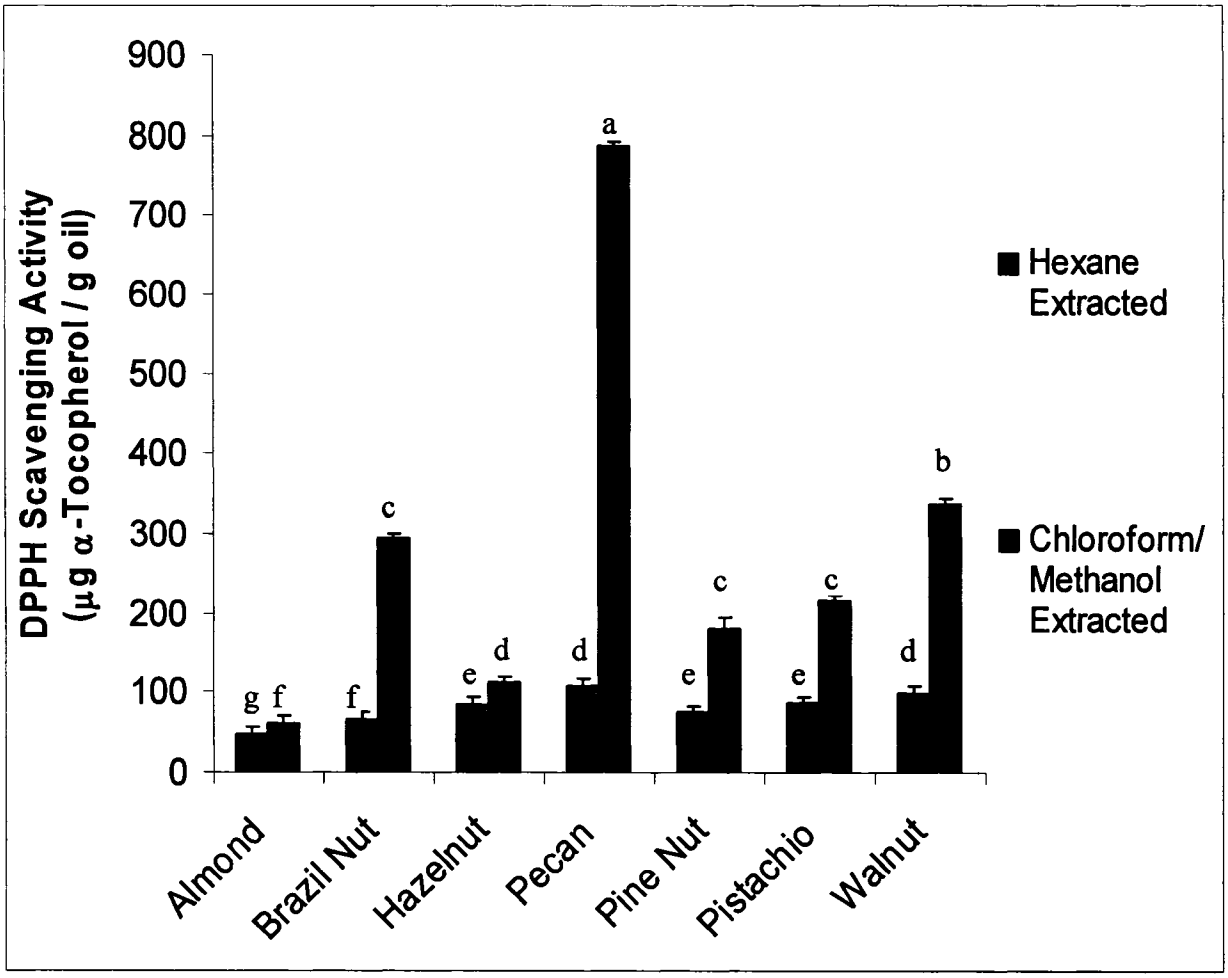


Figure 4.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Capacity of Tree Nut Oil Extracts[1]

[1] Values with different alphabetical identifiers are significantly ($p \leq 0.05$) different.

Table 4.10. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Capacity (µg α-Tocopherol / g Oil) of Tree Nut Oil Extracts[1]

Nut	Hexane Extracted	Chloroform/Methanol Extracted
Almond	46.3 ± 7.4^{g}	62.1 ± 9.9^{f}
Brazil Nut	65.5 ± 10.5^{f}	295.0 ± 23.6^{c}
Hazelnut	84.3 ± 13.5^{e}	112.4 ± 18.0^{d}
Pecan	107.3 ± 17.2^{d}	786.7 ± 31.5^{a}
Pine Nut	76.1 ± 12.2^{e}	181.6 ± 29.0^{c}
Pistachio	87.4 ± 14.0^{e}	214.5 ± 34.3^{c}
Walnut	98.3 ± 15.7^{d}	337.1 ± 27.0^{b}

[1] Values bearing different superscripts are significantly ($p \leq 0.05$) different.

The relatively strong antioxidant activities of pecan and walnut oil extracts may be due to their high tocopherol content that is rich in γ-tocopherol. However, the α-tocopherol equivalence values of tree nut oil extracts obtained using the DPPH radical scavenging assay do not strictly correlate with their actual tocopherol contents since the chloroform/methanol extracts exhibited significantly higher antioxidant activities than their hexane extracted counterparts, although both contained similar tocopherol contents. This indicates that non-tocopherol components present in the chloroform/methanol extracts also contributed to the total antioxidant capacity of the extracts. Besides tocopherols, other antioxidative minor components of tree nut oils include phospholipids [204], phytosterols and phytosterol conjugates [205], and possibly non-tocopherol phenolics, among others [206]. Synergistic antioxidant activities have been reported between tocopherols and nitrogen-containing phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine [207], all of which are present in tree nut oils and their minor component extracts and may help explain the high antioxidant activities observed in this report, particularly of the chloroform/methanol extracted oil extracts.

The DPPH radical scavenging capacity of tree nut oil extracts have not previously been reported. The DPPH radical scavenging activity of black cumin (Nigella *sativa* L.), coriander (Coriandrum *sativum* L.), and Niger (Guizotia *abyssinica* Cass.) oil extracts have been studied, showing that coriander oil exhibited the greatest DPPH radical scavenging activity, followed by black cumin oil and then Niger oil [190]. The antioxidant activities were attributed to both phenolic and non-phenolic compounds such as phospholipids present in the minor component extracts.

4.10. Inhibition of β-Carotene Bleaching of Tree Nut Oil Extracts

The β-carotene bleaching test is a convenient test used to measure the ability of a compound or a mixture to inhibit the oxidation of β-carotene. In this assay, β-carotene is subjected to decolourization due to oxidation by free radicals formed from polyunsaturated fatty acids added exogenously to the assay medium. Thus, antioxidant activity in this assay is related to the ability of compounds to inhibit the initiation and/or propagation steps in oxidation of linoleic acid in aqueous media. The retention of β-carotene over 120 min of assay was used to evaluate the antioxidant activities of tree nut oil extracts. The control assay devoid of any antioxidant lost 99% of its initial β-carotene after 120 min of assay. Among nut oil extracts tested, extracts of chloroform/methanol extracted oils possessed higher antioxidant activities when compared to the extracts of their hexane extracted counterparts (Figure 4.5 and Figure 4.6). Results showed that the extract of chloroform/methanol extracted pecan oil exhibited the highest antioxidant activity, with 79% of β-carotene remaining after 120 min of assay (Table 4.8). The chloroform/methanol extracted walnut oil possessed the second highest activity (70% β-carotene remaining after 120 min assay), followed by chloroform/methanol extracted Brazil nut oil (63% β-carotene remaining after 120 min assay). Hexane extracted almond oil exhibited the lowest antioxidant activity (7% β-carotene remaining after 120 min assay).

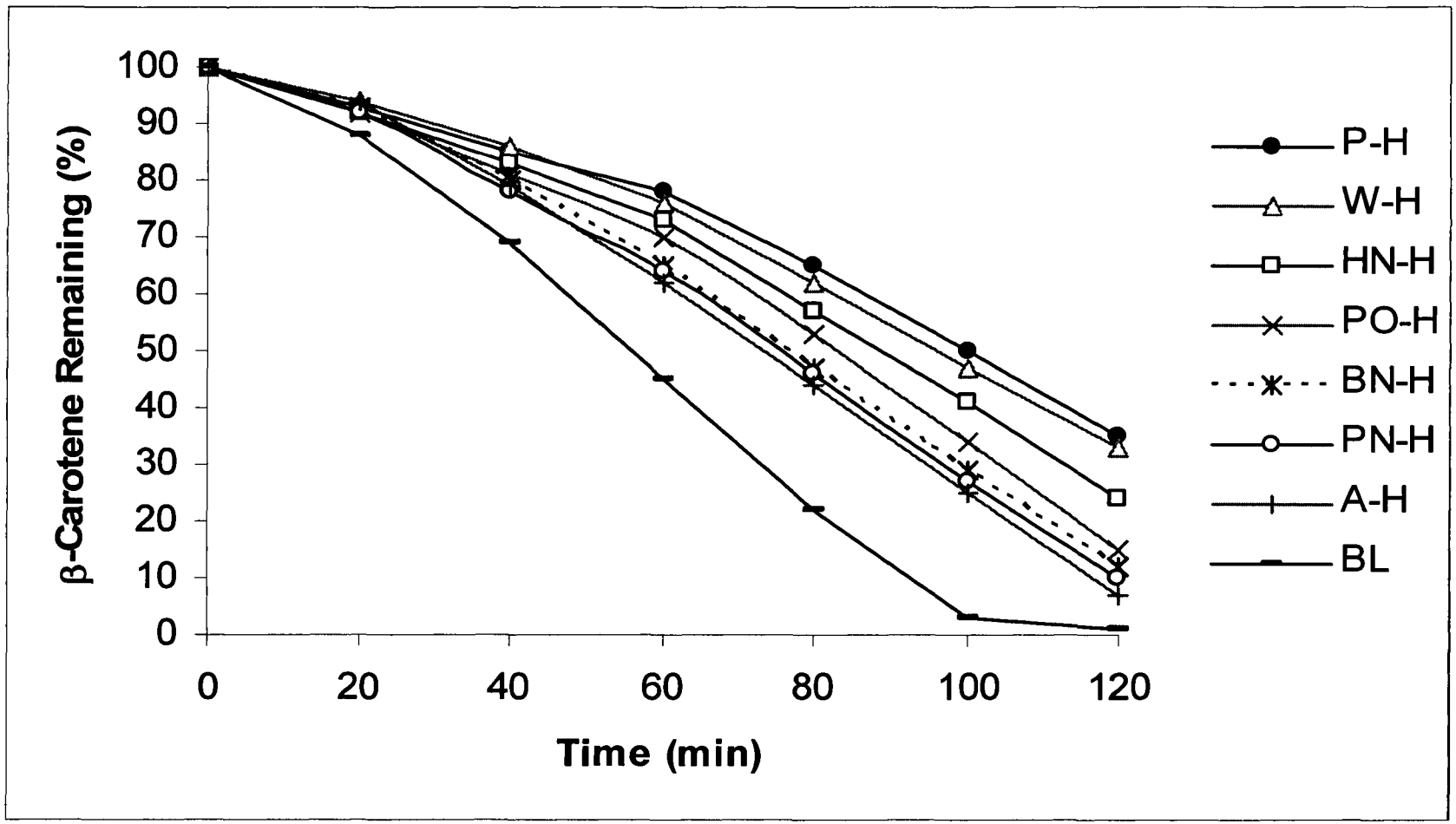


Figure 4.5. Effect of Hexane Extracted Tree Nut Oil Extracts on the Retention of β-Carotene over a 120 min β-Carotene Bleaching Test[1]

[1] Symbol widths represent standard deviations for each data point.

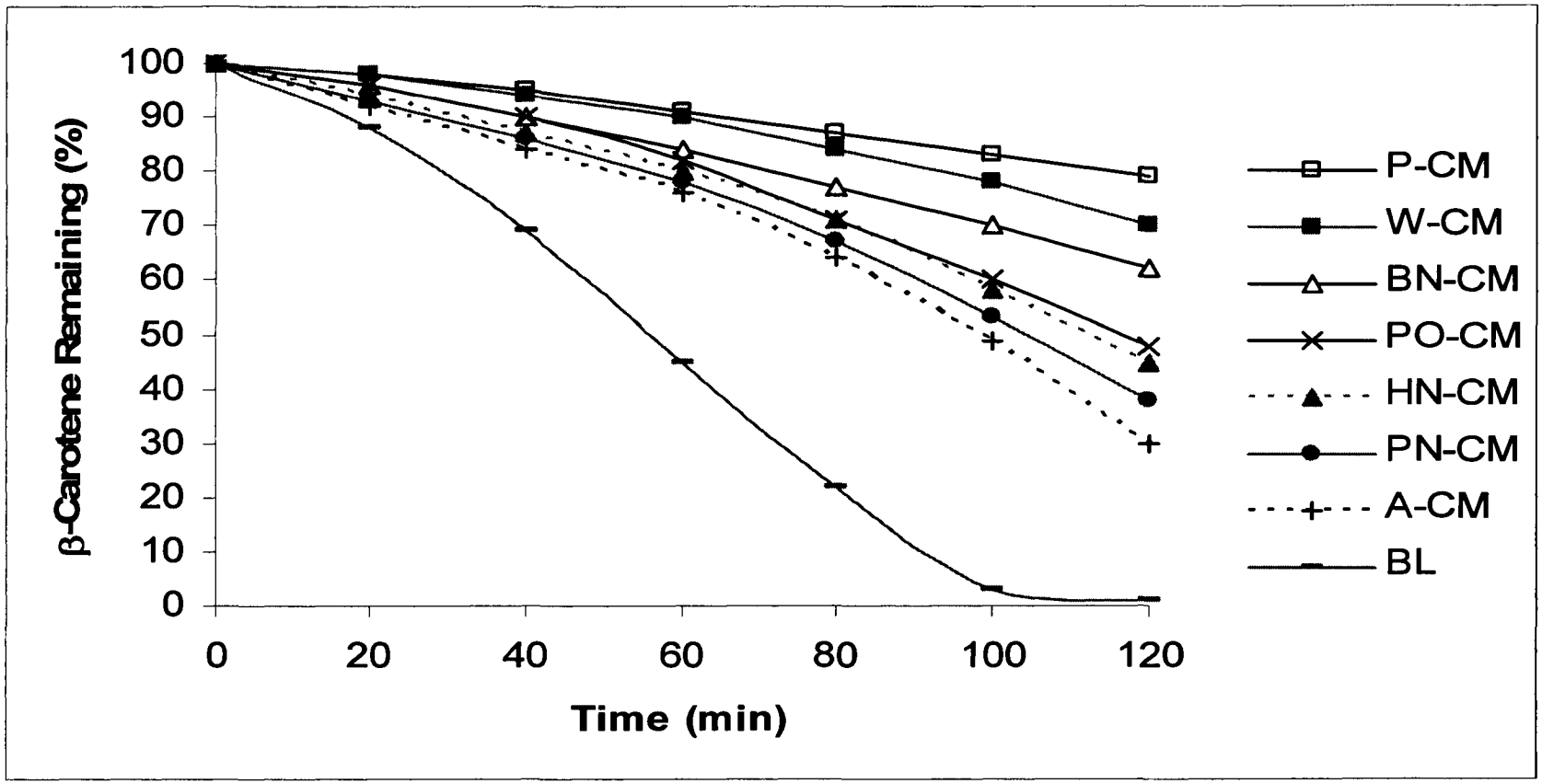


Figure 4.6. Effect of Chloroform-methanol Extracted Tree Nut Oil Extracts on the Retention of β-Carotene over a 120 min β-Carotene Bleaching Test[1]

[1] Symbol widths represent standard deviations for each data point.

Table 4.11. β-Carotene Retention (%) after 120 min β-Carotene Bleaching Test with Tree Nut Oil Extracts[1]

Sample	Hexane Extract (%)	Chloroform-methanol Extract (%)
Almond	7.4 ± 0.1[l]	30.1 ± 0.9[h]
Brazil Nut	12.3 ± 0.5[j]	62.5 ± 1.1[c]
Hazelnut	24.2 ± 0.4[i]	45.2 ± 1.3[e]
Pecan	35.6 ± 0.8[g]	79.2 ± 1.2[a]
Pine Nut	10.2 ± 0.2[k]	38.6 ± 0.8[f]
Pistachio	15.0 ± 0.4[i]	48.5 ± 0.7[d]
Walnut	33.6 ± 0.8[g]	70.0 ± 1.4[b]

[1] Values with different superscripts are significantly ($p \leq 0.05$) different.

The β-carotene bleaching test is similar to an oil-in-water emulsion system; differences in the solubilities of antioxidant compounds influence their activity in this assay. Hydrophobic antioxidants are reported to perform more efficiently than hydrophilic antioxidants in the β-carotene bleaching test by orienting themselves in the lipid phase and the lipid-water interface, thus directly combating lipid radical formation and β-carotene oxidation [208]. The strong activity of chloroform/methanol extracted oil minor components may be due to their higher level of hydrophobic antioxidants such as tocopherols and phospholipids.

4.11. Oxygen Radical Absorbance Capacity of Tree Nut Oil Extracts

The ORAC assay is a free radical scavenging assay; it is based on the time resolved fluorescence of an oxidizable compound, namely fluorescein. In the presence of antioxidants, the oxidation and subsequent loss of fluorescence by fluorescein is inhibited and the extent of this inhibition is directly related to antioxidant activity and/or antioxidant concentration, usually expressed in Trolox or α-tocopherol equivalents. The ORAC values of samples are derived by calculating

the area-under-curve (AUC) of the time resolved fluorescence graph for the assay containing the sample, which is then used along with a standard curve to calculate the ORAC value as equivalents of a pure antioxidant. Results showed that extracts of chloroform/methanol extracted oils possessed higher ORAC values than their hexane extracted counterparts (Figure 4.8, Table 4.11) ($p \leq 0.05$). Among chloroform/methanol extracted oils, pecan oil extract possessed the highest ORAC value (4.04 μmol α-tocopherol equivalents / g oil equivalent), followed by walnut oil (3.41 μmol α-tocopherol equivalents / g oil equivalent), hazelnut oil (2.93 μmol α-tocopherol equivalents / g oil equivalent), pistachio oil (2.24 μmol α-tocopherol equivalents / g oil equivalent), almond oil (2.12 μmol α-tocopherol equivalents / g oil equivalent), and finally, pine nut oil and Brazil nut oil (1.99 and 1.96 μmol α-tocopherol equivalents / g oil equivalent, respectively) ($p \leq 0.05$). Among the hexane extracted oils, walnut oil, hazelnut oil, pine nut oil and Brazil nut oil had similar ORAC values (1.53, 1.48, 1.39 and 1.34 μmol α-tocopherol equivalents / g oil equivalent, respectively), followed by pistachio oil and almond oil (1.08 and 0.97 μmol α-tocopherol equivalents / g oil equivalent, respectively) and finally, pecan oil (0.75 μmol α-tocopherol equivalents / g oil equivalent) ($p \leq 0.05$).

The ORAC of hexane/dichloromethane (1:1, v/v) extracts of tree nuts have previously been reported by Wu et al. [116], showing that lipidic extract of Brazil nut possessed the highest antioxidant activity (5.6 μmol of Trolox equivalents / g), followed by walnut (4.8 μmol of Trolox equivalents / g), then cashew (4.7 μmol of Trolox equivalents / g), pecan (4.2 μmol of Trolox equivalents / g), hazelnut (3.7 μmol of Trolox equivalents / g), pine nut (2.8 μmol of Trolox equivalents / g), and

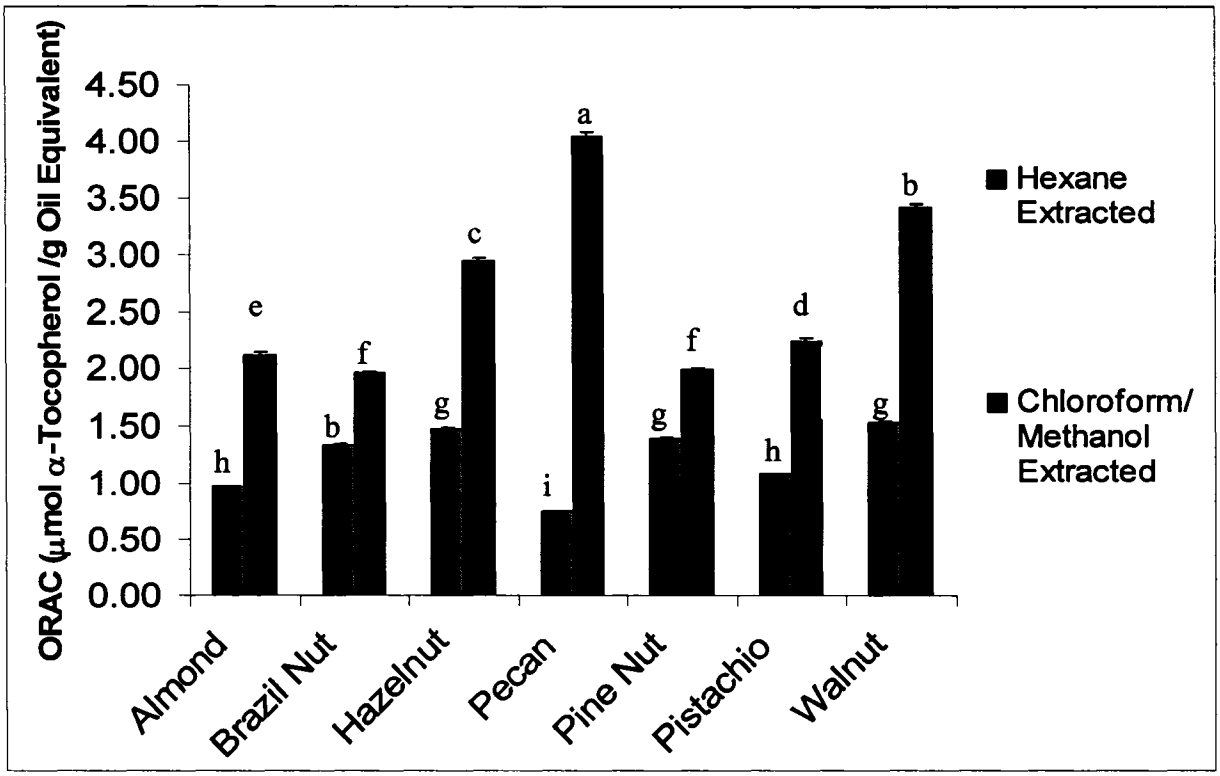


Figure 4.7. Oxygen Radical Absorbance Capacity (ORAC) of Tree Nut Oil Extracts[1]

[1] Values with different alphabetical identifiers are significantly ($p \leq 0.05$) different.

Table 4.12. Oxygen Radical Absorbance Capacity (µmol α-Tocopherol Equivalents/ g Oil Equivalent) of Tree Nut Oil Extracts[1]

Nut	Hexane Extracted	Chloroform/Methanol Extracted
Almond	0.97 ± 0.03^{h}	2.12 ± 0.05^{e}
Brazil Nut	1.34 ± 0.05^{b}	1.96 ± 0.04^{f}
Hazelnut	1.48 ± 0.04^{g}	2.93 ± 0.09^{c}
Pecan	0.75 ± 0.07^{i}	4.04 ± 0.08^{a}
Pine Nut	1.39 ± 0.02^{g}	1.99 ± 0.04^{f}
Pistachio	1.08 ± 0.07^{h}	2.24 ± 0.06^{d}
Walnut	1.53 ± 0.07^{g}	3.41 ± 0.03^{b}

[1] Values bearing different superscripts are significantly ($p \leq 0.05$) different.

finally, almond (1.7 μmol of Trolox equivalents / g) [116]. Since Wu et al. [116] used a different antioxidant extraction procedure and ORAC standard, therefore direct comparison of ORAC values obtained here with their results is impossible; however, when comparing the overall trends among samples some striking similarities exist. These include relatively high antioxidant activities for lipidic extracts of Brazil nuts, walnuts and pecans and lowest activities for almond extracts. The observed differences between this work and that of Wu et al. [116] may be attributable to cultivar for nut varieties studied and/or cultivation and climatic conditions.

4.12. Photochemiluminescence (PCL) Inhibition Assay for Evaluation of Antioxidant Activity of Tree Nut Oil Extracts

The PCL inhibition assay measures the superoxide scavenging capacity of tree nut oil extracts. In the early PCL inhibition methods, superoxide generation was mediated by the xanthine oxidase system [209]; however, problems associated with enzyme activity and method reproducibility led to the development of PCL inhibition methods using photogenerated superoxide [195]. In this work, an automated version of the PCL inhibition assay was used with luminol acting as both the photosensitizer and the superoxide radical detection agent.

Among oils tested, extracts of chloroform/methanol extracted oils possessed a higher antioxidant activity in the PCL inhibition assay compared to hexane extracted oils (Figure 4.8, Table 4.13). Among chloroform/methanol extracted oils, pecan oil extract exhibited the highest PCL inhibition activity (8.63 μmol α-tocopherol / g oil), followed by walnut oil extract (6.88 μmol α-tocopherol / g oil), pistachio oil extract (3.89 μmol α-tocopherol / g oil), pine nut oil extract (2.47 μmol

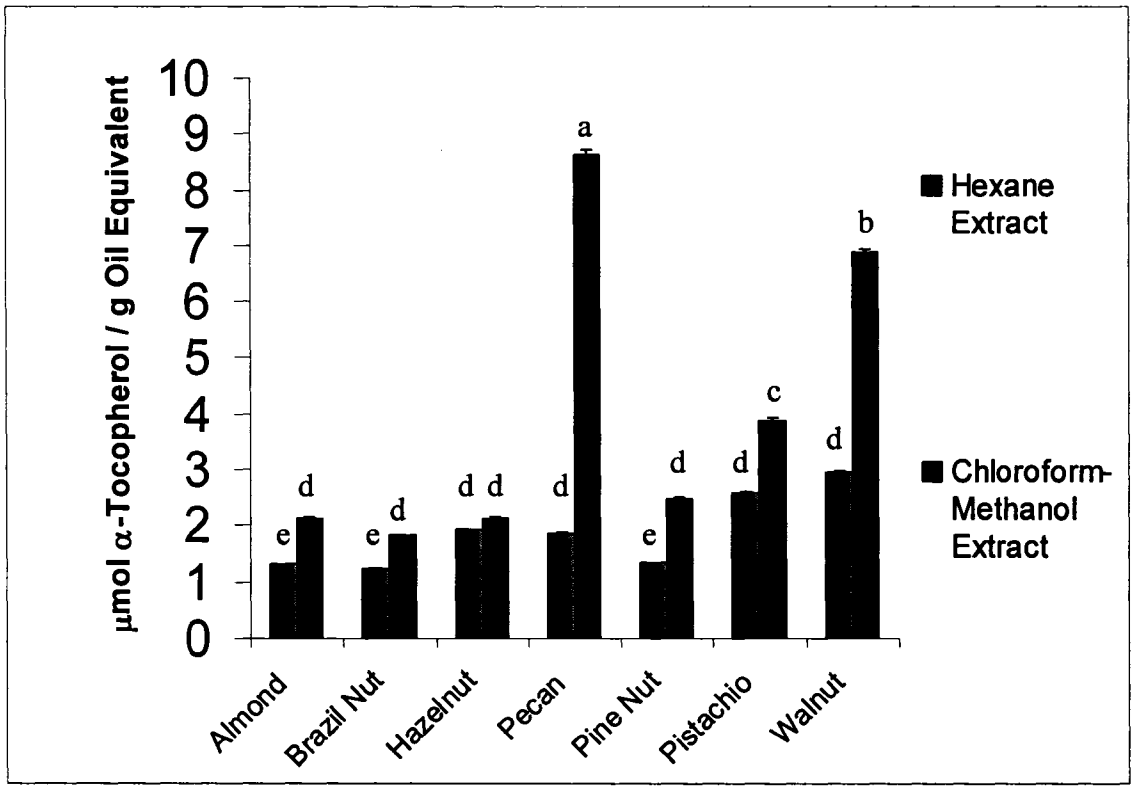


Figure 4.8. Photochemilumenescence (PCL) Inhibition Capacity of Tree Nut Oil Extracts[1]

[1] Values with different alphabetical identifiers are significantly ($p \leq 0.05$) different.

Table 4.13. Photochemilumenescence Inhibition Capacity (µmol α-Tocopherol / g Oil Equivalent) of Tree Nut Oil Extracts[1]

Nut	Hexane Extracted	Chloroform/Methanol Extracted
Almond	1.30 ± 0.14^{e}	2.11 ± 0.22^{d}
Brazil Nut	1.22 ± 0.18^{e}	1.81 ± 0.12^{d}
Hazelnut	1.92 ± 0.19^{d}	2.12 ± 0.11^{d}
Pecan	1.85 ± 0.17^{d}	8.63 ± 0.25^{a}
Pine Nut	1.33 ± 0.12^{e}	2.47 ± 0.20^{d}
Pistachio	2.57 ± 0.19^{d}	3.89 ± 0.21^{c}
Walnut	2.96 ± 0.26^{d}	6.88 ± 0.24^{b}

[1] Values bearing different superscripts are significantly ($p \leq 0.05$) different.

α-tocopherol / g oil), hazelnut and almond oil extracts (2.12 and 2.11 μmol α-tocopherol / g oil, respectively), and finally, Brazil nut oil extract (1.81 μmol α-tocopherol / g oil). Among hexane extracted oils, walnut oil extract had the highest PCL inhibition activity (2.96 μmol α-tocopherol / g oil), followed by pistachio oil extract (2.57 μmol α-tocopherol / g oil), then hazelnut oil extract (1.92 μmol α-tocopherol / g oil), pecan oil extract (1.85 μmol α-tocopherol / g oil), pine nut oil extract (1.33 μmol α-tocopherol / g oil), almond oil extract (1.30 μmol α-tocopherol / g oil), and finally, Brazil nut oil extract (1.23 μmol α-tocopherol / g oil).

The observed differences in PCL inhibition activity between the minor component extracts of chloroform/methanol extracted oils and their hexane extracted counterparts are likely due to compositional differences between the minor component extracts. Since chloroform/methanol extracted oils possessed higher amounts of antioxidative minor components such as tocopherols and phospholipids, their enhanced antioxidant activities can easily be attributed to this difference.

4.13. Oxidative Stability of Tree Nut Oils under Accelerated Autoxidation Conditions

The oxidative stability of non-stripped and stripped tree nut oils were examined using the Schaal oven method at 60 °C over 12 days [197]. The progress of oxidation was monitored using tests for conjugated dienes, peroxide value, *p*-anisidine value and headspace analysis of volatiles. Results showed that chloroform/methanol extracted oils were more resistant to oxidation than hexane

extracted oils. Minor component stripped oils were less stable than their non-stripped counterparts, which shows the importance of oil minor components on their oxidative stability. The stripping process was equally effective for both chloroform/methanol extracted and hexane extracted oils, since both stripped oil types exhibited similar low autoxidative stability. Among oils studied, chloroform/methanol extracted pecan oil showed the highest oxidative stability, with the lowest levels of conjugated dienes, peroxide value, *p*-anisidine value and headspace volatiles after 12 days of accelerated autoxidation. The relatively high resistance of chloroform/methanol extracted pecan oil to autoxidation is likely due to its minor component composition which is rich in tocopherols and phospholipids and in combination with its low degree of unsaturation, as indicated by its low iodine value. The effectiveness of chloroform/methanol extracted pecan oil minor components against lipid oxidation is apparent when comparing its oxidative stability to that of its hexane extracted counterpart, which contained identical fatty acid composition but lower amounts of minor components; however, hexane extracted pecan oil did exhibit the highest oxidative stability among the hexane extracted oils examined. Chloroform/methanol extracted pistachio oil also exhibited high oxidative stability, which was second only to chloroform/methanol extracted pecan oil. Pine nut oils and walnut oils exhibited the lowest autoxidative stabilities among samples examined, which was to be expected since these oils had the highest degree of unsaturation among tree nut oils examined. Interestingly, chloroform/methanol extracted pine nut and walnut oil exhibited higher stability than their hexane extracted counterparts, which further implies that the chloroform/methanol extraction system affords oils with higher amounts of

antioxidative minor components which in turn enhances the stability of the extracted oil. Future studies examining the effects of nut oil minor component extracts on oxidative stability of a single oil system will be of aid when studying their antioxidant activities, since this will allow for direct comparison of the antioxidant activities of the minor component extracts. Oils of almond and hazelnut exhibited intermediate stabilities. Chloroform/methanol extracted Brazil nut oil exhibited very high stability compared to its hexane extracted counterpart; however, hexane extracted Brazil nut oil exhibited similar stability to stripped hexane and chloroform/methanol extracted Brazil nut oil, which implies that the hexane solvent system did not effectively extract the antioxidative components from this nut.

4.13.1. Conjugated Dienes and Peroxide Values of Autoxidized Tree Nut Oils

Conjugated dienes and peroxides are both primary products of oxidation and persist during the early stages of lipid oxidation. Formations of conjugated dienes in tree nut oils during the 12 day autoxidation test are tabulated in Table 4.14. In all oils examined, chloroform/methanol extracted oils were more resistant to the formation of conjugated dienes than hexane extracted oils, and similarly, stripped chloroform/methanol extracted oils were more resistant to conjugated diene formation than stripped hexane extracted oils. Among samples examined, stripped hexane extracted almond oil possessed the highest initial conjugated dienes value (2.6), followed by stripped chloroform/ methanol extracted almond oil (2.3). Hexane extracted pistachio oil had the lowest initial conjugated dienes (0.5). Stripped chloroform/methanol extracted almond oil had lower conjugated diene levels than stripped hexane extracted almond oil after 12 days of autoxidation (15.3 and 15.9, respectively). The chloroform/methanol extract of Brazil nut oil showed

Table 4.14. Formation of Conjugated Dienes in Tree Nut Oils during Autoxidation at 60 °C [1,2]

	Storage Period (Days)					
	0	1	3	6	9	12
Almond Oil						
Hexane Extracted	0.956^{d}	1.095^{g}	1.578^{g}	1.969^{o}	4.796^{p}	6.451^{j}
Chloroform/Methanol Extracted	1.744^{b}	1.812^{e}	2.096^{f}	2.455^{n}	4.863^{p}	6.757^{j}
Stripped Hexane Extracted	2.636^{a}	2.929^{c}	3.519^{d}	9.475^{e}	15.289^{f}	15.914^{c}
Stripped Chloroform/Methanol Extracted	2.270^{b}	2.492^{d}	2.528^{f}	8.832^{f}	12.930^{i}	15.331^{d}
Brazil Nut Oil						
Hexane Extracted	1.768^{b}	2.455^{d}	3.049^{e}	4.163^{l}	7.741^{m}	15.767^{d}
Chloroform/Methanol Extracted	0.692^{e}	0.820^{h}	2.060^{f}	2.421^{n}	2.899^{r}	3.686^{l}
Stripped Hexane Extracted	1.301^{c}	1.793^{e}	2.131^{f}	8.725^{f}	14.783^{g}	15.341^{e}
Stripped Chloroform/Methanol Extracted	1.423^{c}	1.965^{e}	2.464^{f}	7.593^{g}	13.401^{h}	14.430^{f}
Hazelnut Oil						
Hexane Extracted	1.096^{d}	1.858^{e}	2.277^{f}	4.789^{k}	6.951^{n}	7.199^{j}
Chloroform/Methanol Extracted	1.492^{c}	2.422^{d}	2.883^{e}	4.491^{l}	5.789^{o}	6.131^{k}
Stripped Hexane Extracted	1.704^{b}	2.086^{e}	2.339^{f}	6.925^{i}	11.859^{j}	12.033^{g}
Stripped Chloroform/Methanol Extracted	1.467^{c}	2.241^{d}	2.757^{e}	6.374^{j}	10.857^{l}	11.092^{h}
Pecan Oil						
Hexane Extracted	0.502^{e}	0.547^{h}	0.849^{i}	1.305^{p}	1.920^{t}	2.821^{m}
Chloroform/Methanol Extracted	0.267^{f}	0.287^{i}	0.346^{j}	0.394^{q}	1.029^{v}	1.245^{o}
Stripped Hexane Extracted	0.945^{d}	1.183^{g}	1.429^{g}	2.398^{n}	5.829^{o}	6.719^{j}
Stripped Chloroform/Methanol Extracted	0.897^{d}	1.259^{g}	1.709^{g}	2.720^{m}	5.791^{o}	6.036^{k}
Pine Nut Oil						
Hexane Extracted	1.216^{c}	1.537^{f}	2.953^{e}	7.628^{g}	19.865^{e}	24.745^{b}
Chloroform/Methanol Extracted	1.067^{d}	1.058^{g}	1.623^{g}	2.315^{n}	5.829^{o}	7.920^{i}
Stripped Hexane Extracted	1.509^{c}	2.322^{d}	4.043^{c}	17.962^{d}	34.677^{b}	0.493^{p}
Stripped Chloroform/Methanol Extracted	1.524^{c}	2.008^{e}	3.099^{e}	18.472^{c}	37.351^{a}	5.825^{k}
Pistachio Oil						
Hexane Extracted	0.481^{e}	0.473^{i}	0.495^{j}	1.292^{p}	2.379^{s}	3.898^{l}
Chloroform/Methanol Extracted	0.992^{d}	1.002^{g}	1.029^{h}	1.237^{p}	1.401^{u}	1.689^{n}
Stripped Hexane Extracted	1.145^{c}	1.185^{g}	1.219^{h}	1.934^{o}	4.635^{p}	6.945^{j}
Stripped Chloroform/Methanol Extracted	0.936^{d}	1.047^{g}	1.138^{h}	1.789^{o}	4.253^{q}	6.673^{j}
Walnut Oil						
Hexane Extracted	0.554^{e}	0.681^{h}	4.161^{c}	7.247^{h}	13.153^{i}	29.998^{a}
Chloroform/Methanol Extracted	0.535^{e}	0.986^{g}	1.593^{g}	4.279^{l}	11.494^{k}	17.507^{c}
Stripped Hexane Extracted	0.684^{e}	4.834^{a}	13.623^{a}	41.262^{a}	27.953^{d}	5.965^{k}
Stripped Chloroform/Methanol Extracted	0.693^{e}	4.291^{b}	12.315^{b}	39.742^{b}	28.512^{c}	7.452^{j}

[1] Values in the same row bearing different superscripts are significantly ($p \leq 0.05$) different.
[2] Standard deviations did not exceed 0.100 for any data point (data not shown).

very low levels of conjugated dienes after 12 days of autoxidation (3.7), which is very interesting considering that this oil has a high degree of unsaturation compared to other oils examined, and also because its hexane extracted counterpart and stripped counterparts contained 4.5 to 5 times higher conjugated diene levels. Hexane and chloroform/methanol extracted hazelnut oils exhibited similar conjugated diene levels as almond oils after 12 days of autoxidation, however, the stripped hazelnut oils possessed lower conjugated dienes than the stripped almond oils which is likely due to the fact that almond oils have higher degrees of unsaturation than hazelnut oils, as indicated by the significantly ($p \leq 0.05$) higher iodine values of almond oils compared to hazelnut oils (Table 4.1). This implies that the antioxidative components in almond oil are more effective in reducing lipid oxidation than those present in hazelnut oil. Chloroform/methanol extracted pecan oil possessed the lowest level conjugated dienes among samples examined after 12 days of autoxidation (1.2), followed by chloroform/methanol extracted pistachio oil (1.7). Among hexane extracted oils, hexane extracted pecan oil had the lowest conjugated diene level after 12 days of autoxidation (2.8), followed by hexane extracted pistachio oil (3.9). Hexane extracted walnut oil contained the highest level of conjugated dienes after the 12 days of accelerated autoxidation (30.0), followed by hexane extracted pine nut oil (24.7); the chloroform/methanol extracted counterparts of these oils were considerably more stable, with conjugated diene levels of 17.5 and 7.9, respectively, but were still highest among all non-stripped oils examined. The stripped oils of walnuts possessed the highest conjugated diene levels among all samples examined,

followed by the stripped oils of pine nuts; this observation can easily be attributed to the high degree of unsaturation of these oils, in combination with their lack of antioxidative minor components.

The results of the peroxide value tests for tree nut oils subjected to accelerated autoxidation are shown in Table 4.15. Examination of these results show that the rate of formation of peroxides during autoxidation resembles the formation rate of conjugated dienes in oils examined, and the ranking order of oxidative stability, derived using maximum peroxide value levels, were identical to the order obtained using maximum conjugated diene values (pecan oil > pistachio oil > hazelnut oil ≥ almond oil > Brazil nut oil > pine nut oil > walnut oil; chloroform/methanol extracted oils > hexane extracted oils; non-stripped oils > stripped oils).

4.13.2. *p*-Anisidine Values and Headspace Volatile Compositions of Autoxidized Tree Nut Oils

The *p*-anisidine value and headspace analysis are both tests for secondary products of lipid oxidation. The *p*-anisidine value is an empirical test, while headspace analysis can produce quantitative data on oil volatiles formed during lipid oxidation. *Para*-anisidine values of autoxidized tree nut oils are shown in Table 4.16. Results showed that chloroform/methanol extracted oils were more resistant to the formation of *p*-anisidine reactive substances when compared to hexane extracted oils. Also, non-stripped oils were more stable than stripped oils for all nut samples studied. Among samples studied, chloroform/methanol extracted pistachio oil exhibited the lowest *p*-anisidine value after 12 days of accelerated autoxidation, followed by hexane extracted pistachio oil, chloroform/methanol extracted pecan, almond and Brazil nut oils, and chloroform/methanol

Table 4.15. Increase in Peroxide Values (meq oxygen / kg oil) of Tree Nut Oils during Autoxidation at 60 °C [1,2]

	Storage Period (Days)				
Oil	0	3	6	9	12
Almond Oil					
Hexane Extracted	0.040^{a}	0.076^{g}	0.102^{h}	0.153^{i}	0.335^{g}
Chloroform/Methanol Extracted	0.030^{b}	0.044^{g}	0.057^{i}	0.107^{i}	0.164^{h}
Stripped Hexane Extracted	0.023^{b}	0.212^{e}	0.285^{f}	0.393^{g}	0.531^{f}
Stripped Chloroform/Methanol Extracted	0.015^{b}	0.157^{f}	0.246^{f}	0.346^{g}	0.458^{g}
Brazil Nut Oil					
Hexane Extracted	0.047^{a}	0.142^{f}	0.233^{f}	0.370^{g}	0.661^{e}
Chloroform/Methanol Extracted	0.030^{b}	0.061^{g}	0.104^{h}	0.140^{i}	0.197^{h}
Stripped Hexane Extracted	0.015^{b}	0.156^{f}	0.554^{d}	1.195^{d}	1.992^{c}
Stripped Chloroform/Methanol Extracted	0.023^{b}	0.162^{f}	0.446^{d}	0.935^{e}	1.415^{d}
Hazelnut Oil					
Hexane Extracted	0.031^{b}	0.079^{g}	0.125^{g}	0.200^{h}	0.307^{g}
Chloroform/Methanol Extracted	0.059^{a}	0.065^{g}	0.094^{h}	0.150^{i}	0.200^{h}
Stripped Hexane Extracted	0.015^{b}	0.280^{c}	0.389^{e}	0.458^{f}	0.519^{f}
Stripped Chloroform/Methanol Extracted	0.015^{b}	0.252^{d}	0.369^{e}	0.437^{f}	0.467^{g}
Pecan Oil					
Hexane Extracted	0.030^{b}	0.053^{g}	0.085^{h}	0.119^{i}	0.158^{h}
Chloroform/Methanol Extracted	0.023^{b}	0.026^{g}	0.030^{j}	0.036^{j}	0.045^{i}
Stripped Hexane Extracted	0.023^{b}	0.097^{g}	0.149^{g}	0.228^{h}	0.346^{g}
Stripped Chloroform/Methanol Extracted	0.015^{b}	0.073^{g}	0.131^{g}	0.205^{h}	0.309^{g}
Pine Nut Oil					
Hexane Extracted	0.030^{b}	0.206^{e}	0.466^{d}	0.822^{e}	1.317^{d}
Chloroform/Methanol Extracted	0.016^{b}	0.081^{g}	0.155^{g}	0.228^{h}	0.291^{g}
Stripped Hexane Extracted	0.023^{b}	0.296^{c}	0.859^{c}	1.506^{c}	2.305^{b}
Stripped Chloroform/Methanol Extracted	0.015^{b}	0.274^{c}	0.827^{c}	1.428^{c}	2.259^{b}
Pistachio Oil					
Hexane Extracted	0.023^{b}	0.035^{g}	0.065^{i}	0.088^{i}	0.116^{h}
Chloroform/Methanol Extracted	0.015^{b}	0.021^{g}	0.031^{j}	0.036^{j}	0.037^{i}
Stripped Hexane Extracted	0.015^{b}	0.037^{g}	0.098^{h}	0.211^{h}	0.438^{g}
Stripped Chloroform/Methanol Extracted	0.015^{b}	0.037^{g}	0.097^{h}	0.206^{h}	0.428^{g}
Walnut Oil					
Hexane Extracted	0.030^{b}	0.839^{b}	1.711^{b}	1.921^{b}	2.142^{c}
Chloroform/Methanol Extracted	0.015^{b}	0.208^{e}	0.238^{f}	0.287^{h}	0.334^{g}
Stripped Hexane Extracted	0.015^{b}	1.109^{a}	2.276^{a}	3.656^{a}	4.736^{a}
Stripped Chloroform/Methanol Extracted	0.015^{b}	1.065^{a}	2.226^{a}	3.585^{a}	4.629^{a}

[1] Values in the same row bearing different superscripts are significantly ($p \leq 0.05$) different.
[2] Standard deviations did not exceed 0.050 for any data point (data not shown).

Table 4.16. *p*-Anisidine Values of Tree Nut Oils during Autoxidation at 60 °C [1,2]

	Storage Period (Days)				
Oil	0	3	6	9	12
Almond Oil					
Hexane Extracted	0.120^{f}	1.437^{h}	2.335^{n}	3.247^{s}	3.923^{s}
Chloroform/Methanol Extracted	0.561^{b}	1.095^{h}	2.015^{n}	2.464^{t}	3.061^{t}
Stripped Chloroform/Methanol Extracted	0.267^{d}	4.839^{f}	11.739^{e}	19.743^{e}	29.058^{c}
Stripped Hexane Extracted	0.465^{b}	5.294^{e}	11.201^{f}	17.493^{g}	24.389^{f}
Brazil Nut Oil					
Hexane Extracted	0.189^{e}	1.936^{h}	3.853^{l}	6.059^{p}	8.576^{p}
Chloroform/Methanol Extracted	0.821^{a}	1.272^{h}	1.780^{n}	2.392^{t}	3.327^{t}
Stripped Hexane Extracted	0.264^{d}	4.429^{g}	9.221^{g}	13.948^{h}	19.746^{g}
Stripped Chloroform/Methanol Extracted	0.371^{c}	3.700^{g}	7.139^{i}	11.137^{j}	16.048^{i}
Hazelnut Oil					
Hexane Extracted	0.592^{b}	2.138^{h}	4.120^{l}	6.023^{p}	8.262^{p}
Chloroform/Methanol Extracted	0.288^{d}	1.695^{h}	3.274^{m}	4.893^{r}	6.555^{r}
Stripped Hexane Extracted	0.255^{d}	3.943^{g}	8.193^{h}	12.294^{i}	16.428^{i}
Stripped Chloroform/Methanol Extracted	0.343^{c}	3.492^{g}	7.419^{i}	11.489^{j}	15.386^{j}
Pecan Oil					
Hexane Extracted	0.433^{b}	2.162^{h}	4.006^{l}	5.589^{q}	7.172^{q}
Chloroform/Methanol Extracted	0.294^{d}	0.702^{i}	1.398^{o}	2.330^{t}	3.243^{t}
Stripped Hexane Extracted	0.344^{c}	2.583^{h}	5.302^{l}	8.153^{m}	11.876^{m}
Stripped Chloroform/Methanol Extracted	0.257	2.683^{h}	5.732^{k}	8.682^{l}	12.686^{l}
Pine Nut Oil					
Hexane Extracted	0.267^{d}	2.081^{h}	4.359^{k}	10.286^{k}	17.567^{h}
Chloroform/Methanol Extracted	0.493^{b}	2.105^{h}	4.423^{k}	8.473^{m}	14.029^{k}
Stripped Hexane Extracted	0.281^{d}	7.043^{c}	14.382^{c}	22.395^{c}	29.711^{b}
Stripped Chloroform/Methanol Extracted	0.552^{b}	6.382^{d}	13.492^{d}	21.014^{d}	28.549^{d}
Pistachio Oil					
Hexane Extracted	0.545^{b}	0.763^{l}	1.017^{p}	1.484^{u}	1.910^{u}
Chloroform/Methanol Extracted	0.635^{b}	0.691^{i}	0.790^{q}	1.285^{u}	1.731^{u}
Stripped Hexane Extracted	0.518^{b}	2.385^{h}	5.092^{l}	7.854^{n}	10.473^{n}
Stripped Chloroform/Methanol Extracted	0.493^{b}	2.372^{h}	4.727^{l}	6.937^{o}	9.361^{o}
Walnut Oil					
Hexane Extracted	0.230^{d}	4.202^{g}	9.535^{g}	18.826^{f}	29.591^{b}
Chloroform/Methanol Extracted	0.462^{b}	1.472	6.640^{j}	13.782^{h}	24.880^{e}
Stripped Hexane Extracted	0.193^{e}	9.736^{a}	20.472^{a}	34.284^{a}	52.562^{a}
Stripped Chloroform/Methanol Extracted	0.237^{d}	8.583^{b}	18.936^{b}	30.847^{b}	48.836^{a}

[1] Values in the same row bearing different superscripts are significantly ($p \leq 0.05$) different.
[2] Standard deviations did not exceed 0.150 for any data point (data not shown).

extracted hazelnut oil. Hexane extracted walnut oil had the highest *p*-anisidine value among non-stripped samples examined after 12 days of accelerated autoxidation. None of the oil samples examined had reached their *p*-anisidine value post-plateau phase after 12 days of accelerated autoxidation; the *p*-anisidine value was still increasing after 12 days of autoxidation, implying that oxidizable substrate was still available in all samples examined.

Hexanal and nonanal were the most widely detected headspace volatiles observed in tree nut oils subjected to accelerated autoxidation; propanal was present only in walnut oil (Table 4.17). Hexanal is an oxidation product of linoleic acid, an omega-6 fatty acid. Its presence in meat and other lipid sources containing linoleic acid has been reported in the literature [199]. In addition, nonanal is an oxidation product of oleic acid, an omega-9 fatty acid [210]. No headspace aldehydes were detected in oil samples before commencement of the autoxidation studies. Chloroform/ methanol extracted oils contained lower amounts of headspace aldehydes compared to their hexane extracted counterparts at each sampling point of the accelerated autoxidation studies. Stripped oils contained 2 to 4 times the amount of headspace aldehydes of their non-stripped counterparts. Hexanal was the most abundant headspace aldehyde in almond, Brazil nut, hazelnut, pecan, pine nut and pistachio oils, corresponding to oxidation of linoleic acid in these samples. Propanal was the predominant headspace aldehyde in walnut oil, which can be attributed to oxidation of α-linolenic acid, an omega-3 fatty acid, present in these samples. Among non-stripped samples, hexane extracted walnut oil contained the

Table 4.17. Headspace Aldehyde Compositions (μg Aldehyde / g Oil) of Tree Nut Oils during Autoxidation at 60 °C [1]

		Storage Period (Days)			
	Aldehyde	Day 3	Day 6	Day 9	Day 12
Almond Oil					
Hexane Extracted	Hexanal	4.1	16.0	32.6	56.0
	Nonanal	0.0	2.1	7.4	17.0
Chloroform/Methanol Extracted	Hexanal	0.0	22.0	29.5	36.0
	Nonanal	0.0	0.0	1.4	9.0
Stripped Hexane Extracted	Hexanal	21.1	60.4	85.4	122.3
	Nonanal	2.5	6.7	11.4	16.6
Stripped Chloroform/Methanol Extracted	Hexanal	25.7	64.4	92.7	134.4
	Nonanal	0.0	3.5	8.4	14.2
Brazil Nut Oil					
Hexane Extracted	Hexanal	7.6	31.7	49.6	81.7
	Nonanal	0.0	0.0	0.0	13.2
Chloroform/Methanol Extracted	Hexanal	2.3	18.7	24.4	37.4
	Nonanal	0.0	0.0	0.0	2.5
Stripped Hexane Extracted	Hexanal	31.5	79.4	109.7	150.4
	Nonanal	2.7	21.4	28.4	42.4
Stripped Chloroform/Methanol Extracted	Hexanal	28.5	73.6	100.5	123.9
	Nonanal	3.4	14.5	26.4	34.7
Hazelnut Oil					
Hexane Extracted	Hexanal	3.2	37.5	49.6	82.6
	Nonanal	0.0	0.0	0.0	7.22
Chloroform/Methanol Extracted	Hexanal	8.1	28.4	42.5	66.1
	Nonanal	0.0	0.0	0.0	2.14
Stripped Hexane Extracted	Hexanal	24.6	61.6	79.4	136.1
	Nonanal	0.0	6.3	11.6	23.2
Stripped Chloroform/Methanol Extracted	Hexanal	17.5	48.3	87.4	124.2
	Nonanal	0.0	7.2	12.6	20.3
Pecan Oil					
Hexane Extracted	Hexanal	1.7	13.8	19.5	27.8
	Nonanal	0.0	0.0	0.0	2.5
Chloroform/Methanol Extracted	Hexanal	0.0	8.5	11.8	19.7
	Nonanal	0.0	0.0	0.0	0.0
Stripped Hexane Extracted	Hexanal	20.5	57.6	86.7	120.5
	Nonanal	0.0	4.8	8.7	12.4
Stripped Chloroform/Methanol Extracted	Hexanal	14.6	57.6	72.8	116.5
	Nonanal	0.0	4.8	8.4	15.3

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Table 4.17 continued...

Oil	Aldehyde	Storage Period (Days) Day 3	Day 6	Day 9	Day 12
Pine Nut Oil					
Hexane Extracted	Hexanal	2.5	64.7	126.7	166.3
	Nonanal	0.0	19.6	28.4	48.5
Chloroform/Methanol Extracted	Hexanal	1.7	42.7	64.7	120.0
	Nonanal	4.6	19.5	29.6	37.6
Stripped Hexane Extracted	Hexanal	42.7	138.7	216.8	302.1
	Nonanal	5.1	18.6	26.8	37.8
Stripped Chloroform/Methanol Extracted	Hexanal	48.3	127.9	201.3	286.5
	Nonanal	3.7	17.5	23.5	34.4
Pistachio Oil					
Hexane Extracted	Hexanal	3.5	18.5	31.3	43.5
	Nonanal	0.0	1.7	5.6	13.3
Chloroform/Methanol Extracted	Hexanal	1.4	14.3	26.9	34.7
	Nonanal	0.0	2.8	7.5	10.8
Stripped Hexane Extracted	Hexanal	14.7	41.4	63.5	94.6
	Nonanal	0.0	3.7	5.8	12.5
Stripped Chloroform/Methanol Extracted	Hexanal	10.4	39.6	52.7	92.4
	Nonanal	0.0	2.3	5.1	9.3
Walnut Oil					
Hexane Extracted	Propanal	43.8	69.5	118.6	188.3
	Hexanal	31.6	49.4	78.4	72.7
	Nonanal	9.5	19.8	31.8	43.2
Chloroform/Methanol Extracted	Propanal	21.6	58.3	84.8	137.5
	Hexanal	5.9	20.6	34.6	53.2
	Nonanal	2.1	12.3	21.5	29.4
Stripped Hexane Extracted	Propanal	43.8	118.6	163.6	262.4
	Hexanal	31.6	78.4	116.7	167.3
	Nonanal	9.5	31.8	53.7	82.6
Stripped Chloroform/Methanol Extracted	Propanal	39.8	119.6	149.7	251.5
	Hexanal	20.8	68.3	102.6	154.3
	Nonanal	12.3	29.5	52.6	68.0

[1] Sample means were calculated from triplicate analyses; standard deviations were with the range of 0.1 to 9.0 μg/g, with higher mean values having larger standard deviations (data not shown).

highest amount of total aldehydes after 12 days of accelerated autoxidation, followed by chloroform/ methanol extracted walnut oil, hexane extracted pine nut oil and chloroform/methanol extracted pine nut oil. Chloroform/methanol extracted pecan oil contained the lowest amount of total aldehydes at each sampling point. Surprisingly, chloroform/methanol extracted Brazil nut oil contained the third lowest level of headspace aldehydes at day 12, after chloroform/methanol extracted pecan oil and hexane extracted pecan oil, which was unexpected considering its high level of linoleic acid. Nonanal was detected in all samples at day 12 except chloroform/methanol extracted pecan oil, and was the least predominant aldehyde in all samples in which it was detected.

4.14. Photooxidative Stability of Tree Nut Oils

The photooxidative stability of stripped and non-stripped tree nut oils over 72 h were examined using previously described protocols [198]. Conjugated dienes and headspace volatiles were monitored to assess the deterioration of oil quality throughout the photooxidation period. Results of the photooxidation studies showed that chloroform/methanol extracted oils were more resistant to conjugated diene formation than hexane extracted oils. None of the non-stripped oils had reached their post-plateau phase of conjugated diene formation after 72h, which implies that the length of the photooxidation test may not have been long enough to adequately assess the photooxidative stability of the samples examined. Stripped oils were less resistant to conjugated diene formation than their non-stripped counterparts (Table 4.18). Among non-stripped samples, hexane extracted walnut oil had the highest

Table 4.18. Formation of Conjugated Dienes in Tree Nut Oils during Photooxidation[1,2,3]

Sampling Period (h)	0	4	8	12	24	48	72
Non-Stripped Oils							
A-H	0.956^{d}	1.862^{i}	2.683^{g}	3.347^{p}	8.153^{q}	10.967^{n}	14.257^{o}
A-BD	1.744^{b}	4.950^{c}	3.563^{f}	4.174^{o}	8.267^{q}	11.487^{m}	14.933^{n}
BN-H	1.768^{b}	4.174^{d}	5.183^{e}	7.077^{m}	13.160^{n}	26.804^{e}	34.845^{e}
BN-BD	0.692^{e}	1.394^{j}	3.502^{f}	4.116^{o}	4.928^{t}	6.266^{r}	8.146^{r}
HN-H	1.096^{d}	3.159^{f}	3.871^{f}	8.141^{k}	11.817^{o}	12.238^{k}	15.910^{l}
HN-BD	1.492^{c}	4.117^{d}	4.901^{e}	7.635^{l}	9.841^{p}	10.423^{o}	13.550^{p}
P-H	0.502^{e}	0.930^{k}	1.443^{i}	2.219^{r}	3.264^{v}	4.796^{s}	6.234^{s}
P-BD	0.267^{f}	0.488^{l}	0.588^{k}	0.670^{s}	1.749^{w}	2.117^{u}	2.751^{u}
PN-H	1.216^{c}	2.613^{h}	5.020^{e}	12.968^{g}	33.771^{e}	42.067^{b}	54.686^{b}
PN-BD	1.067^{d}	1.799^{i}	2.759^{g}	3.936^{o}	9.909^{p}	13.464^{l}	17.503^{j}
PO-H	0.481^{e}	0.804^{k}	0.842^{j}	2.196^{r}	4.044^{u}	6.627^{q}	8.615^{q}
PO-BD	0.992^{d}	1.303^{j}	1.749^{h}	2.103^{r}	3.382^{v}	3.971^{t}	4.733^{t}
W-H	0.554^{e}	1.158^{j}	7.074^{c}	12.320^{h}	22.360^{i}	50.997^{a}	66.296^{a}
W-BD	0.535^{e}	1.676^{i}	2.708^{g}	7.274^{m}	19.540^{l}	29.762^{c}	38.690^{c}
Stripped Oils							
A-H	2.636^{a}	4.979^{c}	5.982^{d}	16.108^{e}	25.991^{f}	27.054^{d}	35.170^{d}
A-BD	2.270^{b}	4.236^{d}	4.298^{f}	15.014^{f}	21.981^{j}	26.063^{f}	33.882^{f}
BN-H	1.301^{c}	3.048^{g}	3.623^{f}	14.833^{f}	25.131^{g}	26.080^{f}	33.904^{f}
BN-BD	1.423^{c}	3.341^{f}	4.189^{f}	12.908^{g}	22.782^{h}	24.531^{g}	31.890^{g}
HN-H	1.704^{b}	3.546^{f}	3.976^{f}	11.773^{i}	20.160^{k}	20.456^{h}	26.593^{h}
HN-BD	1.467^{c}	3.810^{e}	4.687^{e}	10.836^{j}	18.457^{m}	18.857^{i}	24.514^{i}
P-H	0.945^{d}	2.011^{i}	2.429^{g}	4.077^{o}	9.909^{p}	11.422^{m}	14.849^{n}
P-BD	0.897^{d}	2.140^{i}	2.905^{g}	4.624^{n}	9.845^{p}	10.261^{o}	13.340^{p}
PN-H	1.509^{c}	3.947^{e}	6.873^{c}	30.535^{d}	63.497^{a}	0.838^{v}	1.090^{u}
PN-BD	1.524^{c}	3.414^{f}	5.268^{e}	31.402^{c}	58.951^{b}	9.903^{p}	12.873^{q}
PO-H	1.145^{c}	2.015^{i}	2.072^{h}	3.288^{p}	7.880^{r}	11.807^{l}	15.348^{m}
PO-BD	0.936^{d}	1.780^{i}	1.935^{h}	2.948^{q}	7.230^{s}	11.344^{m}	14.747^{n}
W-H	0.684^{e}	8.218^{a}	23.159^{a}	70.145^{a}	48.520^{c}	10.141^{o}	13.183^{p}
W-BD	0.693^{e}	7.295^{b}	20.936^{b}	67.561^{b}	47.470^{d}	12.668^{j}	16.469^{k}

[1] Abbreviations used: A-H, almond oil – hexane extracted; A-CM, almond oil - chloroform/methanol extracted; BN-H, Brazil nut oil – hexane extracted; BN-CM, Brazil nut oil – chloroform/methanol extracted; HN-H, hazelnut oil – hexane extract; HN-CM, hazelnut oil – chloroform/methanol extracted; P-H, pecan oil – hexane extracted; P-CM, pecan oil – chloroform/methanol extracted; PN-H, pine nut oil – hexane extracted; PN-CM, pine nut oil – chloroform/methanol extracted; PO-H, pistachio oil – hexane extracted; PO-CM, pistachio oil – chloroform/methanol extracted; W-H, walnut oil – hexane extracted; W-CM, walnut oil – chloroform/methanol extracted.

[2] Values in the same column with different superscripts are significantly ($p \leq 0.05$) different.

[3] All samples were analysed in triplicate. Standard deviations did not exceed 0.100 for any data point (data not shown).

levels of conjugated dienes after 72h, followed by hexane extracted pine nut oil, and then hexane extracted Brazil nut oil. Chloroform/methanol extracted pecan oil had the lowest levels of conjugated dienes after 72h of photooxidation, followed by chloroform/methanol extracted pistachio oil. Interestingly, chloroform/methanol extracted Brazil nut oil was considerably more stable than its hexane extracted counterpart; this trend was also observed for conjugated diene formation in Brazil nut oil during the autoxidation studies and may indicate the presence of antioxidants that render stabilities to the oil under both autoxidation and photooxidative conditions.

Results of headspace analyses of photooxidized tree nut oils are given in Table 4.19; hexanal and nonanal were the most prevalent aldehydes present, with propanal existing only in walnut oil. Chloroform/methanol extracted pecan oil contained the lowest level of total headspace aldehydes among all samples after 72h, followed by hexane extracted pecan oil, chloroform/methanol extracted Brazil nut oil, and then chloroform/methanol extracted pistachio oil. Among the non-stripped samples, hexane extracted walnut oil contained the highest amount of total headspace aldehydes after 72h of photooxidation, followed by chloroform/methanol extracted walnut oil, and then hexane extracted pine nut oil. The headspace aldehyde compositions of photooxidized tree nut oils were similar to those observed for autoxidized oils, but higher amounts of total headspace aldehydes were detected during the accelerated autoxidation studies; future studies

Table 4.19. Headspace Aldehyde Compositions (μg Aldehyde / g Oil) of Tree Nut Oils during Photooxidation [1]

		Storage Period (Hours)			
Oil	Aldehyde	12	24	48	72
Almond Oil					
Hexane Extracted	Hexanal	3.0	15.9	26.9	37.4
	Nonanal	0.0	1.5	4.8	11.4
Chloroform/Methanol Extracted	Hexanal	1.2	12.3	23.1	29.8
	Nonanal	0.0	2.4	6.5	9.3
Stripped Hexane Extracted	Hexanal	12.6	35.6	54.6	81.4
	Nonanal	0.0	3.2	5.0	10.8
Stripped Chloroform/Methanol Extracted	Hexanal	8.9	34.1	45.3	79.5
	Nonanal	0.0	2.0	4.4	8.0
Brazil Nut Oil					
Hexane Extracted	Hexanal	6.5	27.3	42.7	70.3
	Nonanal	0.0	0.0	0.0	11.4
Chloroform/Methanol Extracted	Hexanal	2.0	16.1	21.0	32.2
	Nonanal	0.0	0.0	0.0	2.2
Stripped Hexane Extracted	Hexanal	27.1	68.3	94.3	129.3
	Nonanal	2.3	18.4	24.4	36.5
Stripped Chloroform/Methanol Extracted	Hexanal	24.5	63.3	86.4	106.6
	Nonanal	2.9	12.5	22.7	29.8
Hazelnut Oil					
Hexane Extracted	Hexanal	2.8	32.3	42.7	71.0
	Nonanal	0.0	0.0	0.0	6.2
Chloroform/Methanol Extracted	Hexanal	7.0	24.4	36.6	56.8
	Nonanal	0.0	0.0	0.0	1.8
Stripped Hexane Extracted	Hexanal	21.2	53.0	68.3	117.0
	Nonanal	0.0	5.4	10.0	20.0
Stripped Chloroform/Methanol Extracted	Hexanal	15.1	41.5	75.2	106.8
	Nonanal	0.0	6.2	10.8	17.5
Pecan Oil					
Hexane Extracted	Hexanal	1.5	11.9	16.8	23.9
	Nonanal	0.0	0.0	0.0	2.2
Chloroform/Methanol Extracted	Hexanal	0.0	7.3	10.1	16.9
	Nonanal	0.0	0.0	0.0	0.0
Stripped Hexane Extracted	Hexanal	17.6	49.5	74.6	103.6
	Nonanal	0.0	4.1	7.5	10.7
Stripped Chloroform/Methanol Extracted	Hexanal	12.6	49.5	62.6	100.2
	Nonanal	0.0	4.1	7.2	13.2

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Table 4.19 continued...

Oil	Aldehyde	Storage Period (Days) 12	24	48	72
Pine Nut Oil					
Hexane Extracted	Hexanal	2.2	55.6	109.0	143.0
	Nonanal	0.0	16.9	24.4	41.7
Chloroform/Methanol Extracted	Hexanal	1.5	36.7	55.6	103.2
	Nonanal	4.0	16.8	25.5	32.3
Stripped Hexane Extracted	Hexanal	36.7	119.3	186.4	259.8
	Nonanal	4.4	16.0	23.0	32.5
Stripped Chloroform/Methanol Extracted	Hexanal	41.5	110.0	173.1	246.4
	Nonanal	3.2	15.1	20.2	29.6
Pistachio Oil					
Hexane Extracted	Hexanal	3.0	15.9	26.9	37.4
	Nonanal	0.0	1.5	4.8	11.4
Chloroform/Methanol Extracted	Hexanal	1.2	12.3	23.1	29.8
	Nonanal	0.0	2.4	6.5	9.3
Stripped Hexane Extracted	Hexanal	12.6	35.6	54.6	81.4
	Nonanal	0.0	3.2	5.0	10.8
Stripped Chloroform/Methanol Extracted	Hexanal	8.9	34.1	45.3	79.5
	Nonanal	0.0	2.0	4.4	8.0
Walnut Oil					
Hexane Extracted	Propanal	37.7	59.8	102.0	161.9
	Hexanal	27.2	42.5	67.4	62.5
	Nonanal	8.2	17.0	27.3	37.2
Chloroform/Methanol Extracted	Propanal	18.6	50.1	72.9	118.3
	Hexanal	5.1	17.7	29.8	45.8
	Nonanal	1.8	10.6	18.5	25.3
Stripped Hexane Extracted	Propanal	37.7	102.0	140.7	225.7
	Hexanal	27.2	67.4	100.4	143.9
	Nonanal	8.2	27.3	46.2	71.0
Stripped Chloroform/Methanol Extracted	Propanal	34.2	102.9	128.7	216.3
	Hexanal	17.9	58.7	88.2	132.7
	Nonanal	10.6	25.4	45.2	58.5

[1] Sample means were calculated from triplicate analyses; standard deviations were with the range of 0.1 to 9.0 µg/g, with higher mean values having larger standard deviations (data not shown).

may be required to extend the photooxidation period in order to more accurately assess the photooxidative stability of tree nut oils. However, it appears that the oils examined enjoyed a reasonable photooxidative stability under the conditions examined.

The overall trends observed in the photooxidative stability studies are similar to those observed in the accelerated autoxidation studies; oils that exhibited high photooxidative stability also exhibited high autoxidative stability. This implies that the antioxidative minor components of in oils such as chloroform/methanol extracted pecan, pistachio and Brazil nut oils impart both photooxidative and autoxidative stability to them. Further studies on minor component compositions of these and other tree nut oils are warranted.

CHAPTER 5

Summary and Recommendations

Tree nuts are abundant sources of lipids that are rich in unsaturated fatty acids, lipidic antioxidants and phytosterols, as well as other health promoting substances such as phospholipids and non-tocopherol phenolics. With the exception of walnut and pine nut oils, the predominant fatty acid in tree but oils examined was oleic acid. Brazil nut oil contained approximately equal amounts of oleic and linoleic acids, while pine nut and walnut oils contained predominantly linoleic acid. The fatty acid composition of walnut oil was unique among nut oils examined because it was the only significant source of α-linolenic acid, an n-3 fatty acid.

Triacylglycerols were the main lipid class in tree nut oils examined, phosphatidylcholine and phosphatidylserine were the predominant phospholipids present. Gamma- and α-tocopherols were the predominant tocopherols detected, with walnut and pecan oils containing the highest amounts; no tocotrienols were detected in the tree nut oils examined. Chloroform/methanol extracted Brazil nut oil was the only significant source of δ-tocopherol. The chloroform/methanol extraction system was able to extract more of the minor oil components from raw tree nuts than hexane extraction system. Thus, chloroform/methanol extracted oils possessed higher amounts of lipid phase antioxidants, which imparted higher oxidative stability to these oils and enhanced the antioxidant activity of their minor component extracts compared to hexane extracted oils. The solvent stripping

process developed in this study was capable of extracting antioxidative minor components from the tree nut oils for evaluation of their antioxidant potential.

The tree nut oil minor component extracts were tested for their total phenolic content and their antioxidant activity was examined using the TEAC assay, DPPH radical scavenging assay, β-carotene bleaching test, PCL inhibition assay and ORAC assay. Similar antioxidant activity trends were observed in all tests employed in our studies, with extracts of chloroform/methanol extracted oils exhibiting higher activities than extracts of hexane extracted oils. Minor component extracts of chloroform/methanol extracted pecan and walnut oils possessed the highest antioxidant activities among samples tested, which is likely due to their high amounts of tocopherols and other antioxidative minor components such as phospholipids and possibly other unidentified phenolic and/or non-phenolic components present in these extracts. Minor component extracts of almond oils were the least active among samples examined in this study. Isolating the active components of tree nut oil minor component extracts and identifying their individual contribution to the overall antioxidant activity would be of paramount importance in understanding the reason(s) for the observed high activities of certain nut oil extracts examined. This is particularly important for extracts of chloroform/methanol extracted pecan, walnut and Brazil nut oils.

Non-stripped tree nut oils exhibited greater oxidative stability than their stripped counterparts under both accelerated autoxidation and photooxidation conditions. Hexane extracted oils were less oxidatively stable than their chloroform/methanol extracted counterparts. Oils of pecan exhibited the highest stabilities while oils of walnut were the least stable. The results of the oxidative

stability studies show that oils rich in antioxidants and low in polyunsaturated fatty acids were more stable than those with lower levels of antioxidants and higher degrees of unsaturation.

Future research on the antioxidant activities and minor component compositions of tree nut oils may be of interest in order to identify whether a single compound or a group of compounds acting synergistically are responsible for the enhanced antioxidant activity and oxidative stability of chloroform/methanol extracted oils. These studies may shed light on the physiological and disease modifying functions of these components, and may also help explain the observed health benefits of regular consumption of tree nuts and their oils. Research should also focus on the economic feasibility of large scale tree nut oil production and isolation of active components to assess their absorption, metabolism, removal from the body as well as possible allergic, genotoxic and cytotoxic potencies of these components.

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APPENDIX

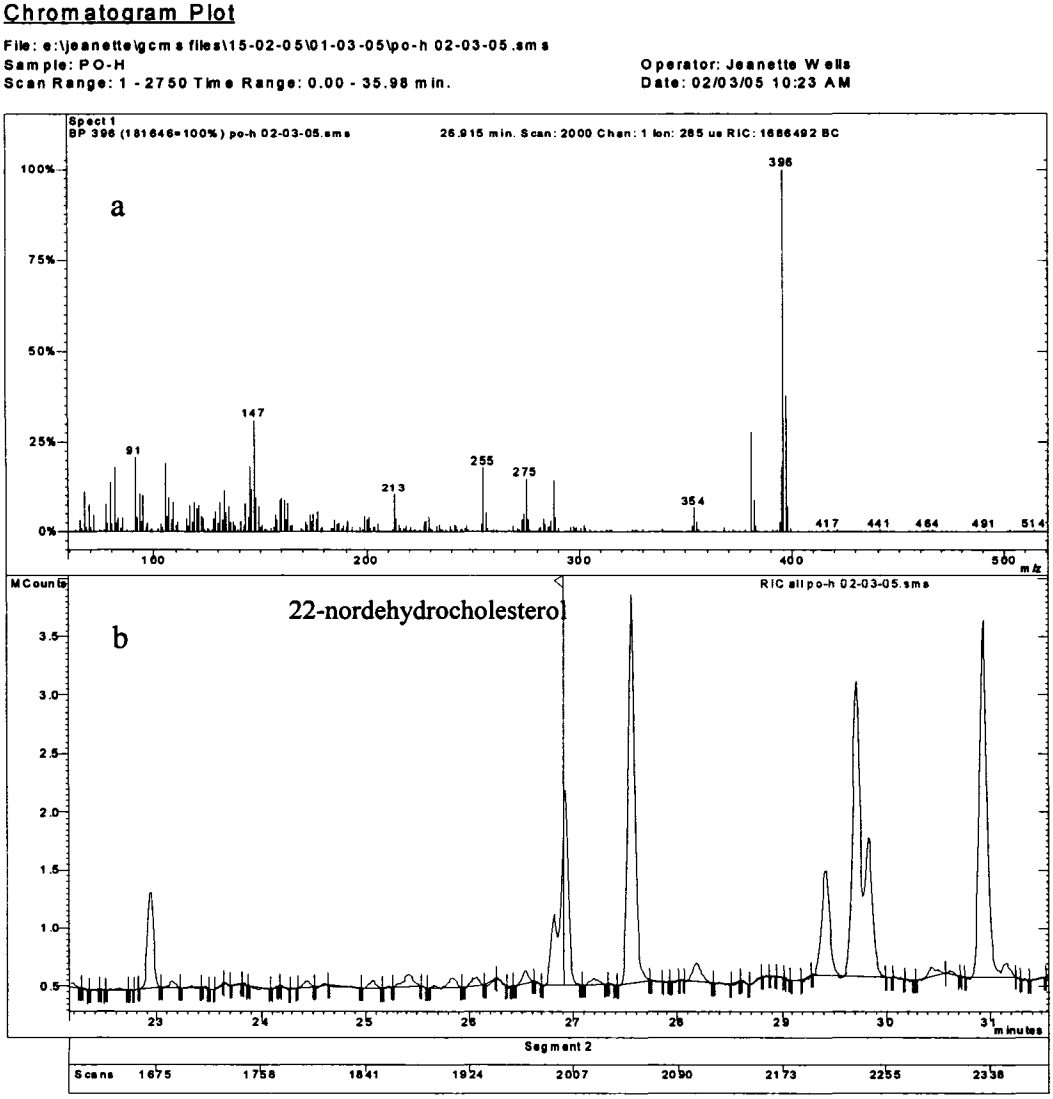

Figure A.1. GC-MS spectrum (a), and chromatographic data (b) of 22-nordehydro-cholesterol

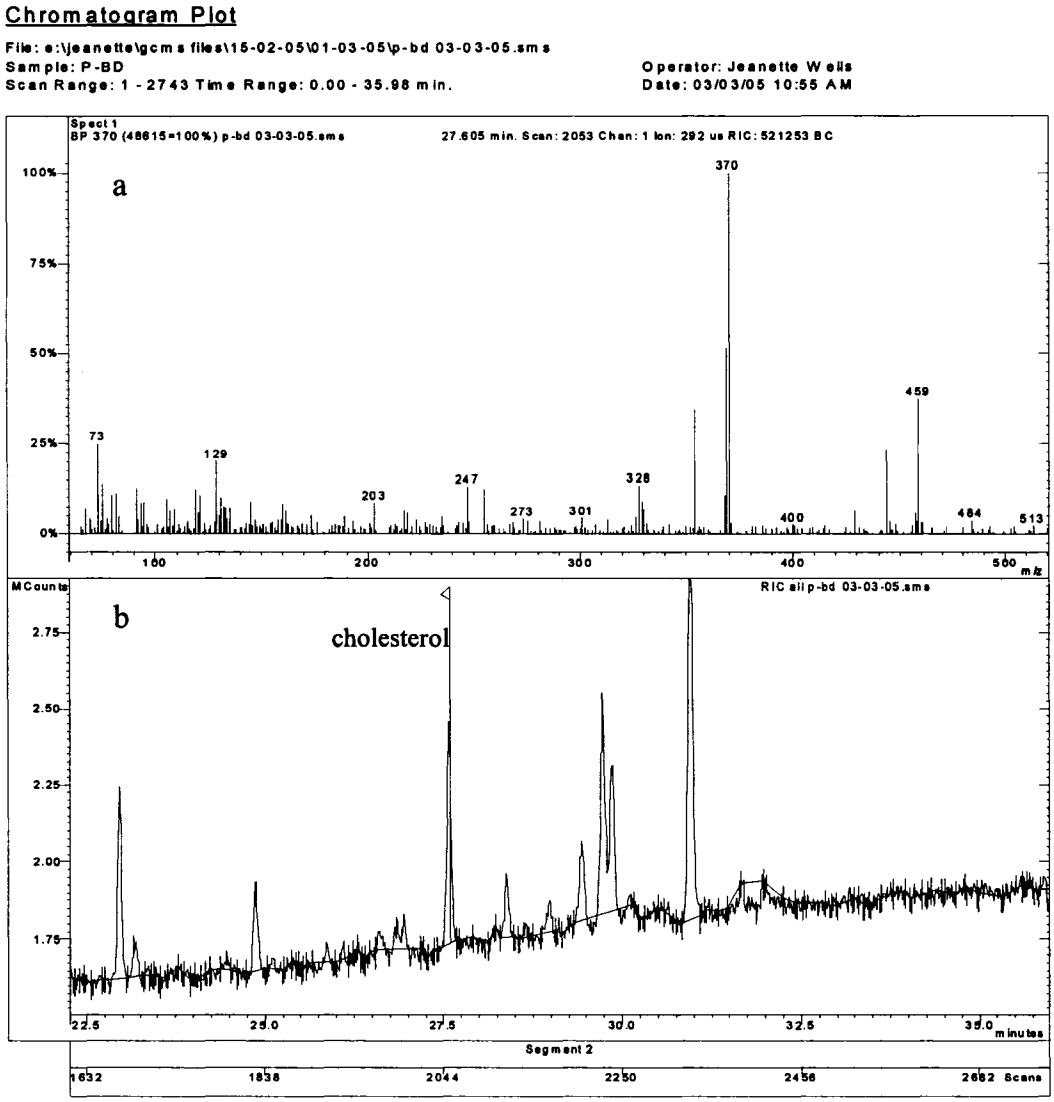

Figure A.2. GC-MS spectrum (a), and chromatographic data (b) of cholesterol

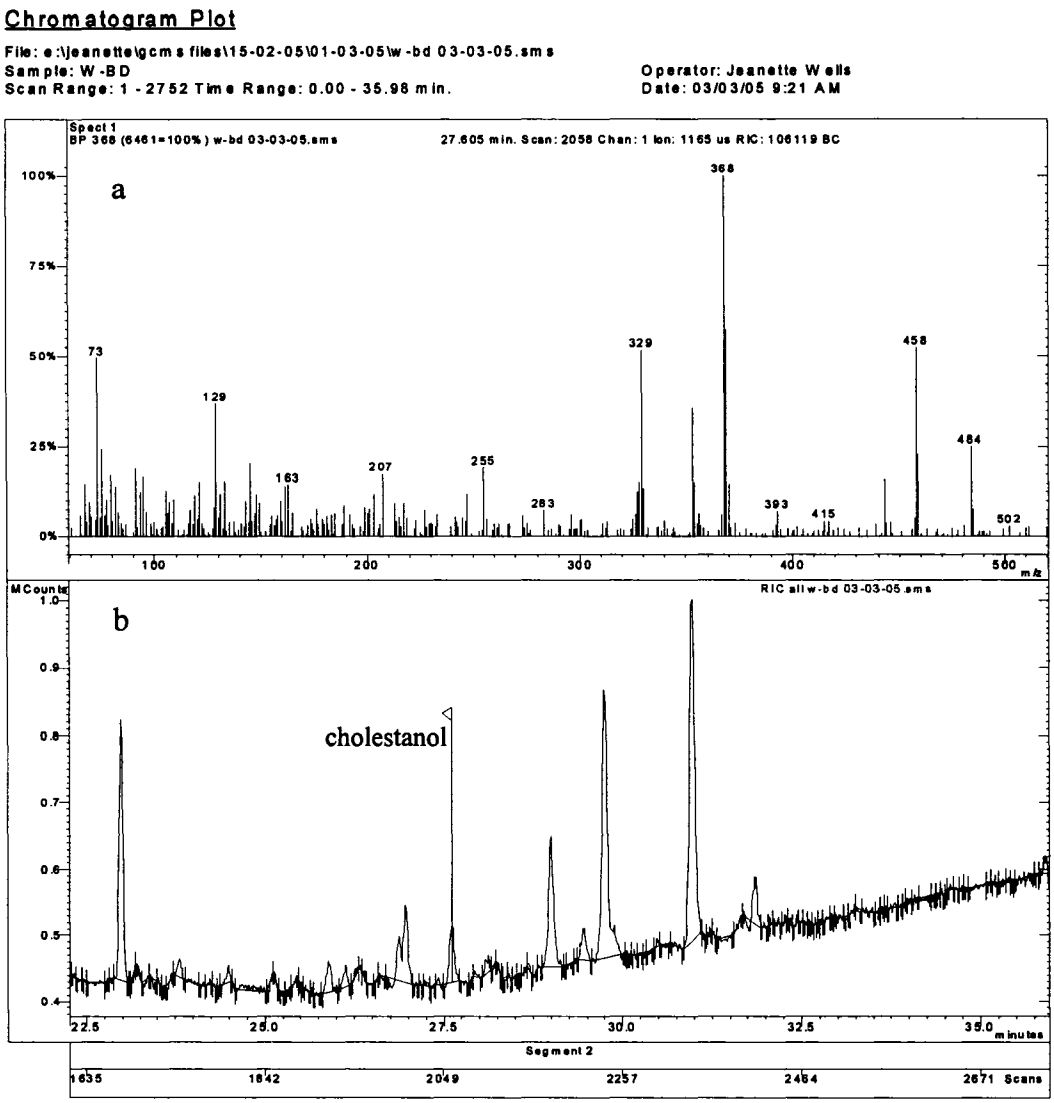

Figure A.3. GC-MS spectrum (a), and chromatographic data (b) of cholestanol

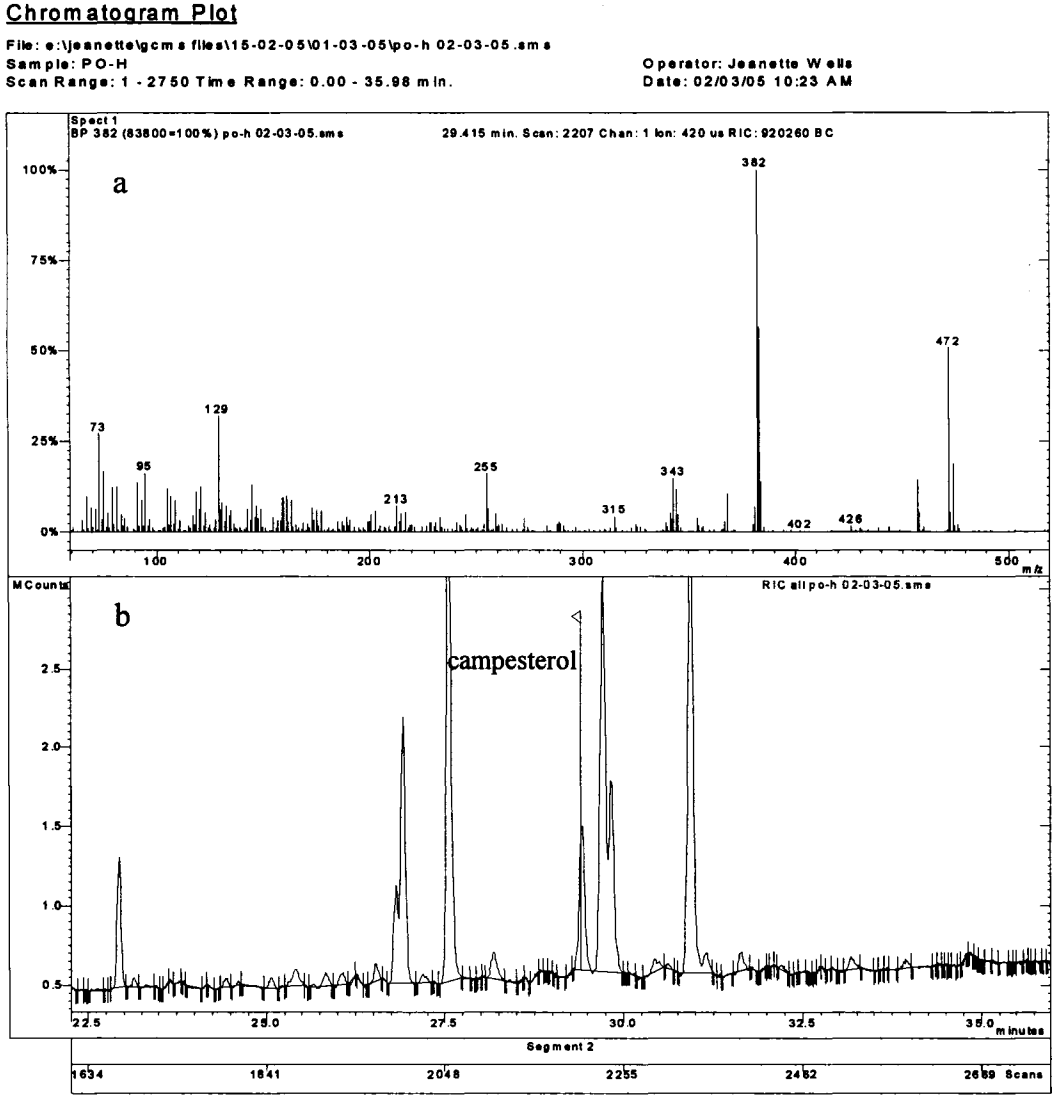

Figure A.4. GC-MS spectrum (a), and chromatographic data (b) of campesterol

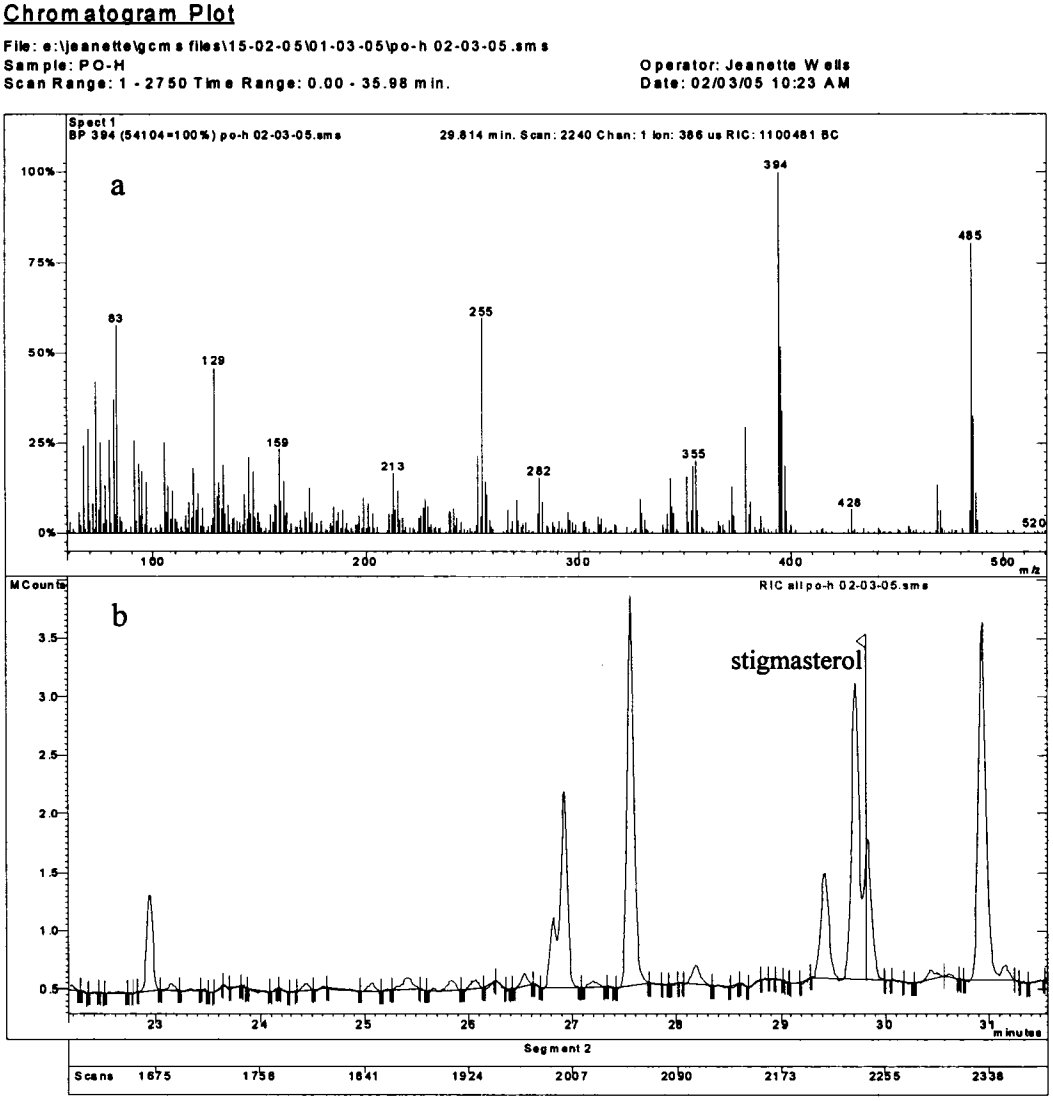

Figure A.5. GC-MS spectrum (a), and chromatographic data (b) of stigmasterol

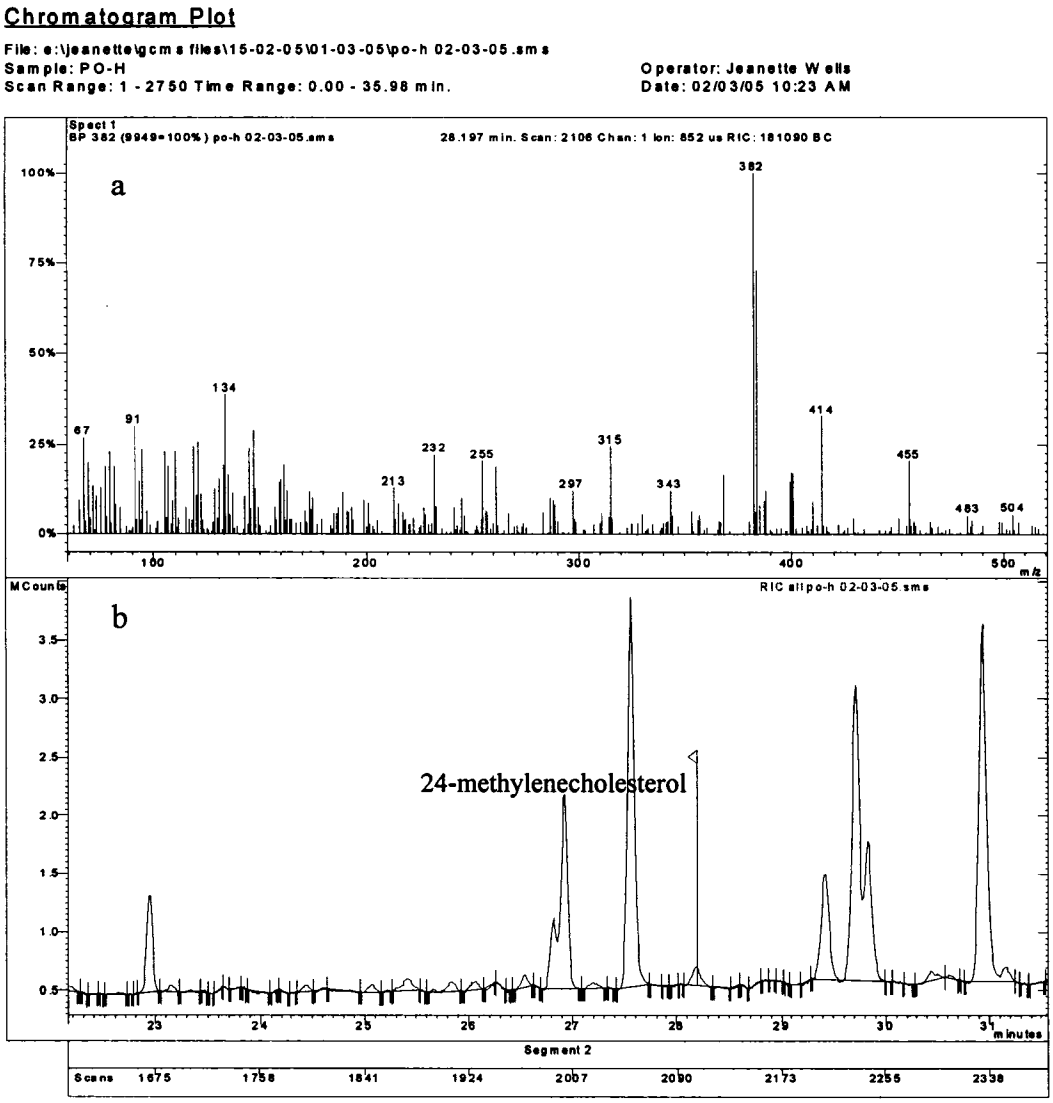

Figure A.6. GC-MS spectrum (a), and chromatographic data (b) of 24-methylene-cholesterol

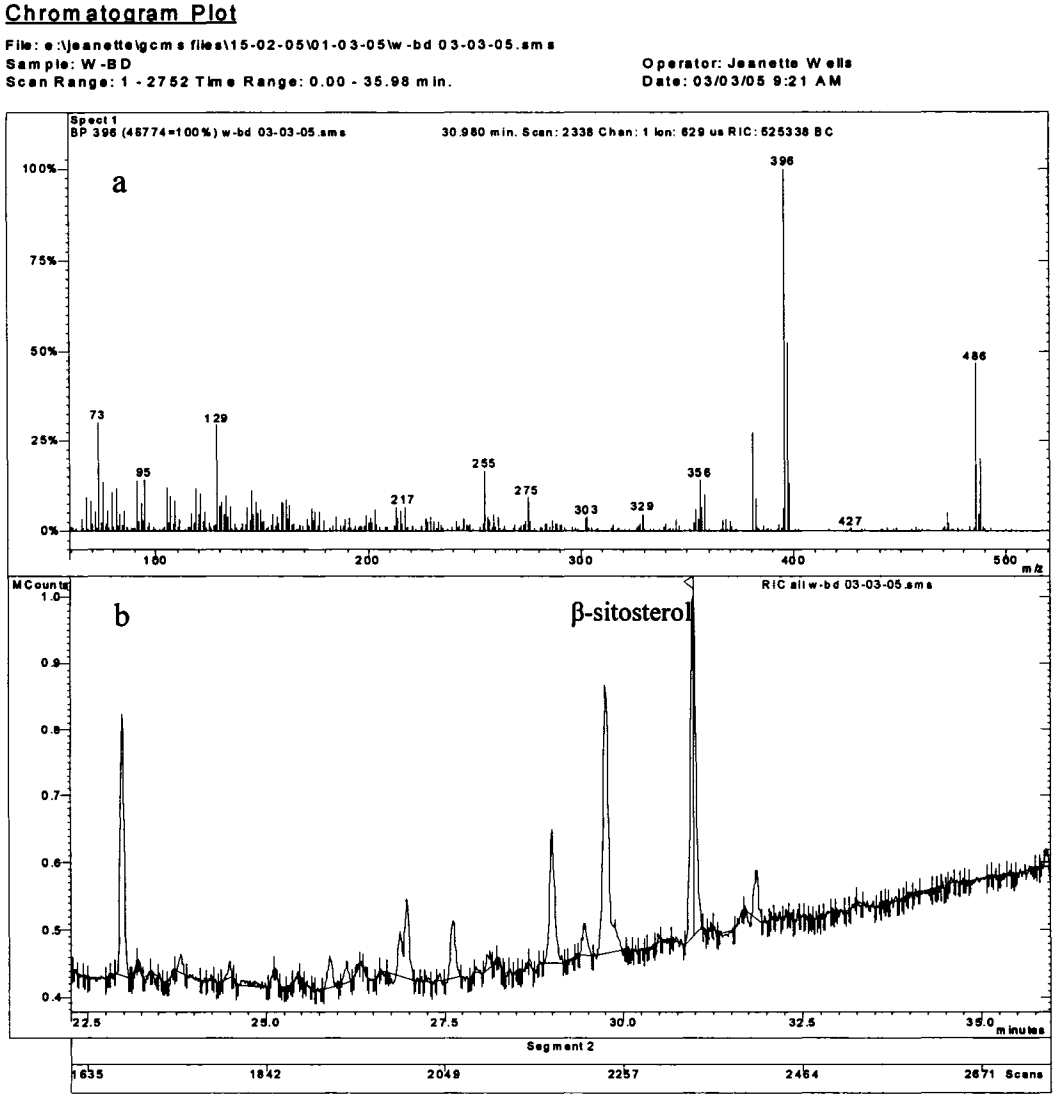

Figure A.7. GC-MS spectrum (a), and chromatographic data (b) of β-sitosterol

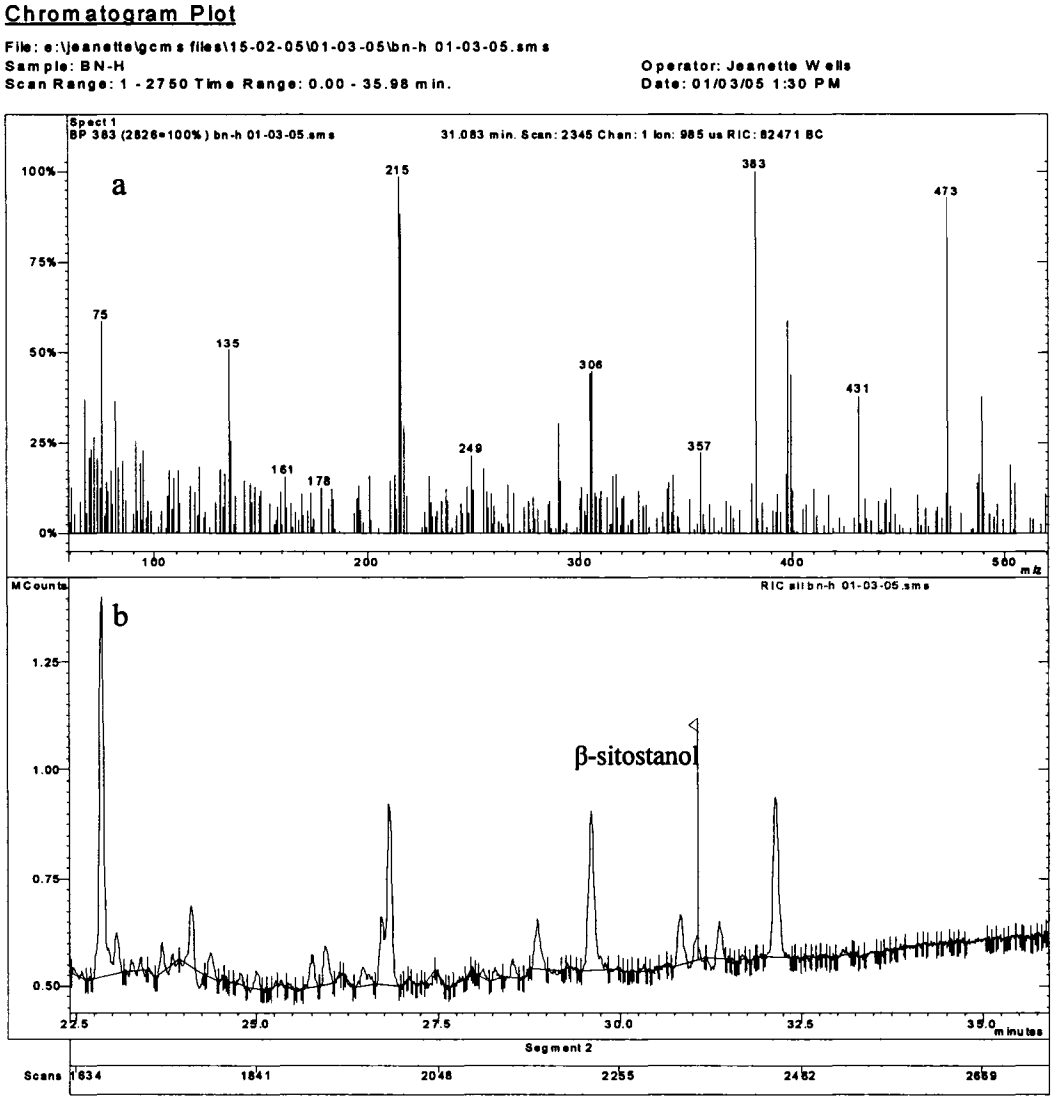

Figure A.8. GC-MS spectrum (a), and chromatographic data (b) of β-sitostanol

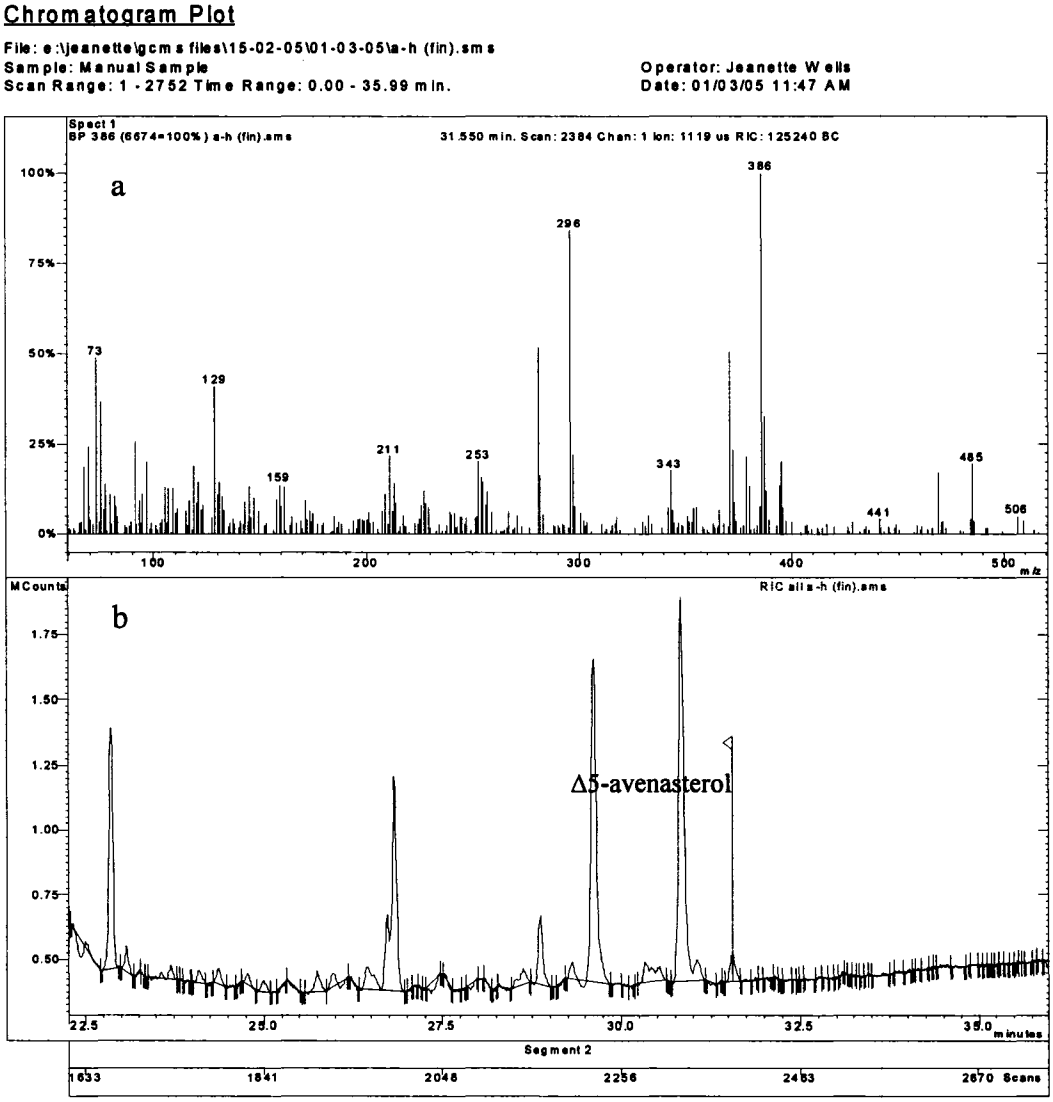

Figure A.9. GC-MS spectrum (a), and chromatographic data (b) of Δ5-avenasterol

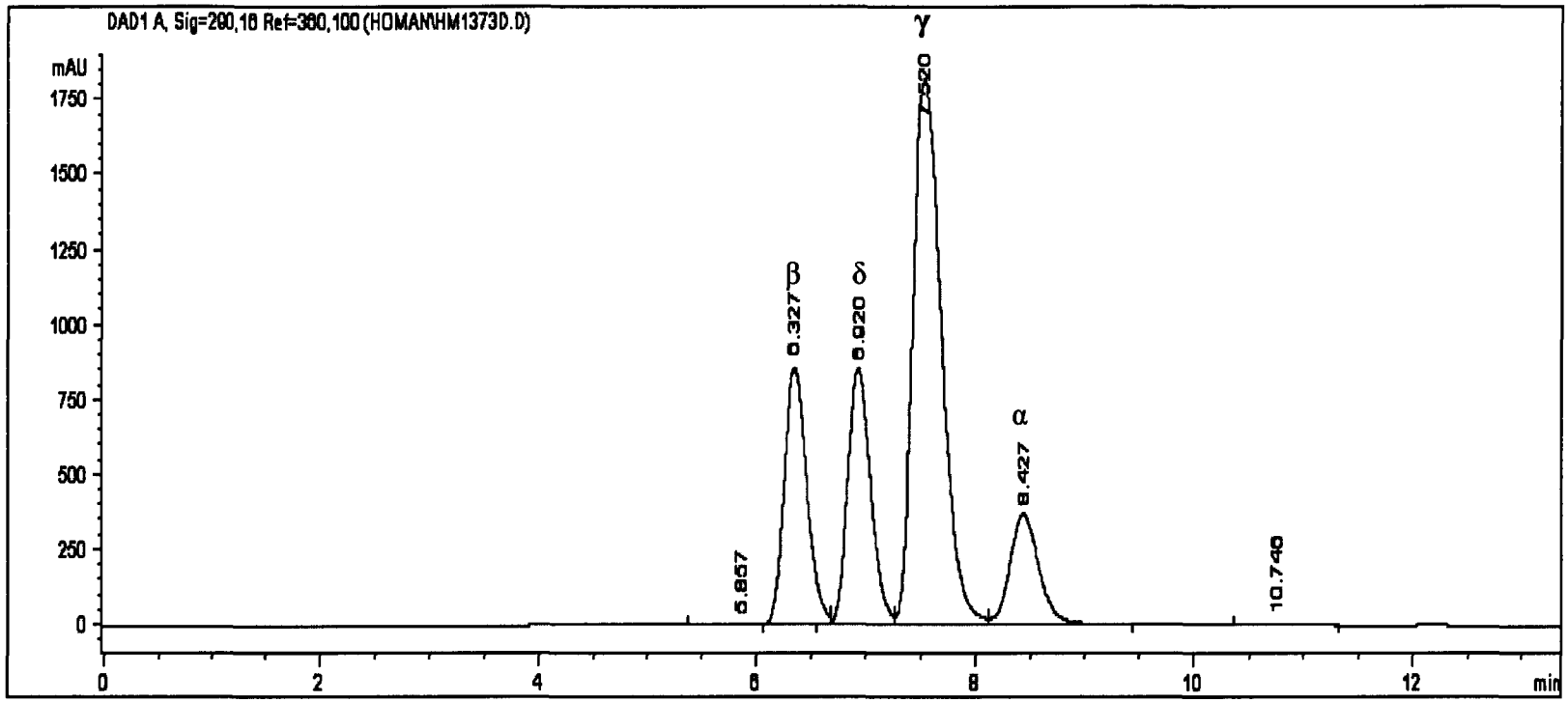

Figure A.10. Representative HPLC-UV chromatogram of tocopherol isomers, showing resolution of α-, β-, γ- and δ-tocopherols

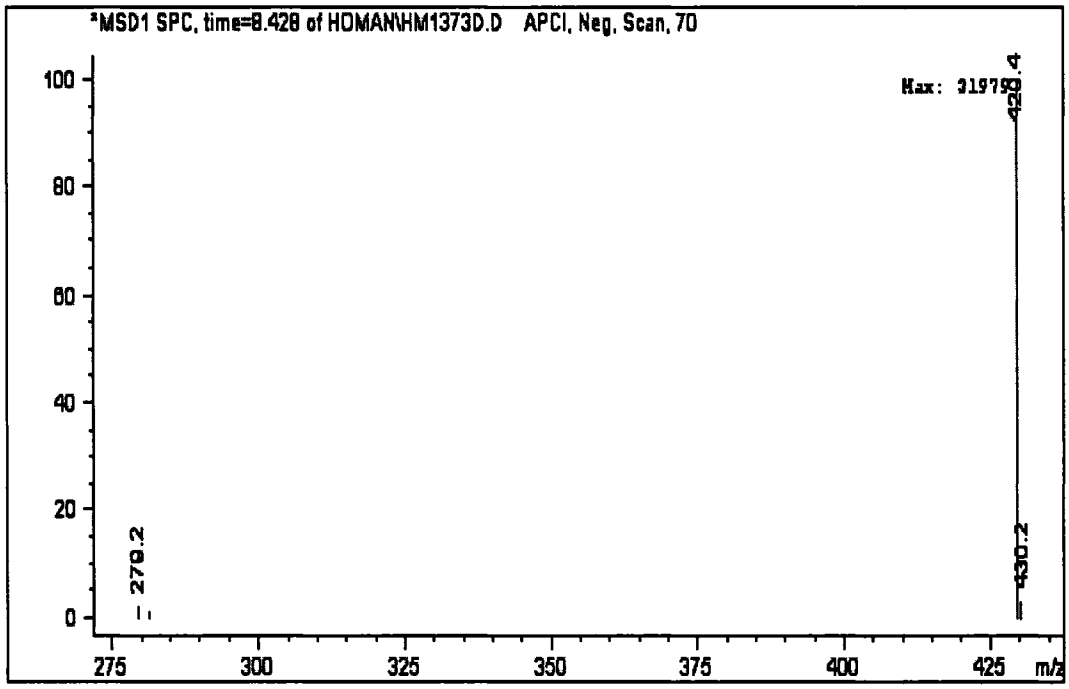

Figure A.11. Mass spectrum of α-tocopherol

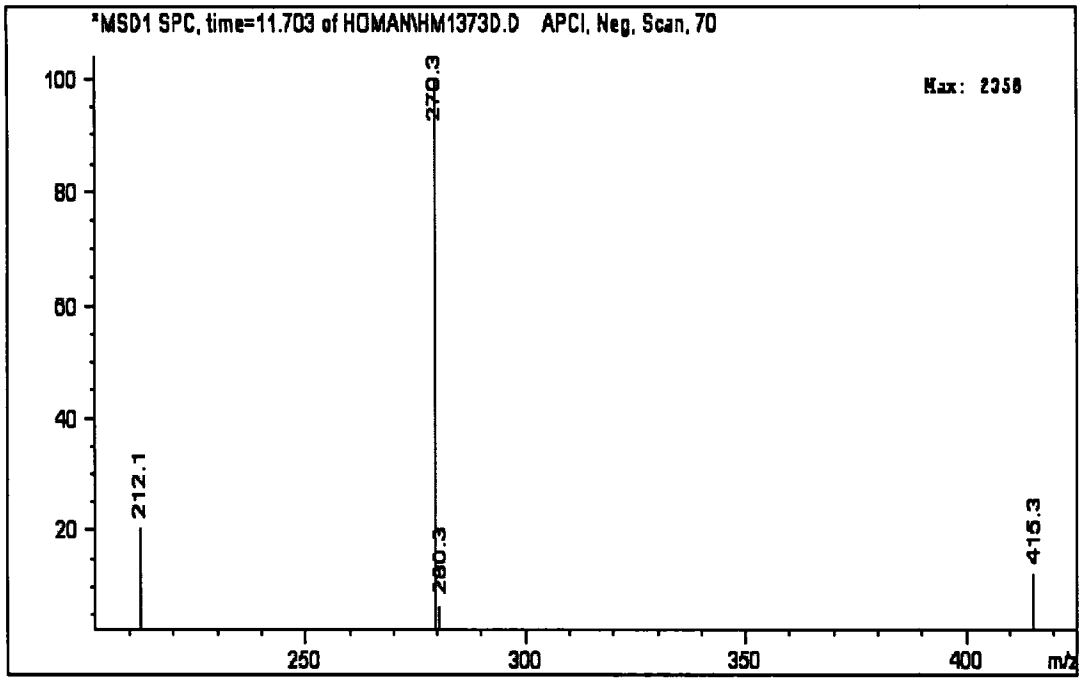

Figure A.12. Mass spectrum of β-tocopherol

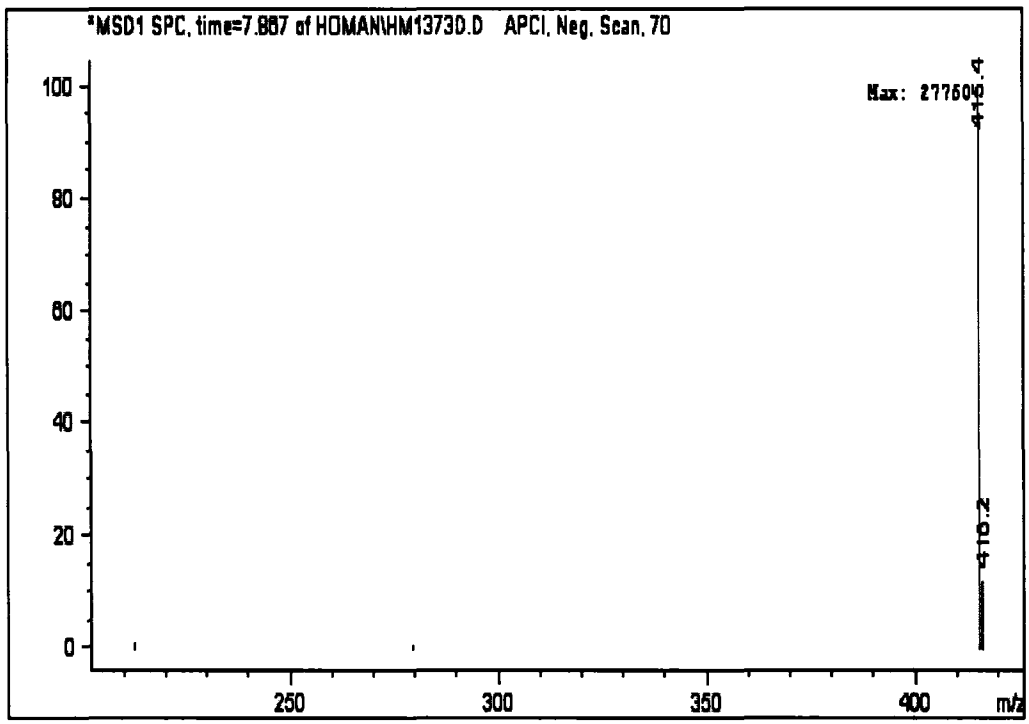

Figure A.13. Mass spectrum of γ-tocopherol

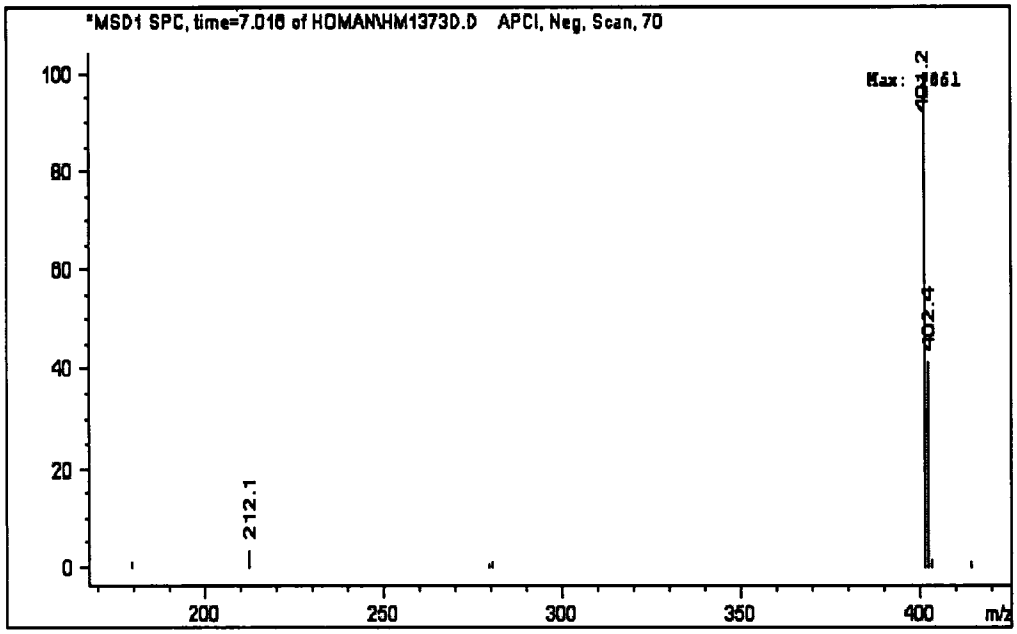

Figure A.14. Mass spectrum of δ-tocopherol

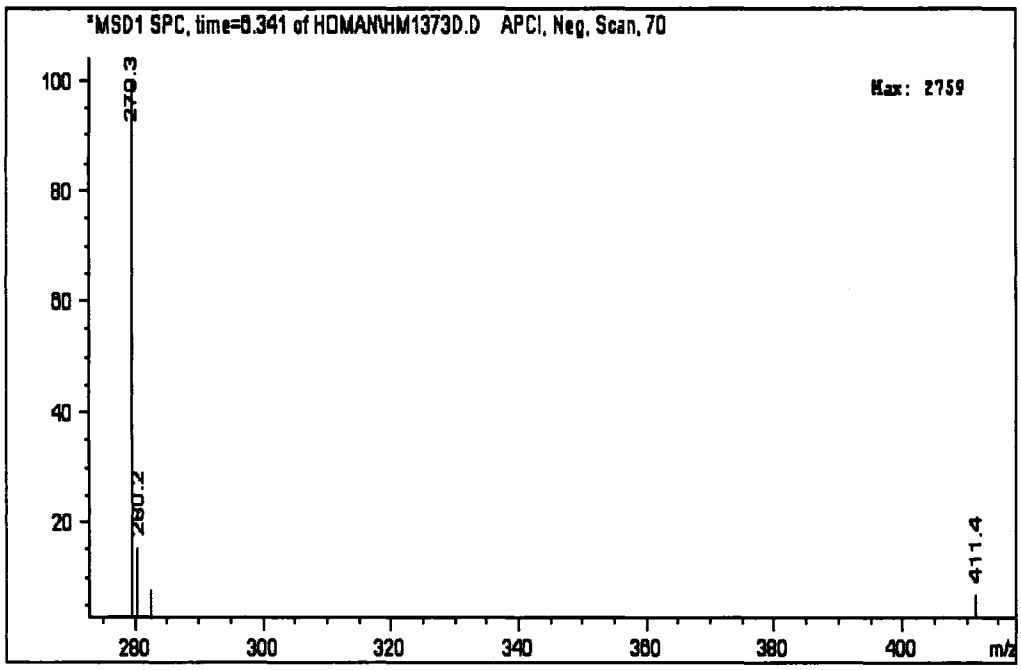

Figure A.15. Mass spectrum of γ-tocotrienol

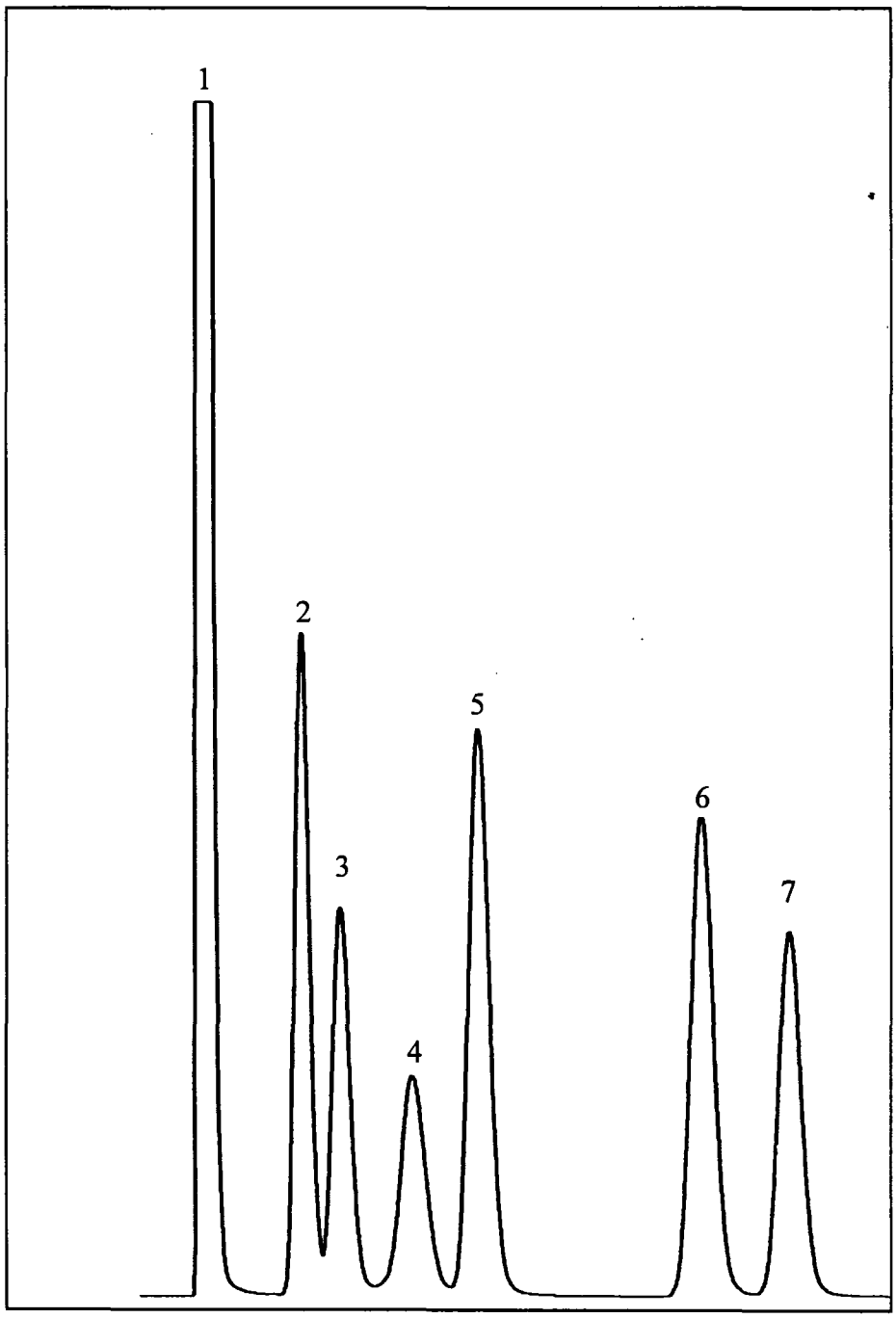

Figure A.16. Representative headspace gas chromatogram of aldehyde standards.
Key: 1, propanal; 2, butanal; 3, pentanal; 4, hexanal; 5, heptanone; 6, octanal; and 7, nonanal.

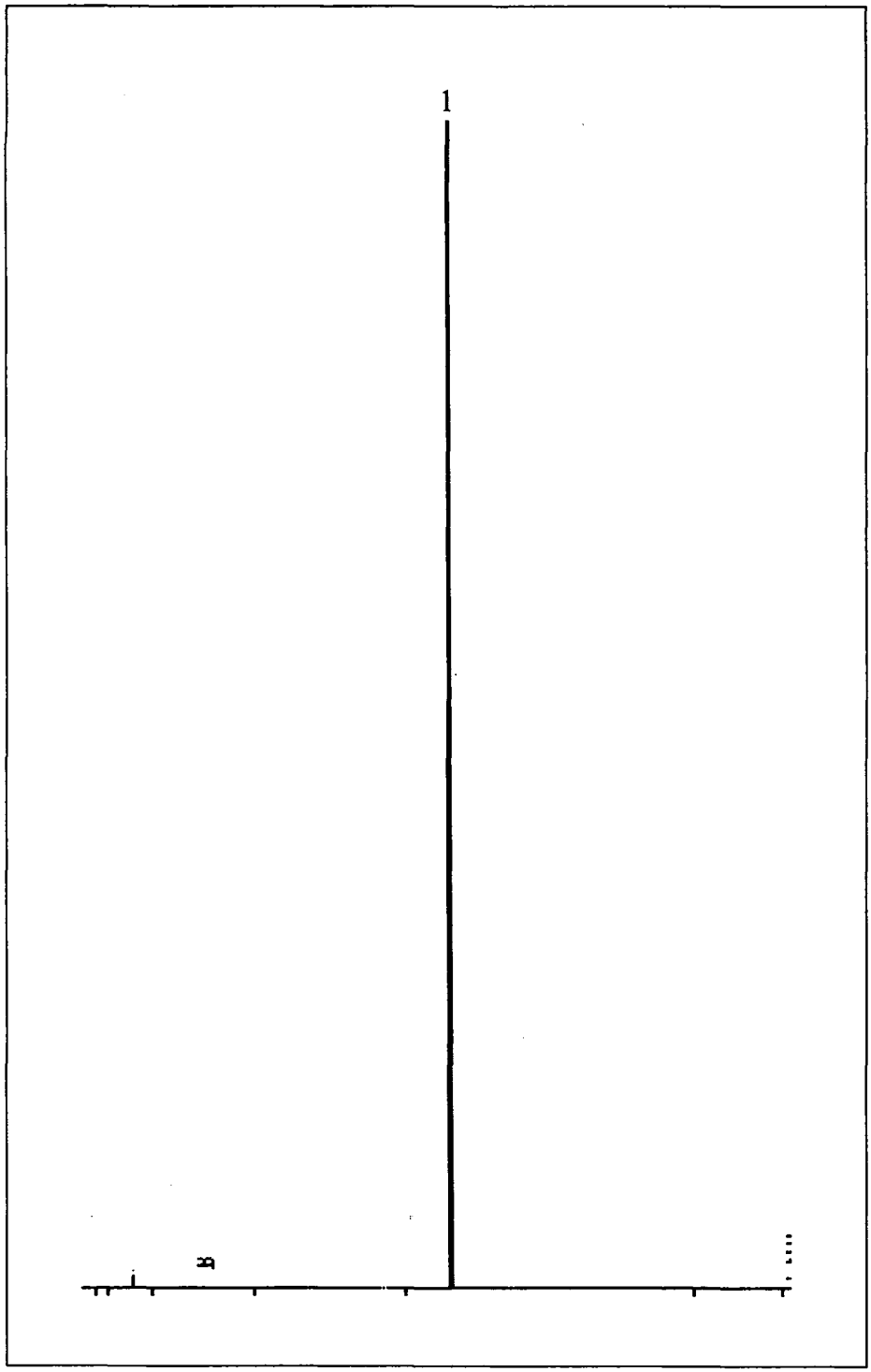

Figure A.17. Headspace chromatogram of unoxidized (day 0) walnut oil.
Key: 1, heptanone (ISD).

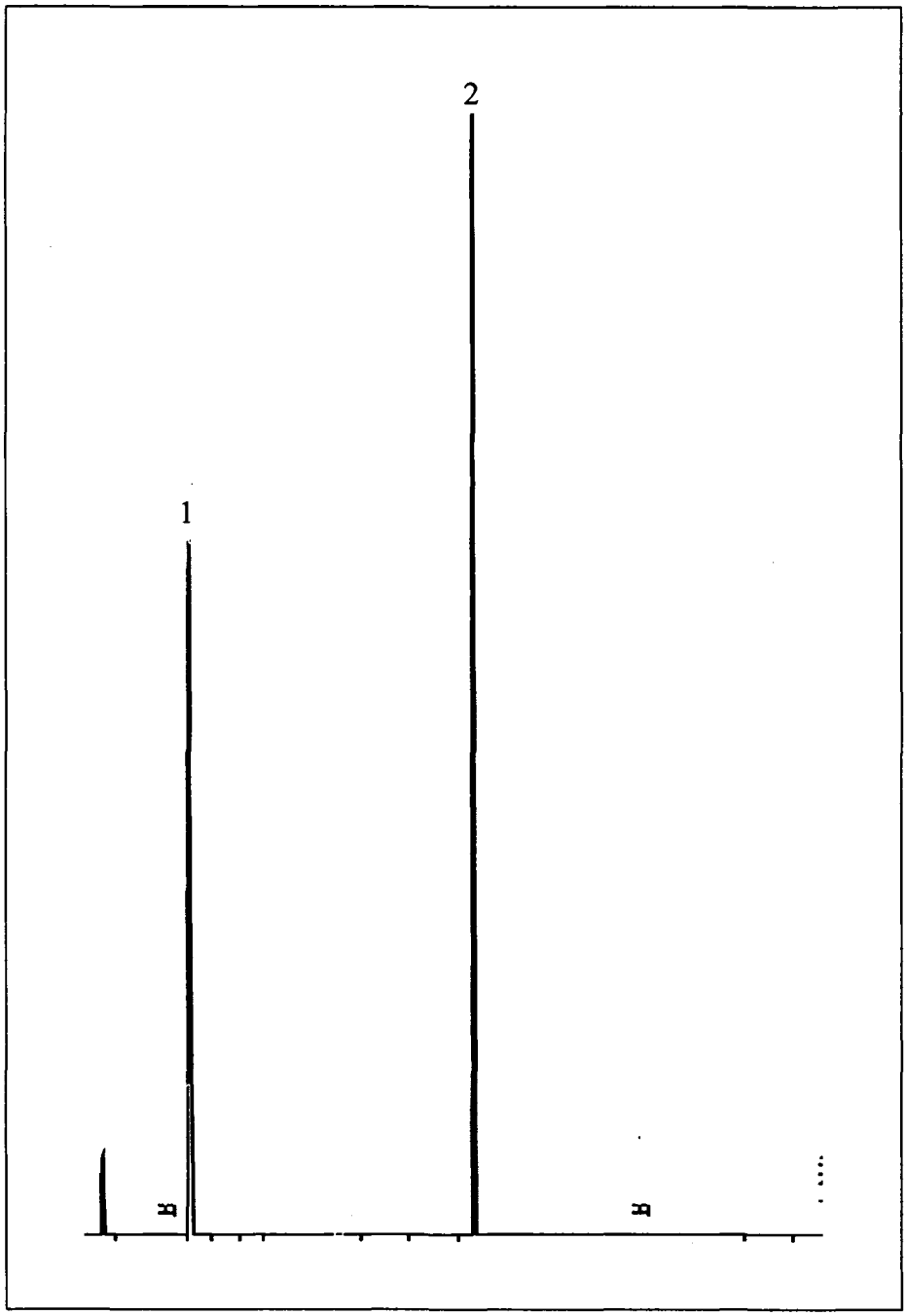

Figure A.18. Headspace gas chromatogram of moderately oxidized (day 3) walnut oil.
Key: 1, propanal; and 2, heptanone (IS).

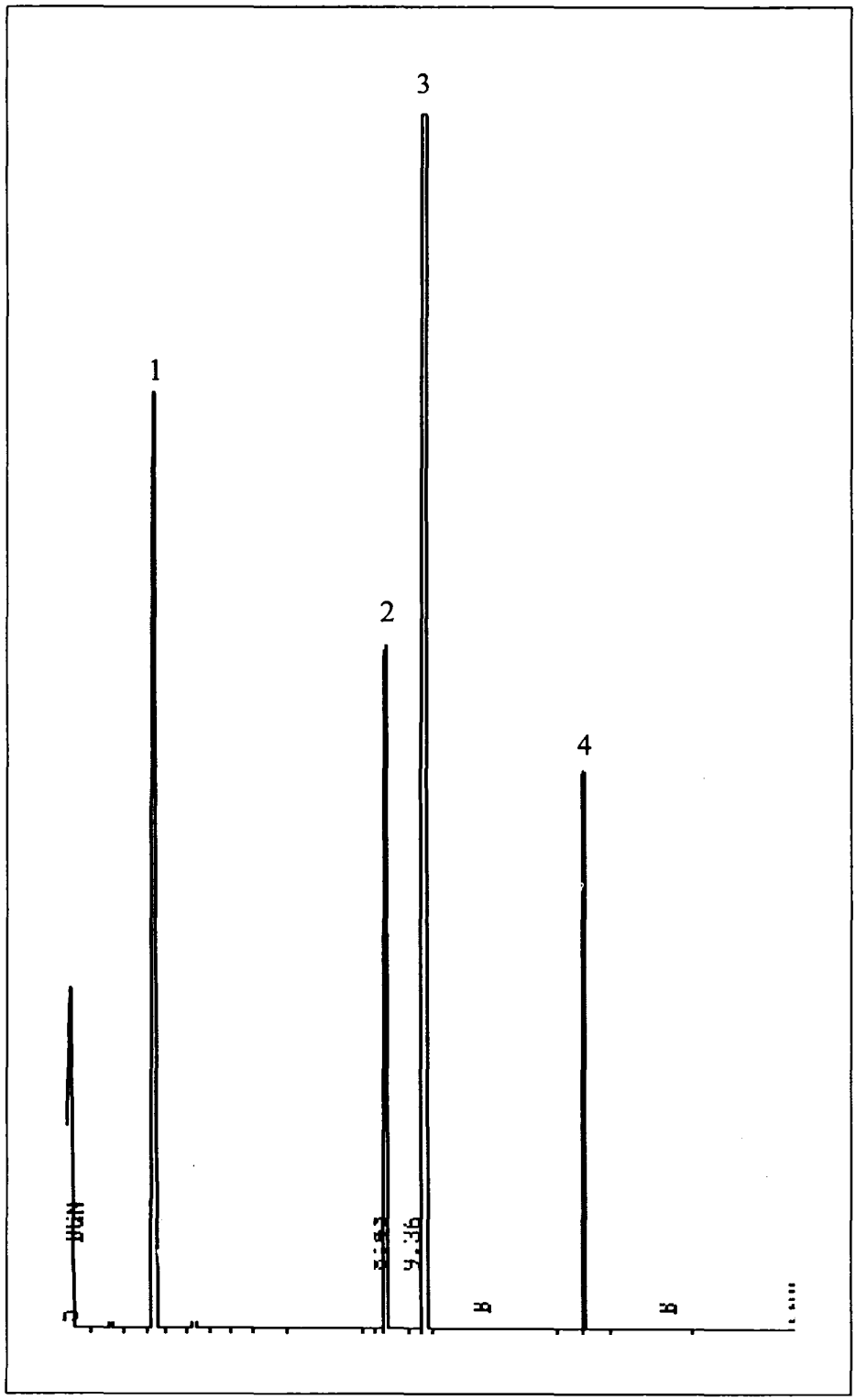

Figure A.19. Headspace gas chromatogram of highly oxidized (day 12) walnut oil.
Key: 1, propanal; 2, hexanal; 3, heptanone (IS); and 4, nonanal.