University of Alberta

CHROMATOGRAPHY APPROACHES FOR ANALYZING FRYING OILS AND ASSESSING THE ANTIOXIDATIVE ABILITY OF SITOSTEROL

by

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Abstract

The novel utility of sitosterol as an antioxidant in frying oils is explained mechanistically based on physical organic chemistry principles. The antioxidative effect of sitosterol is attributed to the conversion of sterol to steradiene by the 1, 2 elimination of water, which may be catalyzed by hydronium ions resulting from atmospheric moisture present at the air–oil interface. Release of protons, allylic to the conjugated diene system in steradiene, may be responsible for interacting with and terminating lipid oxidation chains. A corresponding increase in the extent of triglyceride (TG) ester hydrolysis is further evidence of release of water in the system resulting from the conversion of sterol to steradiene. High Performance Size Exclusion Chromatography (HPSEC) was used to assess the impact of sitosterol on the extent of TG polymerization in frying oils. A reverse phase C30 column, with longer alkyl chain length coupled with thicker and densely bonded phases, was used to further improve separation and identification of lipid oxidation products. A new adsorption chromatography method using hydrated silica cartridges and solvents, acetonitrile, dichloromethane and tetrahydrofuran, was developed for the measurement of polar content as an indicator of the extent of deterioration in frying oils. Concerns are growing over the potential detrimental effects of lipid oxidation products in frying oils and the ineffectiveness of the available antioxidants at frying temperatures. The potential role of sitosterol as a natural, safe and heat–stable antioxidant in frying oils is an important development for the food processing industry.

LIST OF ABBREVIATIONS

ACN	Acetonitrile
APCI	Atmospheric Pressure Chemical Ionization
BDE	Bond Dissociation Energy
CSF	Corn Steryl Ferulates
DCM	Dichloromethane
DG	Diglyceride
ELSD	Evaporative Light Scattering Detector
ESI–MS	Electrospray Ionization – Mass spectrometry
FFA	Free Fatty Acid
FAME	Fatty Acid Methyl Esters
HPSEC	High Performance Size Exclusion Chromatography
HPLC	High Performance Liquid Chromatography
HOSO	High Oleic Sunflower Oil
IP	Induction Period
LLL	Trilinolein
MALDI	Matrix – Assisted Laser Desorption Ionization
MALLS	Multi Angle Laser Light Scattering
MG	Monoglyceride
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance

OSI	Oxidative Stability Index
PV	Peroxide Value
PUFA	Poly Unsaturated Fatty Acid
RBD	Refined Bleached Deodorized
RI	Refractive Index
RP	Reverse Phase
SLS	Static Light Scattering
TG	Triglyceride
THF	Tetrahydrofuran
TOF–MS	Time Of Flight – Mass spectrometry
UV	Ultra Violet

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Chapter 1 Introduction

With increased consumption of fried foods, concerns over the ingestion of non–volatile lipid oxidation products have grown in recent years. There is increasing evidence of their detrimental effects on health which is particularly relevant in oxidative stress related diseases such as liver damage, intestinal tumors, atherosclerosis and cancer (1). Formation of lipid oxidation products at frying temperatures may be limited by the use of antioxidants. However, commercially available antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), tertiary butyl hydroquinone (TBHQ) and tocopherols are heat–sensitive and volatile (2). Also, synthetic additives in edible oils are not desirable, hence there is a need to discover and evaluate natural and heat–stable sources of antioxidants to limit triglyceride (TG) polymerization in frying oils. The ability of sterols to function as an antioxidant may not be readily apparent. Unlike traditional antioxidants like BHA, BHT, TBHQ and tocopherols that possess a phenolic OH group, the hydroxyl group in sterols is not phenolic in nature. Indeed, it was assumed initially that the presence of sterols in vegetable oils may promote polymerization in the form of TG–sterol dimers. Instead, quite the opposite occurred, and preliminary work in this project showed sitosterol significantly reduced TG polymer formation in frying oils. This unexpected beginning became the foundation for the future direction to investigate the ability of sitosterol to limit TG polymerization.

While a number of volatile decomposition products formed in frying oils have been identified, detailed information on the nonvolatile compounds is unavailable

due to the complexities of this fraction and the limitations of the analytical methodologies used for isolation and quantification of such compounds. The simplicity and high reproducibility of High Performance Size Exclusion Chromatography (HPSEC) in combination with adsorption chromatography have contributed significantly to new method development in lipid analysis (3). Application of the HPSEC methodology has enabled a better understanding of the variables that affect frying oil quality based on the extent of TG polymerization in frying oils (4, 5). The combination of adsorption chromatography and HPSEC has enabled the quantification of polar content as an indicator of the extent of deterioration in frying oils and the resulting impact on lipase hydrolysis of TG monomers (6).

The level of polar content in frying oils is considered to be a good indicator of the extent of oxidative degradation. Adsorption chromatography, employing silica cartridges with organic solvents hexane and ether, is normally used for the measurement of polar content in frying oils. A polar content level of 25% is currently being used globally as the limit beyond which frying oil is considered unfit for human consumption. This level is easily reached after a frying duration of about 24 h and it is not uncommon to find oil samples with a level of polar content higher than 25% (7). Hence, it is important to improve existing methods and develop new methods for the quantification and characterization of polar compounds in frying oils. (8)

Objectives and Hypotheses:

The overall purpose of this research was to assess the ability of sitosterol to limit formation of lipid oxidation products in frying oils and to develop new analytical methods using HPSEC to improve separation, identification and quantification of lipid oxidation products in frying oils. The following specific objectives and hypotheses addressed this overall goal.

1) To assess the role of sitosterol as an antioxidant in frying oils. It was hypothesized that sitosterol may undergo a transformation at frying temperatures and acquire structural features that enable it to function as an antioxidant.

2) To estimate molecular weights of heated triolein fractions. It was hypothesized that polystyrene equivalent molecular weights of heated triolein fractions may assist with the characterization of new lipid oxidation products.

3) To use reverse phase C30 stationary phase to improve separation and identification of lipid oxidation products. It was hypothesized that longer alkyl chain length coupled with thicker and densely bonded phases of the C30 stationary phase may improve separation and hence identification of new lipid oxidation products.

4) To develop a new method for the quantification of polar compounds. It was hypothesized that use of a different blend of organic solvents may

enable quantification of components in polar compounds considered physiologically harmful.

5) To assess impact of polymer content on lipase hydrolysis of TG monomers. It was hypothesized that an increase in polymer content may impair the extent of lipase hydrolysis of unaltered TG monomers.

Thesis organization:

This thesis is divided into 8 chapters. After the introduction in chapter 1, chapter 2 reviews the scientific literature on four topics in the following sequence: lipid oxidation, sterol in vegetable oils, the use of HPSEC in lipid analysis and lipase hydrolysis. Chapter 3 provides a mechanistic explanation for the role of sitosterol as an antioxidant in frying oils. Chapter 4 provides molecular weight estimates of various heated triolein fractions. Chapter 5 documents the new use of C30 column and Time of Flight–Mass Spectrometry (TOF–MS) for the separation and identification of non–volatile lipid oxidation products in heated triolein. Chapter 6 includes a new adsorption chromatography method for estimating polar content levels in frying oils. Chapter 7 covers the impact of level of polymer content on TG monomer hydrolysis in frying oils. Finally, chapter 8 provides general conclusions and recommendations.

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Chapter 2 Literature Review

2.1 Lipid Oxidation

2.1.1 Introduction

Frying oil acts as a heat transfer medium and contributes to the crispy texture and desirable flavor of fried foods. Deep–fat frying is also known to impact the nutritional quality of foods. Chemical reactions occurring during the frying process include hydrolysis, oxidation, and polymerization, which result in the formation of volatile and non–volatile compounds. While most volatile compounds volatilize, some get absorbed in fried foods. Non–volatile compounds however get ingested with the food and hence are nutritionally more relevant. Frying temperature and time, frying oil, antioxidants, and the type of fryer are all known to affect the hydrolysis, oxidation, and polymerization reactions during frying.

Oxidative stability of oils, an important indicator of quality and shelf life, can also be affected during processing and storage (1). Autoxidation and photosensitized oxidation are the reaction mechanisms responsible for the oxidation of edible oils during processing and storage depending upon the types of oxygen involved, atmospheric triplet oxygen $^3O_2$, or singlet oxygen $^1O_2$. The triplet oxygen $^3O_2$ is the ground state of the oxygen molecule and has two unpaired electrons in the $2p\pi^*$ anti–bonding orbitals with a permanent magnetic moment and three closely grouped energy states under a magnetic field (1). Photosensitized oxidation of edible oils occurs in the presence of singlet oxygen $^1O_2$, produced in the presence of light, sensitizers, and atmospheric oxygen. In singlet oxygen $^1O_2$, one $2p\pi^*$

anti–bonding orbital has a pair of electrons while the other is empty. Singlet oxygen has only one energy level under a magnetic field and is electrophilic in nature. The electrophilic singlet oxygen can readily react with compounds with high electron densities, such as the double bonds of unsaturated fatty acids (1). Hence, oxidation of edible oils can be influenced by an energy input such as light or heat, composition of fatty acids, types of oxygen, and minor compounds such as metals, pigments, phospholipids, free fatty acids (FFA), monoglycerides (MG) and diglycerides (DG), thermally oxidized compounds, and antioxidants (1).

2.1.2 Autoxidation mechanism

Autoxidation of oils follows free radical chain reaction which includes initiation, propagation, and termination steps.

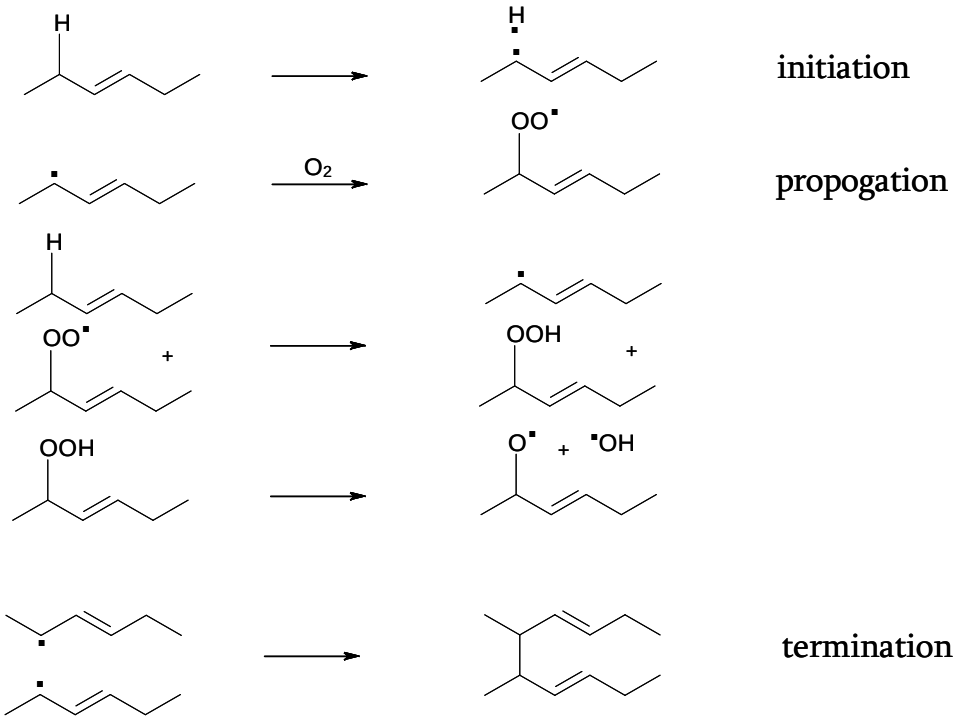


Figure 2.1.1 Autoxidation mechanism (redrawn form reference 4)

Autoxidation of oils requires fatty acids to be in radical forms, which is attained by the removal of a hydrogen atom in the initiation step. Heat, metal catalysts, and ultraviolet and visible light can accelerate removal of a hydrogen atom and free radical formation in fatty acids (1). The ease of removal of the hydrogen atom is dictated by its position in the fatty acid molecule. An allyl hydrogen atom located adjacent to a double bond or one located between two double bonds can be removed easily due to the stabilisation of the resulting free radical by delocalization (2). The energy required for the removal of hydrogen at C11 of linoleic acid located between two double bonds is 50 kcal / mol whereas the energy requirement for the removal of allyl hydrogen at C8 and C14 of linoleic acid is 75 kcal/ mol (2). The autoxidation of linoleic and linolenic acids has been reported to produce only conjugated products (2).

Lipid alkyl radicals react rapidly with triplet oxygen $^3O_2$ to form lipid peroxy radicals hence the concentration of lipid alkyl radical is much lower than that of lipid peroxy radical under normal oxygen pressure (3). The lipid peroxy radicals then abstract hydrogen from other lipid molecules to form hydroperoxides and another lipid alkyl radical. These radicals further catalyze the oxidation reaction resulting in the free radical chain reaction. Oxygen availability and temperature are known to influence the rates of lipid peroxy radical and hydroperoxide formation (4). The termination step of the free radical chain involves reaction between radicals to form nonradical species.

The primary oxidation products, lipid hydroperoxides are relatively stable at room temperature. However, at high temperatures or in the presence of metals,

hydroperoxides readily decompose by a homolytic cleavage between the oxygen – oxygen bond, resulting in the formation of alkoxy and hydroxy radicals, which subsequently undergo homolytic β–scission to generate aldehydes, ketones, acids, esters, alcohols, and short–chain hydrocarbons (5). The activation energy to cleave the oxygen–oxygen bond in hydroperoxide is 46 kcal / mol lower than that to cleave the oxygen–hydrogen bond (6).

2.1.3 Mechanism of photosensitized oxidation of edible oils

Oxidation in edible oils is accelerated by light in the presence of sensitizers such as chlorophyll. Sensitizers in singlet state absorb light energy to become excited and then return to their ground state via emission of light, internal conversion, or intersystem crossing (7). Fluorescence and heat are produced by emission of light and internal conversion, respectively, while intersystem crossing results in excited triplet state of sensitizers. Excited triplet sensitizers may accept hydrogen or an electron from the substrate to produce radicals (type I) or react with $^3O_2$ to produce $^1O_2$ oxygen (type II) (7).

A sensitizer molecule may generate 10^3 to 10^5 molecules of $^1O_2$ before becoming inactive (7). The rate of the type I or II processes depends on the kinds of sensitizers and the type and concentration of substrates and oxygen. Readily oxidized phenols or amines, or readily reducible quinones favour the type I process, while olefins, dienes, and aromatic compounds, which are not so readily oxidized or reduced, favour the type II process. Photosensitized oxidation of edible oil follows the singlet oxygen oxidation pathway wherein the electrophilic $^1O_2$ can directly react with the high–electron–density double bonds

to form hydroperoxides at the double bonds without the formation of alkyl radicals. Hydroperoxide formation is accompanied with double bond migration and generation of *trans* isomers, resulting in both conjugated and non–conjugated products (7).

2.1.4 Factors affecting the oxidation of edible oils

Fatty acid composition of the oil, oil processing, heat or light, concentration and type of oxygen, FFA, MG and DG, transition metals, peroxides, thermally oxidized compounds, pigments, and antioxidants are some of the factors that may impact the oxidative stability of oils.

a. Fatty acid composition of oils

Oils with higher levels of unsaturation are more prone to oxidation than the less unsaturated oils. As the degree of unsaturation increases, both the rate of formation and the amount of primary oxidation compounds accumulated at the end of the induction period increase (9, 10). Soybean, safflower, or sunflower oil with iodine values greater than 130 stored in the dark have a shorter induction period in comparison to coconut or palm kernel oil with iodine value less than 20 (11). Based on oxygen uptake, the relative autoxidation rate of oleic, linoleic, and linolenic acids are 1: 50:100 (12). Similar comparison for $^1O_2$ oxidation rate results in less significant differences as indicated by the reaction rate values for stearic, oleic, linoleic, and linolenic acids of 1.2×10^4, 5.3×10^4, 7.3×10^4, and 10.0×10^4 $M^{-1}s^{-1}$, respectively (13).

b. Oil processing

Oil–processing methods impact oxidative stability of oils. Higher oxidative stability of crude oil in comparison to refined oil has been attributed to higher concentrations of unsaponifiables and tocopherols in crude oil than refined oil. The induction time for hexane–extracted rapeseed oil oxidation at 90 ^{o}C is 10.5 h whereas it is 8.1 h for pressed rapeseed oil (14). Oxidative stability is significantly lower in supercritical carbon dioxide–extracted walnut oil than in pressed oil (15). Improved oxidative stability of safflower and sesame oil by roasting of seeds before extraction may be due to the formation of Maillard products with antioxidant properties (16).

c. Temperature and light

The formation of autoxidation products during the induction period is slower at low temperature. There is an increase in hydroperoxides during the induction period followed by a significant increase in polymerized compounds at the end of the induction period of autoxidation (17). The hydroperoxide decomposition rate of crude herring oil at 50 ^{o}C in the dark is higher than the rate of formation, while the reverse is true at 20 ^{o}C (18). In the case of $^{1}O_{2}$ light is more important than temperature. Light of shorter wavelength can have more detrimental effects on oils compared to longer wavelength (19). Also, the effect of light on oil oxidation has been reported to be less important as temperature increases (17).

d. Oxygen

The effect of oxygen concentration on the oxidation of oil is higher at high temperatures and in the presence of light and metals such as iron or copper. This

higher oxygen dependence of oil oxidation at high temperatures is attributed to the lower solubility of oxygen in the oil at high temperatures (20). At low temperatures during storage when the oil is not stirred, oxygen transfer into the oil may take place by diffusion whereas convection is believed to be the pathway for oxygen penetration into the oil during processing at high temperature when the oil is stirred. The oil oxidation rate is independent of oxygen concentration at oxygen concentrations above 10% in the oxidation of methyl linoleate (21), while at low oxygen content, the oxidation rate is dependent on oxygen concentration and is independent of lipid concentration (20). The autoxidation rate of oil at more than 4% to 5% oxygen in the headspace is independent of oxygen concentration and directly dependent on the lipid concentration (22), whereas the reverse is true at low oxygen pressure of less than 4% in the headspace (23). The type of oxygen significantly influences the reaction rate between lipid and oxygen. The reaction rate of $^1O_2$ with lipid is much higher than that of $^3O_2$ because $^1O_2$ can directly react with unsaturation in lipids while $^3O_2$ reacts with the radical state of lipids. Linoleates can react with $^1O_2$ at a rate 1,450 times faster than with $^3O_2$ (24).

e. Surface to volume ratio

An increase in the surface–to–volume ratio of the oil sample increases the extent of oxidation with the relative rate of oxidation being less oxygen–dependent even at low oxygen content (25). The container surface acts as a reduction catalyst, with an effect proportional to the area of the container in contact with the oils (26).

f. Minor components present in oil

In addition to triglycerides (TG), edible oils also contain minor components such as FFA, MG and DG, metals, phospholipids, peroxides, chlorophylls, carotenoids, phenolic compounds, and tocopherols. Some of these accelerate oil oxidation and others act as antioxidants.

i. FFA, MG and DG: The process of edible oil refining lowers the FFA content present in crude oils. The level of FFA in crude soybean oil has been reported to be about 0.7% which declines to 0.02% after refining (27). The bleaching step during refining can lower the FFA content from 0.72% to 0.56% in sesame oil extracted from roasted sesame seeds (28). FFA are more susceptible to autoxidation than esterified fatty acids and have a pro–oxidant effect in edible oils (29). Given their ampiphilic nature, FFA tend to concentrate at the oil surface, thus lowering the surface tension and increasing the diffusion rate of oxygen from the headspace into the oil to accelerate oil oxidation (30). Similarly, MG and DG, usually present at 0.07% – 0.11% and 1.05% – 1.20% levels respectively in soybean oil, with free hydroxyl groups are also ampiphilic in nature and act as pro–oxidants at 55 ^{o}C in the dark (31, 32).

ii. Metals: Crude oil is known to contain transition metals such as iron or copper at levels of 13.2 ppb and 2.80 ppm of copper and iron respectively in soybean oil. These metals increase the rate of oil oxidation by lowering the activation energy of the initiation step for autoxidation (33) and can directly react with lipids to produce lipid alkyl radicals and produce reactive oxygen species such as $^{1}O_2$ from $^{3}O_2$ and hydroxy radicals from hydroperoxide (20). Metals can

also accelerate autoxidation of oils by the decomposition of hydroperoxides (34). Fe^{2+} is much more active with a rate of 1.5×10^3 $M^{-}1s^{-}1$ than Fe^{3+} in decomposing the lipid hydroperoxides to catalyze autoxidation (35). The iron–binding ability of lactoferrin in fish oil and soybean oil can suppress the pro–oxidant activity of iron (36).

iii. *Phospholipids:* Phospholipids occurring in edible oils include phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidic acid. A typical level of phosphatidylcholine and phosphatidylethanolamine in crude soybean oil has been reported to be 500.8 and 213.6 ppm, respectively (37). However, most of the phospholipids are removed during the degumming step of the refining process and refined, bleached and deodorized (RBD) soybean oil contains much lower levels of phosphatidylcholine and phosphatidylethanolamine (0.86 and 0.12 ppm, respectively) (37). Phospholipids can act as antioxidants or pro–oxidants depending on their concentration and presence of metals. Phospholipids decrease oil oxidation likely by sequestering metals and the nitrogen–containing phospholipids, phosphatidylcholine and phosphatidylethanolamine are efficient antioxidants under most conditions (38). Phospholipids at concentration levels of 3 and 60 ppm can provide maximal antioxidant activity whereas at higher levels may function as pro–oxidants. The pro–oxidant effect of phospholipids may be attributed to their ampiphilic nature which causes a lowering of surface tension and hence an increase in the diffusion of oxygen from headspace into the oil to accelerate oil oxidation (39).

iv. *Chlorophylls*: Chlorophylls are common pigments present in edible oil. The pigment level is drastically reduced during the bleaching step of vegetable oil refining with typical chlorophyll concentrations of 0.30 and 0.08 ppm, respectively in crude and bleached soybean oils (27). Chlorophylls and their degradation products, pheophytins and pheophorbides can act as sensitizers to produce $^1O_2$ in the presence of light and atmospheric $^3O_2$ to accelerate the oxidation of oil (40). Purified soybean oil without any chlorophyll under light at 10 ◦C produced no headspace volatiles resulting from oxidation but soybean oil with added chlorophyll and RBD soybean oil produced headspace volatiles under the same experimental conditions (41). Although strong pro–oxidants under light, chlorophylls can function as antioxidants in the dark possibly by donating hydrogen to free radicals (42).

v. *Thermally oxidized compounds*: Non–volatile lipid oxidation products such as cyclic and noncyclic, non–polar and polar dimers and trimers, resulting from the refining of crude oils at high temperature, can impact oxidative stability of edible oils. Thermally oxidized compounds at a level of 1.2% in RBD soybean oil have been reported to accelerate autoxidation at 55 ^oC (43). Lipid hydroperoxides can also act as pro–oxidants. The products resulting from hydroperoxide decomposition are ampiphilic in nature, containing both hydrophilic and hydrophobic components, hence can lower surface tension and increase introduction of oxygen into the oil to accelerate oil oxidation (44).

2.1.5 Antioxidants

Edible oils naturally contain antioxidants such as phenolic compounds,

tocopherols, tocotrienols, carotenoids, and sterols. Antioxidants are compounds that can extend the induction period of oxidation or slow down the oxidation rate by scavenging free radicals such as lipid alkyl radicals or lipid peroxy radicals, sequestering transition metals, quenching singlet oxygen, and inactivating sensitizers.

a. Phenolic compounds

Monohydroxy or polyhydroxy phenolic compounds represent the major hydrogen donating antioxidants since the aromatic ring enables stabilization of the resulting radical by delocalisation. Compounds with reduction potential lower than that of a free radical can donate hydrogen to that radical. Standard 1– electron reduction potentials for alkoxy, peroxy, and alkyl radicals of polyunsaturated fatty acids have been estimated to be 1600, 1000, and 600 mV, respectively, and that of antioxidants is generally 500 mV or less (45). Hence, antioxidants can react with lipid peroxy radicals before the peroxy radicals produce another free radical by reacting with another lipid molecule. Also, the antioxidant radical produced from the reaction with lipid peroxy radical has lower energy than the lipid peroxy radical itself due to stabilization by delocalisation.

b. Phosphoric acid, citric acid, ascorbic acid, and EDTA

These are known to limit oxidation by sequestering iron or copper ions into insoluble complexes and sterically hinder formation of complexes between metals and lipid hydroperoxides (46). Excited sensitizers or $^1O_2$ may be quenched physically or chemically by antioxidants. Unlike, physical quenching which entails conversion of $^1O_2$ into $^3O_2$ by energy transfer or charge transfer without

oxidizing the antioxidant, chemical quenching of $^1O_2$ results in oxidized antioxidants (47).

c. Tocopherols

Tocopherols are identified as the most important antioxidants in edible oil with relatively high levels in soybean, canola, sunflower, and corn oils. Tocopherols compete with unsaturation in lipids for peroxy radicals and react with lipid peroxy radicals at a much higher rate (10^4 to 10^9 $M^{-1}s^{-1}$) in comparison to lipids (10 to 60 $M^{-1}s^{-1}$). One tocopherol molecule can protect about 10^3–10^8 polyunsaturated fatty acid molecules at low peroxide values (48).

Tocopherols scavenge peroxy radicals by transferring the hydrogen atom at the 6–hydroxy group on its chroman ring to lipid peroxy radicals. With a lower reduction potential of 500 mV, tocopherols can donate hydrogen to lipid peroxy radicals (ROO·) that have a reduction potential of 1000 mV, and produce lipid hydroperoxide (ROOH) and tocopheroxy radicals (48). Tocopheroxy radicals are more stable than lipid peroxy radicals due to greater delocalisation and may react with each other at low lipid oxidation rates and produce tocopheryl quinone and tocopherol. At higher oxidation rates, tocopheroxy radicals may react with lipid peroxy radicals and produce tocopherol–lipid peroxy complexes ([T–OOR]), which can be hydrolyzed to tocopheryl quinone and lipid hydroperoxide (49). The effectiveness of tocopherols as antioxidants depends on the isomers and concentration of tocopherols. Free radical scavenging activity of tocopherols is the highest in δ–tocopherol followed by γ –, β–, and α–tocopherol (50). In addition to their free radical scavenging activity, tocopherols may decrease

soybean oil oxidation under light by $^1O_2$ quenching, which again depends upon tocopherol concentration and type, with the activity decreasing in the order of *α*–, β–, *γ* –, and *δ*– tocopherol (51). At high concentrations, *α*–tocopherol may act as a pro–oxidant in vegetable oils. At low levels of lipid peroxy radicals, the tocopheroxy radical may remove hydrogen from the lipid molecule to produce tocopherol and a lipid alkyl radical, thereby accelerating lipid oxidation. The highest pro–oxidant activity was shown in *α*–tocopherol followed by *γ* – and *δ*–tocopherol in soybean oil autoxidation (52).

d. Carotenoids

Edible oils, especially unrefined oils, contain *β*–carotene. This is one of the most studied carotenoids, consisting of isoprenoid units with double bonds in conjugated and *trans* forms. *β*–Carotene can slow down oil oxidation by light filtering, $^1O_2$ quenching, sensitizer inactivation, and free radical scavenging (53). A reduction in the extent of oxidation of olive oil containing only *β*–carotene under light at 25 ^oC was reported to be due to the filtering of light energy between 400 and 500 nm (53). Carotenoids can quench $^1O_2$ by energy transfer with the excited carotenoids returning to the ground state by giving off heat. The $^1O_2$ quenching effect of carotenoids depends on the number of conjugated double bonds with carotenoids having at least 9 conjugated double bonds being the most effective (45). *β*–carotene has a 1–electron reduction potential of 1060 mV and can donate hydrogen to a hydroxyl radical, which has a high reduction potential (2310 mV), but not to alkyl (600 mV) or peroxy radical (1000 mV) of polyunsaturated fatty acid (54). The resulting carotene radical (Car·) is stabilized

due to delocalization over the conjugated polyene system. At low oxygen concentration, a carotene radical may react with other radicals such as lipid peroxy radicals and form non–radical products (45).

e. Other phenolic compounds

Sesame oil, despite a high iodine value of 109 is quite stable with an autoxidation rate much lower than that of corn oil, safflower oil, and a mixture of soybean and rapeseed oils at 60 ^{o}C (55). The remarkable oxidative stability of sesame oil has been attributed to lignan compounds which include sesamin, sesamol, sesamolin, sesaminol, and sesamolinol (56). Phenolic compounds in olive oil include tyrosol (4–hydroxy phenyl ethanol), hydroxytyrosol (3, 4–dihydroxy phenyl ethanol), hydroxybenzoic acids, oleuropein, caffeic acid, vanillic acid, *p*–coumaric acid, and derivatives of tyrosol and hydroxytyrosol (57). The phenolic compounds in olive oil act as antioxidants mainly at the initial stage of autoxidation by scavenging free radicals and chelating metals (58). Ortho–diphenols such as caffeic acid may get oxidized to quinones by ferric ions and become ineffective in inhibiting iron–dependent free radical chain reactions in oil. However, hydroxytyrosol, tyrosol, vanillic acids, and *p*–coumaric acid remain unaffected by the ferric ions (59). Chlorogenic acid and caffeic acid are the principal phenols found in sunflower oil (60).

f. Antioxidant interactions

Interactions between multi–component antioxidants in edible oils associated with a metal chelator and a free radical scavenger lead to better antioxidant activities compared to using them separately (61, 62). This is caused mainly by the action

of the chelator at the initiation step of oxidation and that of the free radical scavenger at the propagation step. Synergistic effects between α–tocopherol and β– carotene occurring during autoxidation (61) and photosensitized oxidation of soybean oil (62) are attributed to the protection of β–carotene from degradation by α–tocopherol (63). Synergism between tocopherol and ascorbic acid also exists because ascorbic acid can provide hydrogen to the tocopheroxy radical produced from the reaction between the lipid peroxy radical and tocopherol, to regenerate tocopherol (64). The nitrogen moiety of phosphatidylethanolamine and phosphatidylcholine donates a hydrogen or electron to tocopherols and delays the oxidation of tocopherols to tocopheryl quinone (37). Sesamol and sesaminol have synergistic antioxidant activities with γ –tocopherol in the autoxidation of sunflower oil (65). Polyphenols are effective stabilizers of α–tocopherol during heating as higher percentages of α–tocopherol remain in the oil in the presence of increasing concentration of polyphenols (66).

2.1.6 Chemical reactions during deep fat frying

a. *Hydrolysis*

Oxygen, moisture from food and from the atmosphere present at the air–oil interface may initiate the chemical reactions in frying oil. Hydronium ions resulting from moisture at the air–oil interface may attack the ester linkage of TG to produce DG and MG, glycerol, and FFA. The FFA content in frying oil increases with the times of frying and may be used as an indicator to monitor the quality of frying oil (61). Since ester hydrolysis is a reversible process, the extent of hydrolysis may stabilize after an initial increase as was observed with MG and

DG levels in cottonseed oil during frying of potato chips at 155 ^{o}C to 195 ^{o}C (67). Also, sodium hydroxide and other bases used for cleaning a fryer may have an impact on oil hydrolysis (69). FFA and their oxidized compounds can produce off–flavor and make the oil less acceptable for deep–fat frying. DG, MG, FFA and glycerol have all been reported to further increase the extent of hydrolysis (68). A maximum of 0.05% to 0.08% FFA content has been reported in frying oils (69).

b. Oxidation

The chemical mechanism of thermal oxidation is principally the same as autoxidation, involving initiation, propagation, and termination steps. Heat, light, metals, and reactive oxygen species are known to facilitate free radical formation in oil. As in the case of autoxidation, the initiation step involves formation of an alkyl radical by the removal of hydrogen. The site of radical formation in saturated fatty acids is different from those of unsaturated oleic or linoleic acids. The alkyl radical of saturated fatty acids is formed at the position α to the carboxyl group, due to the electron withdrawing effect of the adjacent carboxylic group (70).

c. Polymerization

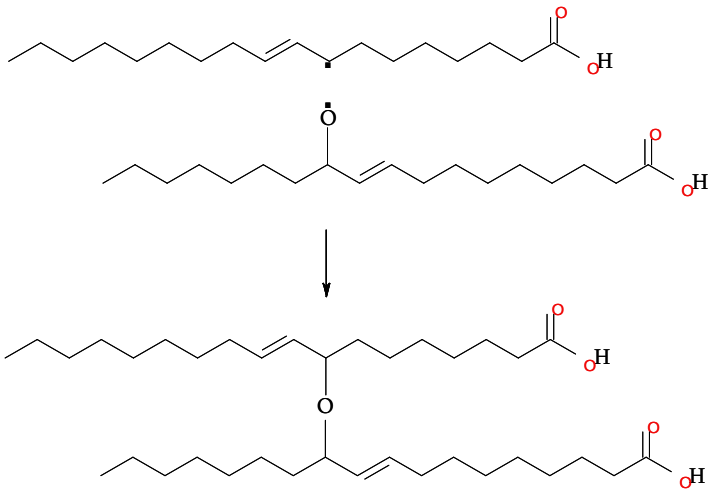
At low or moderate temperatures and atmospheric pressure, solubility of oxygen in oils is high which leads to the generation of alkyl peroxy radicals (ROO$^{\cdot}$) in abundance and as a result, hydroperoxides (ROOH) form the major product. However, as temperature increases, all oxidation reactions are accelerated and the amount of hydroperoxides tends to zero, as their rate of decomposition becomes

higher than their rate of formation (70). At higher temperatures, with reduced oxygen pressure the initiation reaction becomes more important and the concentration of alkyl radicals (R˙) increases. As a result, alkoxy radicals (RO˙) obtained from the decomposition of hydroperoxides and alkyl radicals (R˙) or two alkyl or two alkoxy radicals may react to form polymeric compounds, including monomeric, dimeric, and higher oligomeric TG with modified and unmodified acyl groups (70) (Figure 2.1.2).

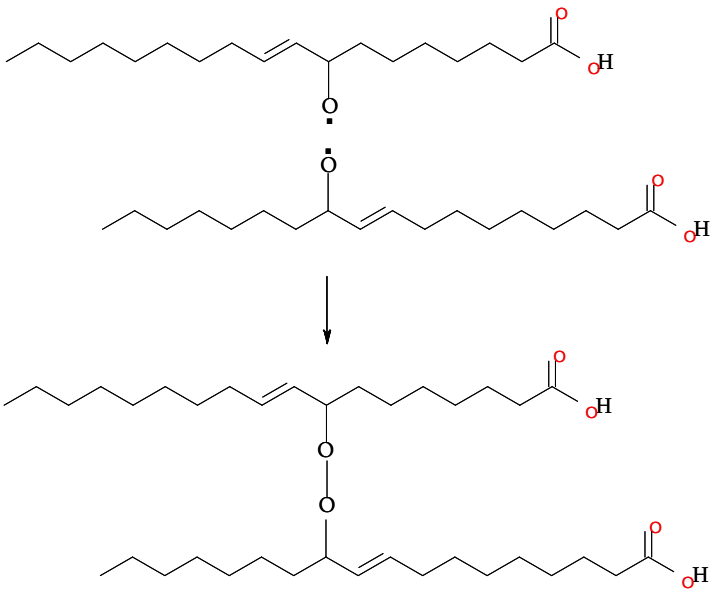
Oxidized TG monomers resulting from the decomposition of hydroperoxides are characterized by the presence of additional oxygen in at least one of the fatty acyl groups of the TG molecule. These include TG containing short chain fatty acyl and short chain n–oxo fatty acyl groups as well as other oxygenated groups like hydroxy, keto and epoxy. Depending on the degree of unsaturation of the fatty acyl group, more than one oxygenated function may be present in the same fatty acyl group and more than one oxidized fatty acyl group may be present in one TG molecule (71). Non–polar dimers are compounds formed through C–C linkages without any extra oxygen in the molecule, mainly from reactions between allyl radicals. Non polar dimers may exist as dehydrodimers resulting from the combination of two allyl radicals. They may also exist in the form of noncyclic dimers by intermolecular addition of the allyl radical to the double bond of an unsaturated molecule or as cyclic dimers by intramolecular addition of an intermediate dimeric radical to a double bond in the same molecule (72). The proportion of different dimers depends on temperature, with the dehydrodimers found in significant amounts at 140 ^{o}C and the mono, bi, and tricyclic dimers

dominating above 250 ^{o}C. In the presence of conjugation, thermal dimers result from the Diels Alder reaction. Non polar dimers are believed to be the most relevant groups among the alteration compounds (72).

Polar dimers may be generated by the oxidation of nonpolar dimers or by the dimerization of two oxidized monomers. Polar dimers have been mainly found with C–C linkages containing monohydroxy, dihydroxy, and keto groups (73). Formation of dimers and polymers depends on the oil type, frying temperature, and number of fryings. The polymer content increases with the number of fryings and an increase in the frying temperature (74). Unlike oleic acid, a higher linoleic acid level impacts polymerization more during deep–fat frying (75). Linolenic acid content greater than 20% promotes the formation of cyclic monomers and polymers (76). The formation of tricyclic dimers and bicyclic dimers of linoleate as well as cyclic monomers during deep–fat frying has been reported in soybean oil (77). Polymers accelerate further degradation of the oil, increase the oil viscosity, reduce the heat transfer, produce foam during deep–fat frying, and develop undesirable color in the food. Polymers also cause the high oil absorption in foods (78).



Ether linkage



Peroxide linkage

Figure 2.1.2 Formation of ether or peroxide linkage polymer in oil during deep–fat frying (redrawn from reference 70)

The volatile compounds in frying oil may also undergo further reactions such as oxidation, dimerization, and polymerization. The amount of volatile compounds present in the oil varies widely depending on the kind of oil, food, and frying conditions. While most volatiles are removed from frying oil along with steam during deep fat frying, these compounds are also known to contribute to the flavor quality of the frying oil and the fried foods (79).

2.1.7 Factors affecting the quality of oil during deep–fat frying

The turnover rate of oil, frying time and temperature, frying oil composition, initial oil quality, composition of food to be fried, type of fryer, antioxidants, and oxygen content affect the deterioration of oil during deep–fat frying.

a. *Replenishment of fresh oil*

Frequent replenishment of fresh oil can decrease the formation of polar compounds, DG, and FFA and increase the frying life and quality of oils. Also, a high ratio of fresh oil to total oil improves frying oil quality (80).

b. *Frying time and temperature*

Longer frying time increases the FFA content and the level of polar compounds such as oxidized TG monomers, non–polar and polar TG dimers and TG polymers (78). High frying temperature accelerates thermal oxidation and polymerization of oils (81). Also, at high frying temperatures, polymers with a peroxide linkage become fewer whereas those with an ether linkage or carbon to carbon linkage increase in concentration (82). Intermittent heating and cooling of oils can cause a greater deterioration of oils than continuous heating due to the enhanced oxygen solubility at lower temperatures (83).

c. *Quality of frying oil*

Higher level of unsaturation can significantly impact thermo oxidative degeneration in frying oils, with a less unsaturated corn oil performing better at frying temperatures in comparison to soybean oil with a higher level of unsaturation (84). Evaluation using rancimat revealed a shorter induction period of 10.1 h for virgin oil with additional oleic acid in comparison to 14.3 h for samples with additional palmitic acid (85). Also, levels of linolenic acid can critically impact frying performance, stability of oil, and the flavor quality of fried food (81).

d. *Composition of foods*

High moisture content of foods may increase oil hydrolysis during deep–fat frying. Starch can increase the degradation of oil whereas the presence of amino acids can protect the oil from degradation during deep–fat frying (86).The presence of transition metals in food, such as iron in meat can increase the rates of oxidation and thermal degradation of oil (87, 88). The application of an edible film, such as hydroxyl propyl methylcellulose to foods can decrease the degradation of frying oil during deep–fat frying (89).

e. *Type of fryer*

A small surface–to–volume ratio of the frying system is recommended to minimize exposure of oil to air during deep–fat frying. Oil oxidation can be reduced by modifying a fryer to have a ratio of oil depth (*D*) to oil area (*A*) with $D/A^{1/2}$ =0.93. Copper or iron fryers may accelerate the oxidation of frying oil because of the leaching of metal ions (90).

f. Antioxidants

Naturally occurring or added antioxidants in oils and foods significantly impact oil quality during deep–fat frying. Tocopherols, BHA, BHT, TBHQ and propyl gallate are less effective at frying temperature due to their loss through volatilization or decomposition. The reported decomposition of tocopherols (12%) in soybean oil, compared to beef tallow (100%) and palm oil (100%) after 8 h frying of steamed noodles at 150 ^{o}C, may be attributed to the higher levels of unsaturation in soybean oil getting preferentially oxidized and thus preventing tocopherol degradation (91). The combination of tocotrienols and carotenes can decrease the oxidation of oil synergistically during frying of potato slices at 163 ^{o}C, likely due to the regeneration of carotenes from carotene radicals by tocotrienols (92).

Lignan compounds in sesame oil such as sesamol, sesamin, and sesamolin are stable during heating and may be responsible for the high oxidative stability of roasted sesame oil heated at 170 ^{o}C (93). A blend of soybean oil with roasted sesame oil was found to have lower levels of conjugated dienoic acids in comparison to soybean oil during frying at 160 ^{o}C (93).

Sterols and their fatty acid esters can improve the oxidative stability of oil during deep–fat frying (94, 95). Silicone can protect oil from oxidation during deep fat frying by the formation of a protective layer at the air–oil interface. The combination of silicone and antioxidants can act synergistically to decrease the oxidation of oil during deep–fat frying (96).

g. Dissolved oxygen content

The level of dissolved oxygen can impact oxidation of oil during frying. Nitrogen or carbon dioxide flushing can decrease the dissolved oxygen content in oil and thus reduce the oxidation of oil during deep–fat frying. CO_2 provides better protection due to its higher solubility and density than nitrogen (97).

h. Effect on nutrients in foods during deep–fat frying

The aldehydes, epoxides, hydroxyl, ketones and dicarboxylic compounds formed during lipid oxidation may react with amines, amino acids, and proteins in fried foods. Carbonyl compounds such as acrolein resulting from lipid oxidation may react with amino acids like asparagine to produce acrylamide and decrease the nutritional value and safety of foods (98).

2.2 Sterols in vegetable oil

2.2.1 Introduction

Phytosterols are important structural components of plant membranes that serve to stabilize phospholipid bilayers just as cholesterol does in animal cell membranes. Sterols in plants exist as free alcohols and as esters with fatty acids, glycosides, and acylated glycosides (99). The most concentrated source of phytosterols is vegetable oils, where they are primarily in the free and esterified forms. Free and esterified sterols may have different physiological effects and the physical properties of free and esterified sterols vary as well. Esterification of phytosterols with long–chain fatty acids is known to increase fat solubility, a characteristic of steryl esters that has been utilized in the preparation of sterol– and stanol–ester fortified margarines (100).

Phytosterols have been widely studied for their hypocholesterolemic, anticarcinogenic, and other health benefits (101, 102). Although they are absorbed in trace amounts, they are known to inhibit the absorption of intestinal cholesterol. Sitosterol usually makes up more than 50% of the phytosterols found in vegetable oils, with stigmasterol, campesterol and brassicasterol being the other significant sterols (Table 2.2.1). In comparison to cholesterol, sitosterol has an additional ethyl group and campesterol an additional methyl group at the C24 position on the side chain. Increased hydrophobicity due to the presence of a longer side chain may be responsible for the relatively poor intestinal absorption of phytosterols when compared with cholesterol. Under similar conditions, in comparison to 20–80% of cholesterol absorption, only 10% of campesterol and 5% of sitosterol absorption has been reported (103). Consumption of 30 g/day of corn oil has been

reported to provide 286 mg of phytosterols, an amount that has been shown to be effective in reducing cholesterol absorption (104). Hence, increasing the aggregate amount of phytosterols consumed in a variety of foods may be an important way of reducing cholesterol levels and preventing coronary heart disease (105).

2.2.2 Nomenclature

Phytosterols may be broadly divided into three main groups based on the number of methyl groups on carbon at 4 position, two (4–dimethyl), one (4–monomethyl) or none (4–desmethyl). (Figure 2.2.1) 4–Dimethylsterols and 4α–mono methylsterols are metabolic intermediates in the biosynthetic pathway leading to the end product 4–desmethyl phytosterols, but they are usually present at low levels in most plant tissues. Cycloartenol and cycloartanol are examples of 4–dimethylsterols, and gramisterol is an example of a 4α–mono methylsterol (99) (Figure 2.2.2).

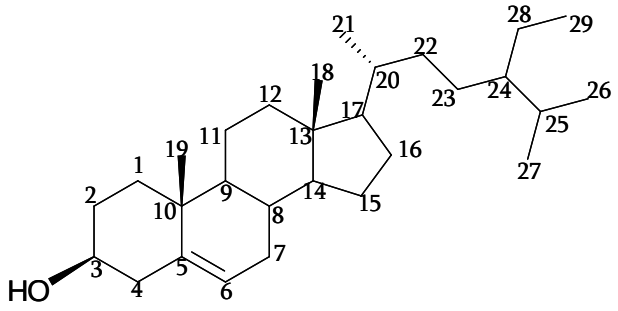


Figure 2.2.1 Nomenclature of phytosterols (Sitosterol = Stigmast–5en–3β–ol = 24R–ethylcholest–5–en–3β –ol) (redrawn from reference 99)

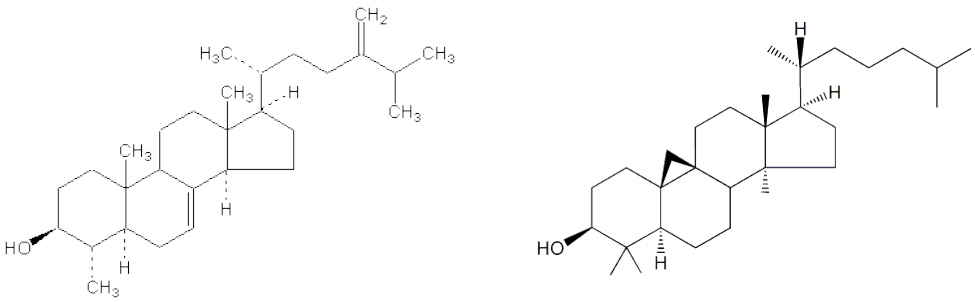


Figure 2.2.2 4α–mono methylsterol (Gramisterol) and 4–dimethylsterol (Cycloartanol) (redrawn from reference 99)

4–Desmethylsterols include the 27–carbon sterol (Figure 2.2.3), cholesterol predominant in animals, but also present in plants at low levels. Also included are 28–carbon (Figure 2.2.4) and 29–carbon (Figure 2.2.5) phytosterols, which are typically major membrane structural components in plant cells. Most 4–desmethyl phytosterols have a double bond between carbons 5 and 6 of the ring system and are thus called Δ5 phytosterols. Another group of desmethyl sterols have a double bond between carbons 7 and 8 and are referred to as Δ7 phytosterols. Both Δ5 and Δ7 desmethyl sterols can include a second double bond in the alkyl side chain between carbons 22 and 23 or carbons 24 and 28 (99).

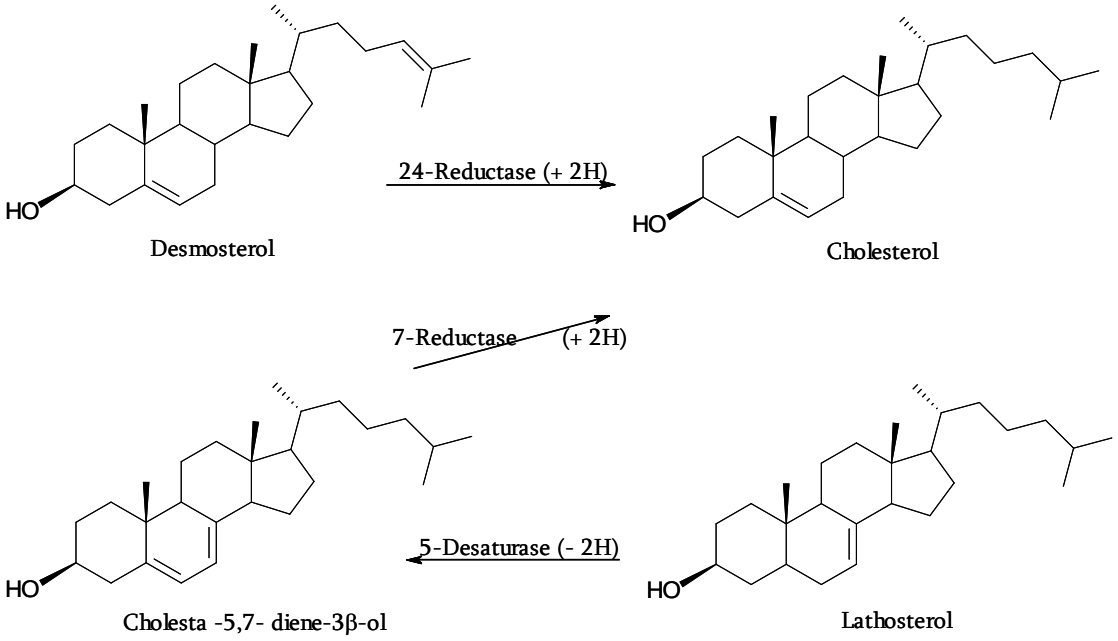
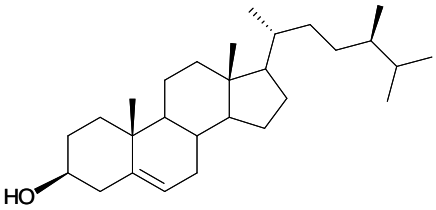
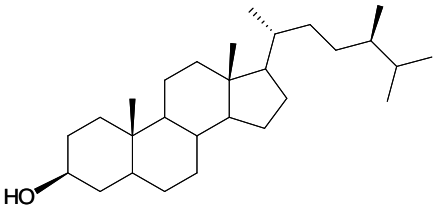


Figure 2.2.3 C27 4–desmethyl sterols (redrawn from reference 99)

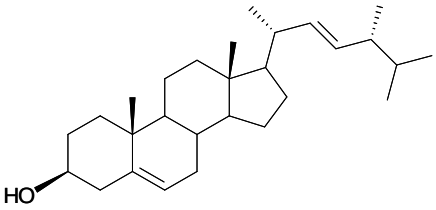
For the C28 and C29 phytosterols the introduction of a methyl or ethyl group at C24 renders this position chiral and thus two epimers are possible. Of the common C28 phytosterols campesterol, epibrassicasterol, and campestanol are 24α epimers while brassicasterol, ergosterol and ergostanol are 24β epimers. (Figure 2.2.4) The 24–methyl epimers are also designated 24R and 24S, which are equivalent to 24α and 24β, respectively, unless there is a double bond at C22,23, in which case the chirality is reversed (24R=24β and 24S=24α) (99).



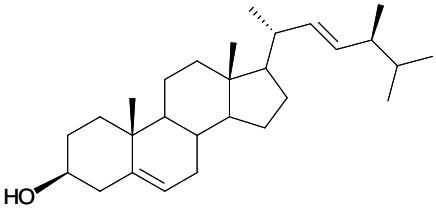
Campesterol



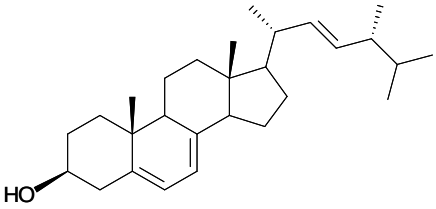
Campestanol



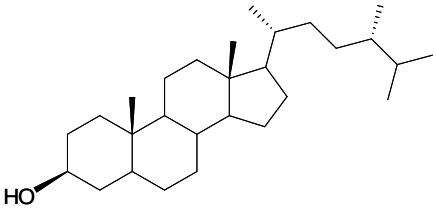
Brassicasterol



Epibrassicasterol



Ergosterol

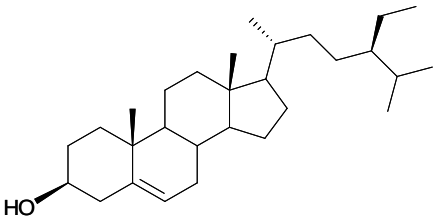


Ergostanol

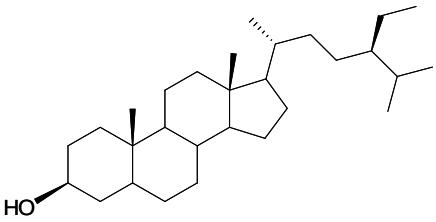
Figure 2.2.4 C28 4–desmethyl phytosterols (redrawn from reference 99)

All C29 phytosterols are 24α–ethyl epimers (Figure 2.2.5). Three of the common C29 phytosterols have a double bond at C24, 28 and the resulting ethylidene group can either be *cis* or *trans*. The C24 ethylidene in fucosterol is the *trans* isomer and is designated as 24E, whereas the C24 ethylidene in Δ5–avenasterol and Δ7–avenasterol is the cis isomer and is designated as 24Z. The C22, 23 double bond in common phytosterols brassicasterol, epibrassicasterol, stigmasterol, and 7–stigmasterol (spinasterol) only occurs as 22E. The common 29–carbon desmethyl sterol stigmasterol, which includes both C5,6 and (*trans*) C22,23 double bonds, is designated as Δ5,22E (99).

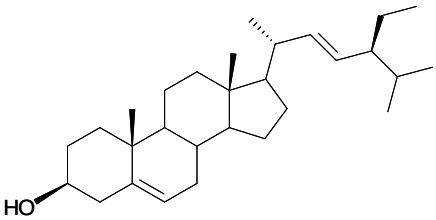
In all plant tissues, phytosterols occur in five common forms: free alcohols, fatty–acid esters, steryl glycosides, acylated steryl glycosides or steryl ferulates. The last four forms are generically called phytosterol conjugates. In free phytosterols, the 3β–OH group on the A–ring of the sterol nucleus is underivatized, whereas in the steryl conjugates the OH is covalently bound with another constituent. The OH group is ester–linked with a fatty acid in steryl esters and linked by a 1–O–β–glycosidic bond with a hexose (most commonly glucose) in steryl glycosides. Acylated steryl glycosides, include a fatty acid esterified to the 6–OH of the hexose moiety. Seeds of corn and rice and other grains contain, phytosteryl hydroxycinnamic–acid esters, in which the sterol 3β–OH group is esterified to ferulic or p–coumaric acid (99). (Figure 2.2.6)



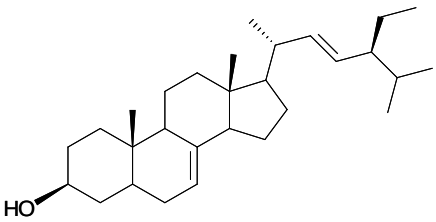
Sitosterol



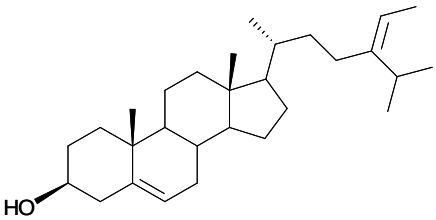
Sitostanol
Stigmastanol



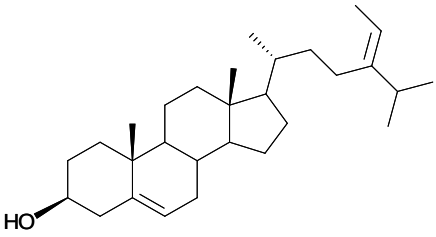
Stigmasterol



Spinasterol
Δ7–Stigmasterol

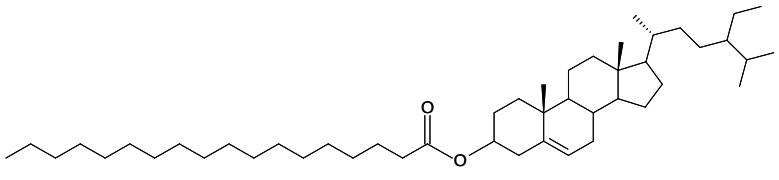


Δ5 Avenasterol
28–Isofucosterol

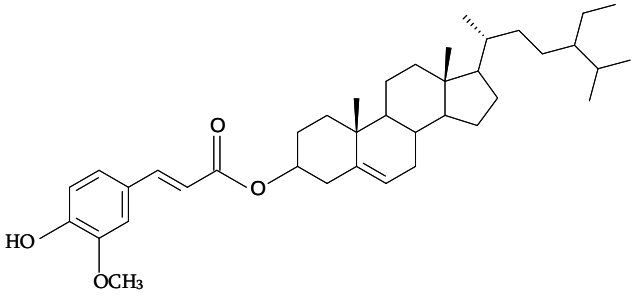


Fucosterol

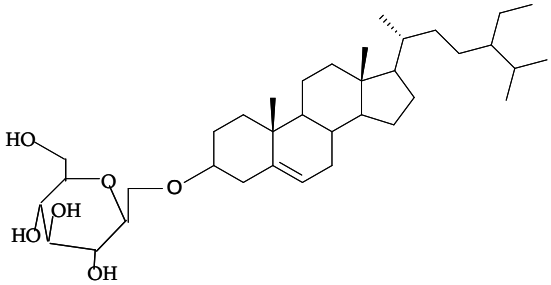
Figure 2.2.5 C29 4–desmethyl phytosterols (redrawn from reference 99)



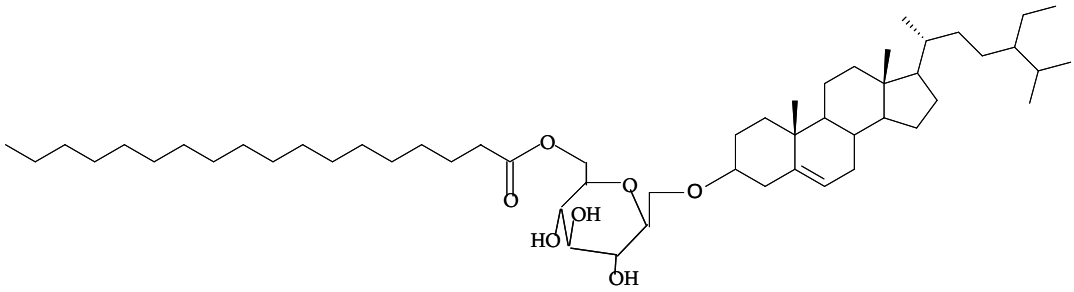
Sitosteryl Stearate



Sitosteryl Ferulate



Sitosteryl Glycoside



Acylated Sitosteryl Glycoside

Figure 2.2.6 Phytosterol conjugates (redrawn from reference 99)

2.2.3 Sterol Oxidation

With the demand for phytosterol enriched food products increasing in the market, concerns have grown regarding the oxidative stability of phytosterols. Just like other unsaturated lipids, sterols and their esters are also prone to oxidation. Autoxidation, a free radical chain reaction, may take place in both steryl and acyl moieties of steryl ester leading to the formation of hydroperoxides as the primary oxidation products. Hydroperoxides decompose into secondary oxidation products which subsequently lead to the formation of polymerized oxidation products, polar and non–polar dimers, trimers and higher oligomers (106).

Esterified sterols oxidize more than the corresponding free sterols at moderate temperatures without an oil matrix and in a saturated matrix system. This has been attributed to the lower melting point of esterified sterols (107). Also, the resulting secondary oxidation product profile is different for sterol esters. The degree of unsaturation of the acyl moiety may influence the oxidation behaviour of the steryl moiety. Sterol esterification with a saturated fatty acid leaves only the steryl moiety prone to oxidation, which increases the formation of steryl hydroperoxides. The acyl moiety of sterols esterified with unsaturated fatty acids oxidizes as well. Increased unsaturation of the acyl moiety increases the formation of primary hydroperoxides of both steryl and acyl moieties. The oxidation of unsaturated steryl esters likely begins by intermolecular radical propagation in the acyl moiety with the resulting acyl carbon radical delocalising into C_7 radical in the steryl moiety (108).

The structural features of the acyl moiety and temperature also affect the extent of polymerization of steryl esters. Both the presence and increased unsaturation of the acyl moiety may increase the rate of polymerization, with the effect of increased temperature being more pronounced. Oligomers form alongside the secondary oxidation products of sterol like hydroxides, epoxides, and ketones, however after prolonged heating, polymerization becomes the predominant reaction. Hence, polymerization is believed to be the termination process in the autoxidation of steryl esters (109).

Oxidation of sterols in foods have been studied by analyzing the major sterol oxides, such as 7–hydroxy, 5, 6–epoxy, and 7–ketosterols. The sterol secondary oxidation product profile formed depends on the rate constants of various reactions such as reduction and dehydration of the primary oxidation product 7–hydroperoxides, dehydrogenation of 7–hydroxysterols, and epoxidation of sterol (110). (Figure 2.2.7) Theoretical calculations based on bond dissociation enthalpies for various C–H and the O–H bonds in sterols have implicated dominant sites of oxidation attack in sterols (111). This is in good agreement with published experimental data on sterol oxidation (111). Δ7–sterols are more susceptible to oxidation attack in comparison to Δ5–sterols. In sterol nuclei, the lowest bond dissociation energy (BDE) was found for C7–H bond in Δ5–sterols and for C14–H in Δ7–sterols (111). In Δ5–sterols with a C=C double bond in the side chain, the lowest BDEs was found for C–H bonds in α–positions to this bond. The BDE of the hydroxyl O–H bond was found to be higher in comparison to the studied C–H bonds in sterol (111).

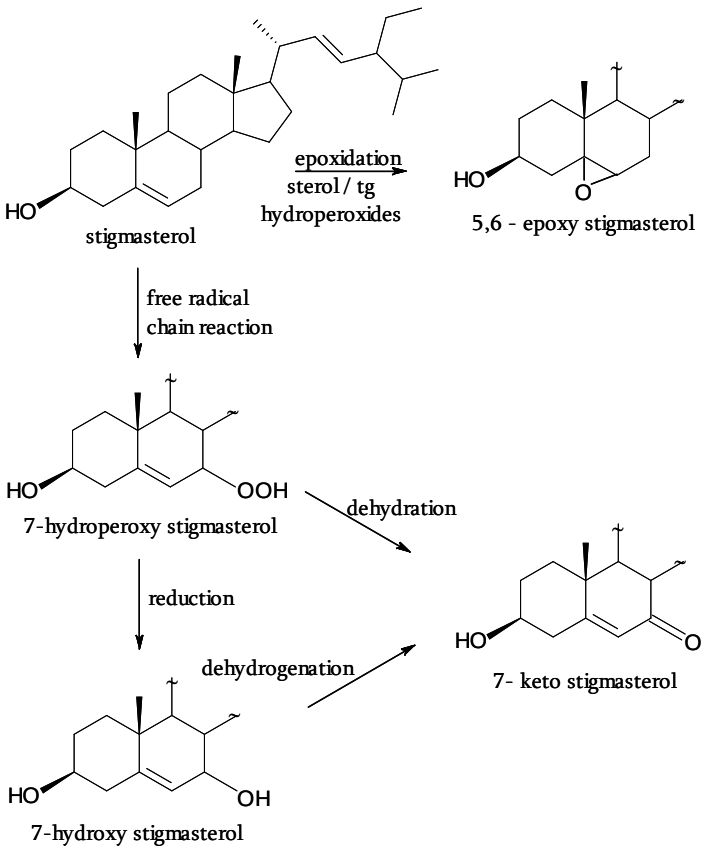


Figure 2.2.7 Reaction pathways for stigmasterol oxide formation

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The formation, analysis, occurrence, and biological effects of the oxidation products of cholesterol, have been extensively studied. However, research on phytosterol oxidation products has been limited (112). Phytosterol oxides exhibit patterns of toxicity similar to those of cholesterol oxides, in cultured macrophage derived cell lines (113) and in cultured mammalian cells (114). As phytosterol–enriched products may be exposed to heat treatments during food processing, temperature is an important factor affecting sterol oxidative stability (115).

Phytosterol stability is also influenced by interactions between lipid components in foods (116) and/or their decomposition products (117). A connection between the oxidation of the lipid matrix and sterol has been suggested with the onset of oxidation in unsaturated lipids leading to the oxidation of sterol (118). The interaction of matrix composition and temperature also likely affects sterol oxidation. Unlike at lower temperatures (<140 °C), at temperatures above 140 °C sterols are more stable in unsaturated than in saturated matrices, a phenomenon attributed to the higher reactivity of the unsaturated lipid matrix that then protects sterols from reacting. However, in a saturated matrix (tripalmitin), sterols tend to be the more reactive lipid component, and hence are more susceptible to oxidation at high temperatures (118).

The extent of sitosterol alteration increases with the unsaturation of the lipid matrix after oxidation at 120 °C for 2 h (119). Cholesterol oxidation is accelerated by the autoxidation of unsaturated TG after 32 days of autoxidation at 25 °C (120). One study assessing the cholesterol decomposition at 130 °C in tristearin, triolein, trilinolein, and milk fat, found tristearin to be the least destructive and triolein to be the most destructive matrix after 3 h of heating (117). However, sitostanol oxidized more in an unsaturated lipid matrix (purified rapeseed oil) than in a saturated matrix (tripalmitin) at all temperatures. This means that the matrix composition and heating temperature interactions are more effective with unsaturated sterols. Oxidation pathways for phytosterol oxide formation have been reported to be different in the saturated tripalmitin medium at 80–140 ^{o}C and at 180 ^{o}C (119).

2.2.4 Sterols as Antioxidants

Phytoserols constitute the major components of unsaponifiable matter in vegetable oils. The oxidative stability of vegetable oils at frying temperature has been reported to be influenced by the unsaponifiable matter content (121). Phytosterols with an ethylidene side chain can be effective as antioxidants at frying temperature (121). The antioxidant effect of ethylidene side chain sterols has been attributed to its ability to release unhindered protons from the C–29 allylic carbon that interfere with lipid oxidation chains, with the resulting free radical isomerizing to a relatively more stable tertiary free radical (122). Further evidence of the formation of tertiary free radical in sterols with an ethylidene side chain at high temperatures was obtained from the reported formation of 24–hydroperoxide in fucosterol upon air oxidation, with a shift in the double bond from the 24–28 to the 28–29 position (123) and the finding that the oxidation of Δ5–avenasterol at 180 ^{o}C led to the formation of 5,24(25),28–stigmasta–trien–3β–ol which did not form in cholesterol oxidised under similar conditions (124).

In addition to Δ5–avenasterol, the most abundant ethylidene side–chain sterol in vegetable oils, a number of other plant sterols with an ethylidene side chain including Δ7–avenasterol, citrostadienol and vernosterol have also been reported to inhibit oxidative degradation of unsaturated fatty acids during high temperature heating (125–127). Vegetable oil sterols such as sitosterol, stigmasterol and campesterol have been reported to be either inactive or slightly pro–oxidant (124, 125,128). However, in contradiction to earlier reports, studies assessing the effect on thermal polymerization of purified high oleic sunflower TG at 180 ^{o}C have reported only marginal stabilising effects for fucosterol, a sterol with an

ethylidene side chain, and slight pro–oxidant effects for sitosterol and stigmasterol (129). In comparison to α–tocopherol, fucosterol was found to have no antipolymerization effect. Also, no significant difference was found in the percent residual sterol left between the three sterols, fucosterol, sitosterol and stigmasterol, after heating (129). This discrepancy between the fucosterol study and earlier reports on sterols with an ethylidene side chain has been attributed to the differences in the nature of fatty acid substrate, analytical methodology and experimental conditions.

Variables such as surface–to–volume ratio and the type of heating can influence the thermo oxidative stability of oils (130). Under similar conditions of temperature and surface–to–volume ratio, High Oleic Sunflower Oil (HOSO) oxidizes much faster when heated on a hot plate than when incubated in an oven. Higher temperatures and surface–to–volume ratios can enhance the oxidation upon heating in both the hot plate and the oven (130). Δ5–Avenasterol has been reported not to stabilize oils heated in an oven but was found to be effective when the same samples were heated on a hot plate at the same temperature (130). This was attributed to the efficient oxygen recirculation environment created by oil movement caused by a temperature gradient effect in hot plate heating whereas the system is more static under oven heating (130).

Also, the degree of unsaturation of the substrate may impact the effectiveness of sterols with an ethylidene side chain as antioxidants, as was shown in the case of linalyl acetate, a non–sterol containing an ethylidene like group, which was an effective antioxidant when used to stabilise heated soybean oil but not when used

with heated lard (127, 132). Sitosterol at 5% level was reported to function as an antioxidant in sunflower oil and lard TG but as a pro–oxidant in tristearate (133). A more recent study (134) reported that the anti–polymerization activity of phytosterols in oils heated at 180 ^{o}C over a period of 8 h is more dependent on the number and location of double bonds in the phytosterol ring structure rather than on the presence of an ethylidene group in the side chain. Ergosterol with two endo cyclic double bonds but without an ethylidene group in the side chain was found to be the most effective in reducing the extent of polymer formation (134). Phytosterols with two double bonds, one in the ring structure and one in the side chain such as stigmasterol, brassicasterol and fucosterol had only slight anti–polymerization activity but the ethylidene side chain did not appear to confer any increased activity for fucosterol. However, fucosterol loss was slightly greater than that of stigmasterol and brassicasterol, indicating that the hydrogen on allylic carbons in the ethylidene group may have been more labile (134). Also, monounsaturated sitosterol and saturated sitostanol were reported to have no effect on the extent of polymer formation (134).

Sterols with unsaturation in the ring structure are more reactive than sitostanol and hence have higher losses in comparison to sitostanol under similar conditions (135). A study involving the use of a phytosterol blend at 0.25–0.5% level, reported a slight decrease in thermal polymerization in soybean oil stripped of tocopherols and most of the free phytosterols using a short–path thin–film evaporator unit, but no impact on thermal polymerization in stripped high oleic sunflower oil (135). At higher levels (1 and 2.5%), the phytosterol blend

decreased polymerization of stripped soybean oil, while at 2.5% level it increased polymerization in stripped high oleic sunflower oil. Residual phytosterols were higher in stripped soybean oil compared to stripped high oleic sunflower oil. At both 1 and 2.5% levels, phytosterol negatively impacted the Oxidative Stability Index (OSI) values at 110 ^{o}C for both oils, but the pro–oxidant effects were more pronounced in stripped high oleic sunflower oil. The difference in the activity of phytosterols in these two oils was attributed to the differences in their fatty acid composition (135).

In vegetable oils low in saturates, factors other than the fatty acid composition of the medium may play a role in phytosterol destruction during frying, which may include the concentration of natural antioxidants such as tocopherols in the oils. Tocopherols are known to degrade or volatilize during the frying process, but their loss often varies depending on the homologue and the composition of the oil (142). Preferential oxidation of tocopherols over phytosterols may explain the enhanced stability of phytosterols (143).

Rice steryl ferulates (γ–oryzanol) possess good antioxidant activity. Extracts of steryl ferulates from rye and wheat also have good antioxidant activity in the bulk lipid system. The radical scavenging activity is similar to that of non–esterified ferulic acid, indicating that the ferulic acid moiety may be responsible for the antioxidant effect (144). Corn steryl ferulates (CSF) and oryzanol have been reported to confer different antipolymerization activity in soybean oil used for frying, with CSF reducing the polymerization of soybean oil during frying by as much as 85% after the first day of frying and by 68% after the second day of

frying (145). At similar levels of oryzanol or ferulic acid, reduction in soybean oil polymerization was 12% on the first day and 5% after the second day of frying. Different interactions with tocopherols naturally present in soybean oil have been proposed as an explanation for the effectiveness of CSF in limiting polymerization of soybean oil in comparison to oryzanol or ferulic acid (145). While CSF can have a protective effect on the tocopherols, oryzanol and especially ferulic acid increased tocopherol degradation. Hence, with the ability to increase tocopherol retention in addition to protecting the oil, CSF may have potential for use as frying oil additives to protect highly unsaturated oils such as soybean oil.

Several studies (136–139) have examined the formation of phytosterol oxidation products and loss of phytosterols in model heating systems in order to understand the effects of temperature, structure, and the degree of saturation of the medium on phytosterol oxidation. The analysis of sterol oxidation products does not always account for total sterol losses (136–139). It has been reported that almost one third of sterol content was unaccounted for when sitosterol was heated in tripalmitin at 180 ^{o}C for 3 h (146). Also, the gap between the amount of initial sterol before heating and the amounts of unaltered sterol and its oxides measured after heating, appeared to be much larger at frying temperatures. It was concluded that this was at least partly due to thermal reactions that prevail at temperatures close to 200 ^{o}C (143).

While the interaction of sterols with atmospheric oxygen has been extensively studied, the interaction with atmospheric moisture has not received much

attention. Given the ampiphilic nature of the sterol molecule it is reasonable to expect the OH group in sterol to be closer to the air–oil interface and thus be available to interact with the energized hydronium ions generated from the atmospheric moisture at the air–oil interface. Hydrolysis of the ester functionality in TG would precede any interaction of sterol OH group due to the higher proton affinity of the ester functionality (148). However, when TG hydrolysis attains equilibrium, the hydronium ions at the air – oil interface would become available to interact with the OH group in sterols. This interaction would lead to an acid catalyzed dehydration of the sterol molecule resulting in the formation of steradiene with a conjugated diene system. A concerted release of protons from allylic carbons at both ends of the conjugated diene system may explain the antioxidative effect observed in sterols at high temperatures. Sitosterol at 5% level has been reported to significantly limit polymer formation in triolein heated at frying temperature continuously for 72 h. This may also explain the reported enhanced effectiveness of ergosterol with two endocyclic double bonds as an antioxidant in comparison to stigmasterol, brassicasterol and fucosterol under similar conditions (134). Dehydration of ergosterol would generate a conjugated triene system which would provide much greater delocalization for the radicals and hence an enhanced release of protons from the allylic carbons.

While sterols with a single endocyclic double bond represent a (4n+2) closed ring system, steradiene with 2 double bonds represents a (4n) closed ring system, which is known to be highly unstable and hence would easily decompose and volatilize at frying temperatures. This provides a more probable explanation for

the gap found between the amount of initial sterol before heating, and those of unaltered sterol and its oxides measured after heating. While the formation of sterol dimers, oligomers and polymers cannot be ruled out at high temperatures in the absence of matrix, there may be thermodynamic and steric restrictions in an actual oil system that may limit the formation of sterol dimers and oligomers.

2.3 Analysis of Lipids by HPSEC

2.3.1 Introduction

High Performance Size Exclusion Chromatography (HPSEC) is a separation technique in which molecules are separated on the basis of hydrodynamic molecular volume or molecular size. Using a calibration curve obtained by plotting retention volume vs. the logarithm of molecular weight (MW) for a series of known standards, the MW of unknown molecules can be estimated, provided they adopt a conformation in solution similar to that of the standard. Calibration methods include use of instruments like light scattering, mass spectrometry, nuclear magnetic resonance, as well as universal calibration using viscometry (148, 149).

HPSEC column consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes. These pores may be depressions on the surface or channels through the bead. Differences in elution time are based solely on the time spent by the analyte within the pore system of the column. A small molecule that can effectively penetrate the pore system of the stationary phase elutes later. On the other hand, very large molecules that cannot penetrate the pore system elute earlier.

Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of this range, which includes molecules that are too large to penetrate the pores of the stationary phase. The permeation limit defines the molecular weights at the lower end of the range and includes molecules small enough to penetrate into the pores of the stationary phase completely. Molecules below the permeation limit

elute as a single band.

The total volume of a SEC column includes three components, *Vo, Vi* and *Ve*. The void volume *Vo* represents the volume outside of the beads, which may be determined using a very large molecule such as thyroglobulin that is larger than the exclusion range for the gel. The space within the beads is called inclusion or internal volume, *Vi* which may be determined using a small molecule such as an amino acid. The actual volume required to elute a biomolecule from a SEC column is called the elution volume, *Ve*. For an efficient separation using SEC, *Vo* > *Ve* > *Vi*. The most important parameters in SEC influencing separation capacity and resolution efficiency are the pore volume, pore–size distribution, and particle size (150).

Originally, size exclusion chromatography was used mainly for characterization of high–MW molecules, either synthetic polymers or biopolymers, but applications soon expanded to other areas, including lipids. The simplicity and high reproducibility of HPSEC in combination with adsorption chromatography have contributed significantly to the development of new analytical methods in lipid analysis (151).

HPSEC stationary phases commonly used in lipid analysis include copolymers of styrene divinyl benzene with pore sizes, 50, 100, and 500 Å, considered essential porosities for low–MW separations (100–20,000 MW), and particle sizes of 5 and 10 μm. Packed columns with dimensions ~30 cm × 0.8 cm i.d. are normally used in series. Spherically shaped, mono–sized particles contribute considerably to improving particle–size and pore–size distribution which significantly impact

efficiency and separation capacity (152).

Tetrahydrofuran (THF) is the most commonly used solvent as the mobile phase, although toluene and dichloromethane may be used for certain applications. Flow rates between 0.5 and 1.5 ml/min are the most common enabling an analysis time of less than 30 min. The commonly used detectors include refractive index detector (RI), and evaporative light scattering detector (ELSD). The RI detector is limited to isocratic separations, while ELSD has excellent gradient capabilities and a slightly superior sensitivity (153).

2.3.2 HPSEC and Adsorption Chromatography

Figure 2.3.1 provides a schematic representation of different methods based on HPSEC used for the quantification of non–volatile oxidation compounds in frying oils.

a. *HPSEC and Silica column:* The method combining adsorption chromatography using silica columns and HPSEC for the quantitative analysis of oxidized and hydrolytic compounds in lipids was introduced and validated for the first time in 1988 (154). Separation of nonpolar and polar fractions was achieved by eluting 1 g sample of oil with 150 ml of a mixture hexane:diethyl ether 90:10 and 150 mL diethyl ether, respectively. After gravimetric determination of polar compounds, the polar fractions were further analyzed by HPSEC, using two columns (100 and 500 Å, particle size: 5 μm), connected in series and THF as the mobile phase at a flow rate of 1 mL/min. with refractive index / ELSD detection.

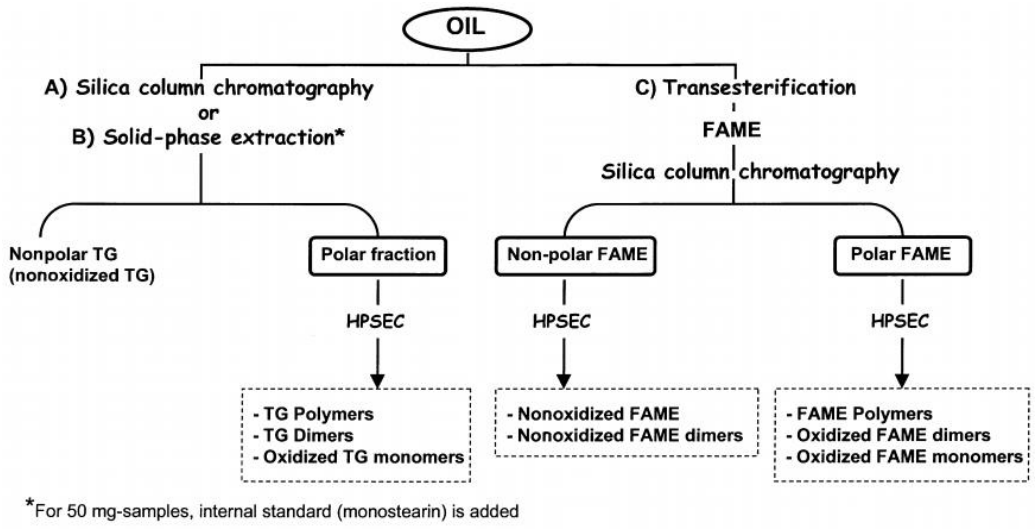

Figure 2.3.1 Representation of methodologies based on HPSEC for quantitation of non–volatile oxidation compounds. FAME: Fatty Acid Methyl Esters

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Application of HPSEC to the concentrated fraction of polar compounds after the removal of the non–polar fraction enabled the separation and quantitation of different groups of oxidized and hydrolytic compounds in the polar fraction including, TG polymers, TG dimers, oxidized TG monomers, DG, MG and FFA (Figure 2.3.2). The method combining adsorption chromatography with HPSEC can be applied to samples at different stages of oxidation which may include unused unoxidized oils to thermally oxidized oils.

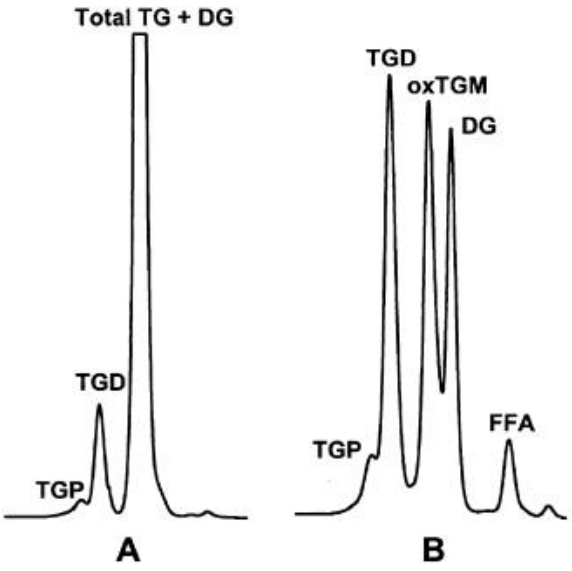

Figure 2.3.2 HPSEC chromatograms of (A) total used frying oil (B) its polar fraction

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Oxidized TG monomers and DG in the polar fraction serve as markers of oxidation and hydrolysis, respectively. Oxidized TG monomers represent all monomeric TG containing at least one oxidized fatty acyl group including oxygenated functionalities such as peroxides, the primary oxidation products, or those resulting from decomposition of hydroperoxides, like epoxy, alcohols, or ketones. Quantitation of oxidized TG monomers provides an excellent global measurement of the non–volatile oxidation products formed during the initiation reactions. Concentration of the polar fraction enables a substantial increase in sensitivity for the quantification of polymerization compounds, TG dimers and TG polymers. Formation and increase of TG dimer and TG polymer content is indicative of the onset of the accelerated oxidation stage at high temperatures.

b. *HPSEC and SPE:* Solid phase extraction (SPE) makes use of silica gel cartridges (155) or NH_2 cartridges (156) in place of silica columns. Shorter analysis time and reduced solvent consumption are the advantages of using silica cartridges. No significant differences have been reported between the mean values obtained for polar content measurements of samples with polar content ranging from 3.7 to 24.3% using the SPE–HPSEC and the silica column–HPSEC methods (4). However, the SPE method may be better suited for samples with lower polar content as indicated by smaller standard deviation values.

c. *HPSEC and Transesterification:* Oxidized fatty acyls included in TG molecules may be used to obtain a measure of the polar content in oil samples. FAME are obtained by transesterification of 1 g of sample with sodium methoxide and hydrochloric acid/methanol, recovered quantitatively and subjected to separation by silica column chromatography, using 150 mL hexane/diethyl ether (88:12, v/v) to elute the nonpolar fraction and 150 mL diethyl ether to obtain the polar fraction (151). The separated nonpolar and polar fractions are further analyzed by HPSEC. (Figure 2.3.3)

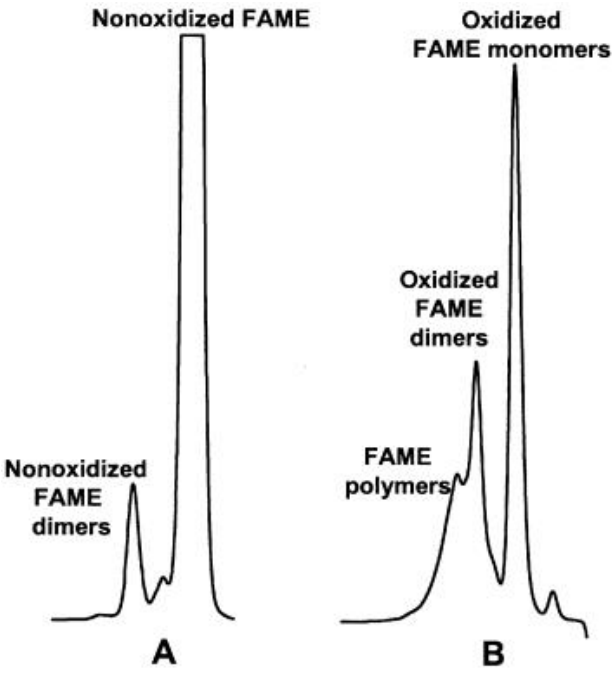

Figure 2.3.3 HPSEC chromatograms of (A) non–polar FAME (B) polar FAME fractions of used frying high oleic sunflower oil

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The unoxidized FAME and the nonpolar or unoxidized FAME dimers, linked by C–C bonds and lacking extra oxygenated functions are eluted in the first fraction. FAME polymers, oxidized FAME dimers, and oxidized FAME monomers are eluted in the polar fraction, which also provides a measure of the total oxidized fatty acyl groups included in TG molecules. Use of hexane/diethyl ether at 95:5 (v/v) may enable elution of only unoxidized FAME in the nonpolar fraction (151). The peak of FAME dimers in the polar fraction would then include both the nonpolar and oxidized types of FAME dimers. However, given the high level of

unchanged fatty acyl groups in oxidized TG molecules, sensitivity of the method for samples of low oxidation level may be limited (157).

2.3.3 HPSEC for analysis of lipid oxidation compounds

Autoxidation of unsaturated lipids, which proceeds *via* a free radical mechanism, leads to the formation of hydroperoxides, which upon further oxidation, decomposition, and polymerization result in a complex mixture of volatile and non–volatile oxidation compounds differing in polarity and MW. Although volatile compounds are important for sensory evaluation and consumer acceptance, non–volatile products are nutritionally more relevant because they get ingested with food.

a. Use of HPSEC *to assess oxidative status of crude and refined oils*

HPSEC can be used to obtain valuable information on the quality and oxidative status of crude and refined oils. Edible oils are refined to remove unwanted minor components such as phospholipids, FFA, color, and volatile components. Figure 2.3.4 represents HPSEC chromatograms of virgin olive oil and the polar fractions of the virgin and refined olive oil. Given the low levels of polar content, detection of the components of polar fraction in crude and refined fats and oils is not possible; however, separation of the polar fraction using adsorption chromatography followed by further analyses using HPSEC enables identification of four peaks in the polar fraction which include TG dimers, oxidized TG monomers, DG, and FFA (151).

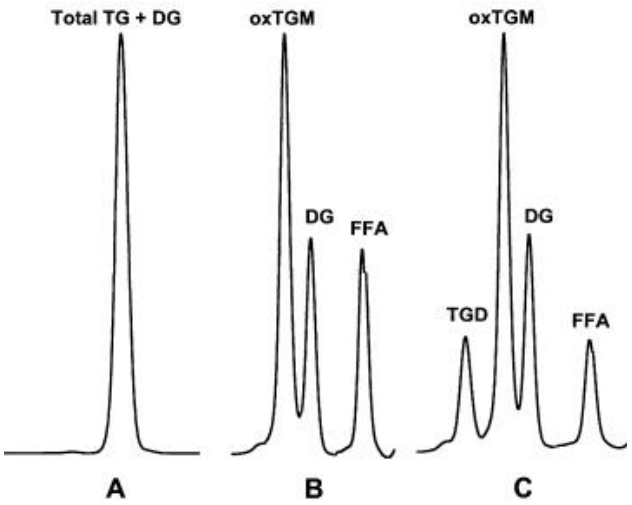

Figure 2.3.4 HPSEC chromatograms of (A) total virgin olive oil (B) polar fractions of virgin olive oil (C) refined olive oil

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Quantitation of these compounds in crude oils and at different stages of the refining process indicate that TG dimers are absent in crude oils but can form during the deodorization step of refining; levels of oxidized TG monomers and DG in refined oils remain unchanged from crude oil levels because they are non–volatile under refining conditions and are neither formed nor eliminated in any of the processing stages; due to the neutralization step in the refining process FFA levels in refined oils are lower in comparison to crude oils. Hence, overall quality related information on refined oils which may impact their storage stability and utilization for food preparation, can be obtained using HPSEC (158–160).

b. Use of HPSEC to follow oxidation during storage

Silica column–HPSEC or the SPE–HPSEC methodologies may be used for the evaluation of oxidative stability and quantitation of non–volatile oxidation compounds formed during storage of oils. HPSEC chromatograms in Figure 2.3.5 illustrate the changes in the polar fractions of sunflower oil observed at different stages of oxidation at 25 °C; at the starting point; during the early stage of oxidation or Induction Period (IP) and at the end of the IP. Oxidized TG monomer and TG dimer values for the starting oil are 0.9% and 0.5% (w/w) respectively, levels commonly found in refined oils (4). As illustrated by the chromatogram in dashed line (Figure 2.3.5) during the early stage of oxidation, oxidized TG monomers are the only group of compounds increasing with an oxidized TG monomer content of 2.9%. The chromatogram in solid line represents a sample at the end of IP with exhausted tocopherols and a significant increase in oxidized TG monomer content of 17.7% and a TG dimer content of 1.4%, indicating the onset of the period of advanced oxidation. As oxidized TG monomers represent the group of compounds with the largest increase during early oxidation, they may be used as a measure of the onset of rancidity in fats & oils (161–164).

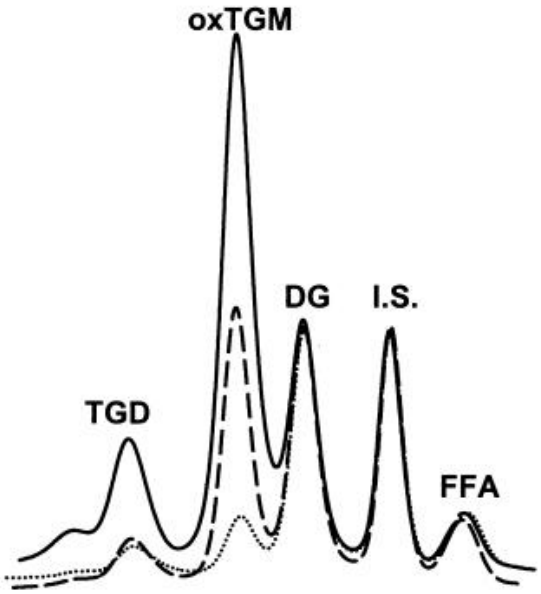

Figure 2.3.5 HPSEC chromatograms of polar fractions of sunflower oil during oxidation at 25 ºC: at the starting point (dotted line), during the early oxidation stage (dashed line), and at the end of the induction period (solid line).

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c. *Use of HPSEC to follow oxidation kinetics*

HPSEC can be used to follow changes during lipid oxidation and gain insight into the oxidation kinetics (165, 166). Oxidation of trilinolein (LLL) was used as the basis to follow changes in concentration of three groups of nonvolatile oxidation compounds, oxidized TG monomers, TG dimers, and TG polymers, and the influence of both temperature (25 °C, 60 °C, and 100 °C) and the addition of α–tocopherol on oxidation kinetics was examined (166). A considerable delay in the formation of non–volatile oxidation compounds in the presence of antioxidants

enables a clear distinction between the two stages, an induction period (IP) and an accelerated oxidation stage (Figure 2.3.6). Oxidized TG monomers, comprised mainly of hydroperoxides, are the only group of compounds increasing during the early oxidation stage. At the end of the IP, oxidation accelerates as is indicated by the sharp increase in oxidized TG monomers, significant formation of polymerization products, and exhaustion of antioxidants (165). (Figure 2.3.6)

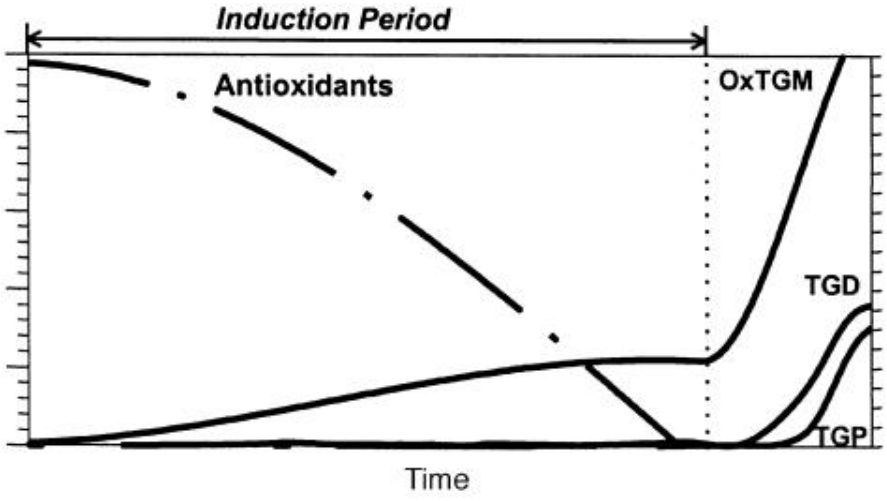

Figure 2.3.6 General oxidation profile found in model TG and oils oxidized at low temperatures.

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An increase in temperature is accompanied with a decrease in the IP which impacts the slope of the initial linear stage of oxidation as shown by the decreasing levels of primary oxidation compounds (oxidized TG monomers) at the end of the induction period (165). This may be attributed to enhanced

antioxidant degradation at high temperatures. Hence, depending on temperature, the polymerization step commenced at different levels of primary oxidation products which was evident in the ratio of oxidized monomers–to–polymerization compounds, 20:1, 9:1, and 3:1, obtained at 25 °C, 60 °C, and 100 °C respectively (19). The kinetic parameters calculated based on experimental data indicates that the increase in oxidized TG monomers is linear during the induction period in the presence of an antioxidant. The influence of temperature on the oxidation rate during the induction period based on the Arrhenius law, is a linear relation between ln IP and 1/T (T = absolute temperature).

In a recent study (167) to determine changes in non–volatile oxidation compounds during long–term storage at room temperature involving samples differing exclusively in degree of unsaturation, including conventional high–linoleic sunflower oil (HLSO), genetically modified high–oleic sunflower oil (HOSO) and a 1:1 mixture of the two, the oxidation pattern was similar to that obtained for LLL shown in Figure 2.3.6. The length of IP depended on the degree of unsaturation, and the amount of oxidized TG monomer accumulation at the end of the IP increased as the unsaturation degree of the oil increased (167). Also, an excellent correlation was observed between peroxide value (PV) measurements and those of oxidized TG monomers, which are to known to be comprised primarily of hydroperoxides during the early stage of oxidation, for all oil samples within the early oxidation stage with a TG dimer content of ~1% (167). However, once oxidation accelerated, the relationship became more complex due to the formation of secondary oxidation products resulting from the decomposition of

hydroperoxides and the contribution of functions other than hydroperoxide, such as epoxy, keto, or hydroxy to the amount of oxidized TG monomers. Also, hydroperoxide functionalities have been reported to be present not only in primary oxidation compounds but are also involved in dimeric linkages of polymerization compounds (168).

d. Use of HPSEC to follow oxidation at high temperatures

During the frying process, thermal, oxidative and hydrolytic reactions take place resulting in the formation of a complex mixture of new compounds. Quantification of polar compounds formed during frying using silica columns has been used for quality control of used frying oils (169, 170) and is currently included in some European regulatory protocols to limit polar compounds for human consumption to ~25% (171). In addition, quantification of TG polymers, which represent the major components of degradation compounds, by HPSEC has also been used for quality control of used frying oils for samples containing > 3% polymers (172, 173). Good correlations have been reported between the polymer and polar content in used frying oils (174, 175). The methodology based on silica column–HPSEC provides an excellent alternative for the evaluation of used frying oils by enabling the determination of both the polar and polymer contents and also the identification of the different groups of non–volatile oxidation compounds and hydrolytic products (176 –178). The advantages of the combined technique are clearly evident in the HPSEC chromatograms for used frying oil and the polar compound fractions obtained from it. (Figure 2.3.2) These include an increased sensitivity in polymer quantitation overcoming the limitation of a minimum of 3%

content (IUPAC 1992) and differentiation of thermally oxidized compounds (oxidized TG monomers, TG dimers and TG polymers) from hydrolytic products (DG and FFA). This establishes the nutritional relevance of a 25% polar compound limitation on frying oils, since only the thermally oxidized components of polar compounds are associated with negative physiological effects, whereas hydrolytic compounds are products naturally released from lipolysis in the gut before absorption. Application of the silica column–HPSEC methodology has permitted a better understanding of some of the important developments during the frying process which include, impact of duration of heating, temperature, surface–to–oil volume ratio and degree of unsaturation on the frying process (179); connection between loss of antioxidants and formation of degradation compounds (180 –182); impact of additional mixed phytosterols on the thermal polymerization in frying oils (183); effect of the degree of unsaturation and presence of ethylidene group on the anti–polymerization activity of phytosterols (184); role of hydrolysis in the formation of compounds during the frying process (185, 186); lipid exchange between oil and food during the frying process and composition of oil absorbed by the fried food (187, 188); effect of the addition of dimethyl polysiloxane to frying oil (189, 190); differences between continuous and batch frying (189); and the thermal stability and frying performance of oils from genetically modified sunflower seeds (187, 191).

In addition to the effective use in the study of frying oils, the technique combining adsorption chromatography with HPSEC has proven useful in the study of different stages of lipid oxidation by enabling the quantitation of primary and

secondary oxidation products in a single analysis and providing oxidation profiles in different lipid systems. Future applications of the HPSEC method may involve use as a preparative technique to isolate and concentrate fractions of the more stable high molecular weight lipid oxidation products as standards for the identification and molecular weight determination of unknown high molecular weight lipid oxidation products.

2.4 Lipase Hydrolysis

2.4.1 Introduction

Lipases are acyl hydrolases that play a key role in fat digestion by cleaving long–chain TG into polar lipids. Given the hydrophilic nature of the enzyme and a lipophilic substrate, lipase reactions are known to occur at the interface between the aqueous and the oil phases. Substrate inaccessibility at the interface is believed to impact lipase activity more than enzyme denaturation or inactivation, as is often hypothesized. Also, it has been shown that lipolysis during TG digestion follows a self–regulated mechanism (192).

Lipases have only marginal activity towards molecularly dissolved substrates in aqueous solution but show high activity when the substrate concentration is high enough to form micellar aggregates or emulsion droplets, i.e. in the presence of an interface. The activity is independent of the molar concentration in solution, but is controlled by the concentration of substrates at interfaces. Based on their physical properties, substrates, enzymes and products partition themselves among the bulk and surface phases and this distribution changes as lipolysis proceeds (193).

Lipases are recognized as the most important group of biocatalysts in biotechnology with applications in industries such as food, detergent, pharmaceutical, leather, textile, cosmetic, and paper (194). These are ubiquitous enzymes found in most microbes, plant and animals that serve as a powerful tool for catalyzing hydrolysis, esterification and transesterification reactions (195).

The two main categories into which lipase catalyzed reactions may be classified are hydrolysis and synthesis which includes esterification and transesterification comprised of interesterification, alcoholysis and acidolysis (196).

i.	Hydrolysis:	$RCOOR' + H_2O \rightarrow RCOOH + R'OH$
ii.	Synthesis:	
(a)	Esterification	$RCOOH + R'OH \rightarrow RCOOR' + H_2O$
(b)	Interesterification	$RCOOR' + R''COOR \rightarrow R''COOR + R''COOR'$
(c)	Alcoholysis	$RCOOR' + R''OH \rightarrow RCOOR'' + R'OH$
(d)	Acidolysis	$RCOOR' + R''COOH \rightarrow R''COOR' + RCOOH$

Lipases use a catalytic triad of amino acids: serine, histidine and aspartate or glutamate (192). The lipase catalysis mechanism involves activation of serine by deprotonation in the presence of histidine and aspartate. This is followed by a nucleophilic attack on the carbonyl group of the substrate by the hydroxyl residue of serine leading to the formation of an acyl–enzyme intermediate. The presence of an oxianion hole contributes to the charge distribution stabilization and reduction of the ground state energy of the tetrahedral intermediate. In the deacylation step, nucleophiles such as H_2O or MG present at the interface attack the acylated enzyme leading to product release and regeneration of the catalytic site. (Figure 2.4.1)

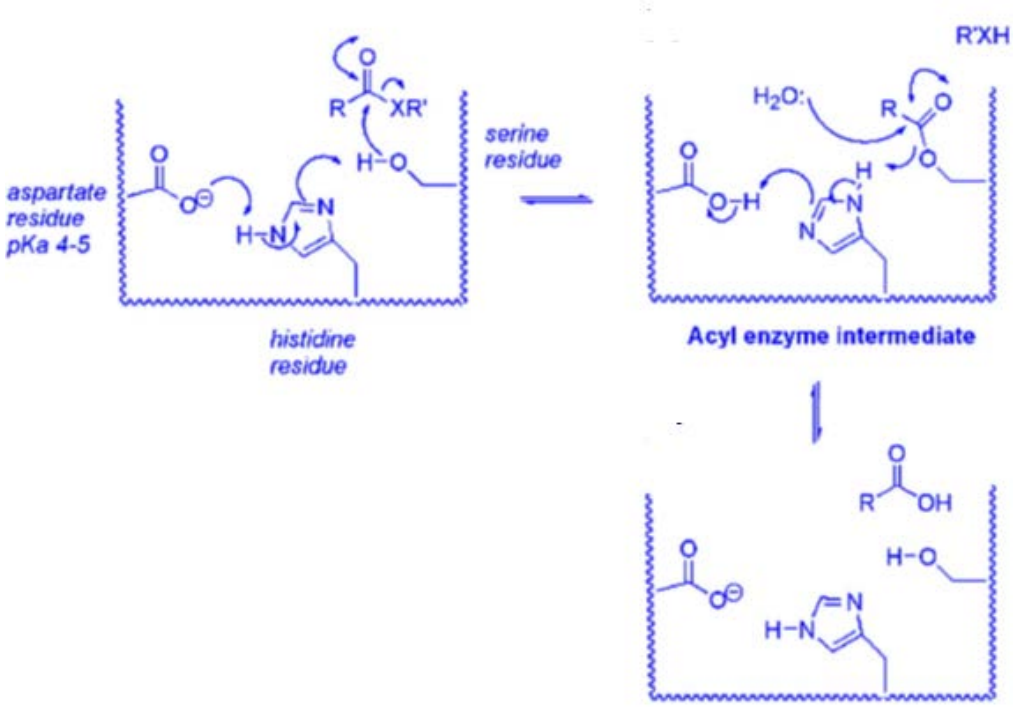

Figure 2.4.1 Lipase catalysis mechanism

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Lipases display varying degrees of selectivity toward their substrates and are able to catalyze reactions with a broad range of substrates with reaction rates varying widely with the structure of the substrate molecules. Steric hindrance and hydrophobic interactions are believed to be two primary forces that determine lipase selectivity (197). Also, substrate availability and suitable orientation at the interface and conformational change in the lipase upon adsorption at the lipid/water interface are considered factors that may influence lipase catalyzed reactions (198).

2.4.2 Enzyme kinetics

Michaelis–Menten kinetics is one of the best known models of enzyme kinetics. The model describes the rate of enzymatic reactions, by relating reaction rate *v* to the concentration of a substrate [*S*] (199). (Figure 2.4.2)

$$v = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_m + [S]}.$$

V_{max} = maximum rate achieved by the system at maximum substrate concentration

K_m = Michaelis constant, represents substrate concentration at which the reaction rate is half of V_{max}

Michaelis–Menten model may be represented by the schematic:

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{\text{cat}}} E + P$$

With the assumption that the enzyme concentration is much less compared to the substrate the rate of product formation is given by

$$v = \frac{d[P]}{dt} = V_{\max}\frac{[S]}{K_m + [S]} = k_{\text{cat}}[E]_0\frac{[S]}{K_m + [S]}$$

The reaction rate increases with increasing substrate concentration [*S*], asymptotically approaching its maximum rate, V_{max} when all enzyme is bound to substrate.

$$V_{\max} = k_{\text{cat}}[E]_0$$

$\boldsymbol{k_{cat}}$ = turnover number, the maximum number of substrate molecules converted to product per enzyme molecule per second

In contrast to esterases which act only on the water–soluble substrates and show normal Michaelis–Menten type dependence on substrate concentration, lipases are characterized by interfacial catalysis with the activity controlled by the concentration of substrates at interfaces and are independent of molar concentration in solution (200). Verger–De Haas' model is the simplest adaptation of the Michaelis–Menten kinetic model for the interfacial hydrolysis of short– and medium chain lipids. (Figure 2.4.3) The first step in this process is the adsorption of a water–soluble enzyme (E) at the lipid–water interface leading to a more favourable energy state of the enzyme (E*). The enzyme present at the interface binds to a substrate molecule (S), resulting in the formation of an enzyme–substrate complex (E* S), followed by a two dimensional catalytic process generating the product (P*) which is then solubilised into the aqueous phase (200).

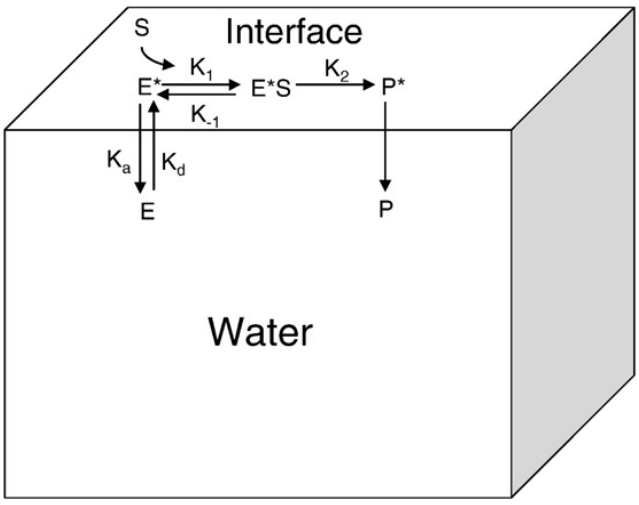

Figure 2.4.2 Verger –De Haas kinetic model for lipase action (201)

However, the natural substrates for lipolytic enzymes are long–chain lipids which generate water–insoluble products. Hence, kinetic models must take into account the processes involved in the interfacial molecular reorganization and segregation of the insoluble products. Water–soluble acceptors such as ß–cyclodextrine may be used to remove lipolysis products from the interface (202). In vivo, lipolytic products such as FFA and MG in the gastrointestinal tract are solubilised in micelles with bile salts while FFA in the blood are bound by serum albumin. Lipolysis is a classic example of heterogeneous biocatalysis where the reaction rate and direction are controlled by the overall composition at the interfacial microenvironment. Lipolytic reactions are strongly dependent on the interfacial activity, kinetics of diffusion and partitioning of substrates, products and inhibitors. Confining a reaction to an interface can considerably speed up the substrate–enzyme association rate by decreasing the entropy through motional constrains, as well as by hydrophobic and electrostatic interactions which are also known to influence the diffusion of substrate and products to and from the active site (203).

2.4.3 Fat digestion and absorption

Dietary intake of fat in the west averages 60 to 100 g/ day, of which 90% is TG while cholesterol esters, plant sterols and phospholipids make up the rest (204). Fat digestion begins with the action of lingual and gastric lipases, which can cleave short and medium chain TG more efficiently than TG of longer chain length but cannot process phospholipids or sterols (205). An estimated10–30% of ingested lipids is believed to be digested in the stomach yielding DG and FFA

(206). The crudely emulsified lipids at low pH then get ejected from the stomach into the duodenum, coming in contact with the duodenal fluid originating from the pancreas, gallbladder and small intestine. In the duodenum, an abrupt increase in pH caused by the secretion of $NaHCO_3$ causes partial ionization of fatty acids and hence an improved emulsification. Also, biliary lipids ejected from the gallbladder in the form of biliary mixed micelles become rapidly diluted further emulsifying the products coming from the stomach. Bile production is necessary for efficient intestinal absorption of dietary lipids and fat soluble vitamins. While bile salts have a high capacity to solubilise phospholipids and the products of pancreatic lipolysis, they are also known to enhance solubility of relatively non–polar molecules such as cholesterol (204).

The pancreas secretes three distinct lipolytic enzymes: colipase–dependent lipase or pancreatic lipase, carboxyl ester hydrolase or bile salt stimulated lipase, and phospholipase A2. Pancreatic lipase has a remarkably high turnover number of 250,000 to 500,000 long–chain TG molecules/min with an activity 100 to 1000 fold in excess of that needed for complete hydrolysis of TG in the upper small intestine (207). Carboxyl ester hydrolase is considered as a minor component of pancreatic juice and is known to catalyse the hydrolysis of water–soluble esters, triacetin, lysolecithin and insoluble esters of cholesterol and lipovitamins A, D and E (207). Long–chain TG emulsified with phospholipids are not directly available for hydrolysis by pancreatic lipase *in vitro* even in the presence of bile salts and colipase. The inhibition can be overcome by a limited hydrolysis of the phospholipid by phospholipase A2, leading to the generation of FFA at the

interface, which enhance the binding of colipase and thereby lipase due to its attachment to colipase. Sn–2 MG and FFA form the final products of TG digestion due to the Sn–1, 3 regiospecificity of the gastro–intestinal lipases (208).

2.4.4 Lipase reaction at interfaces as a self–limiting process

Lipolysis during TG digestion is believed to follow a self–regulated mechanism, which involves the expulsion of lipase from the interface by the accumulation of Sn–2 MG. In order to test the hypothesis of self–regulation of the enzymatic reaction through accumulation of Sn–2 MG, a model biphasic system was studied wherein the substrate p–nitro phenyl palmitate (pNPP) was converted to nitrophenol upon lipase catalyzed hydrolysis (209). The extent of hydrolysis of pNPP was found to be lower in the presence of Sn–2 monolaurin in comparison to the Sn–1/3 isomer. Sn–2 MG has also been shown to decrease lipase catalyzed hydrolysis of pNPP in a biphasic system in the presence of bile salts at physiological concentrations. Spectrophotometry results have confirmed that 95% of the initial substrate was consumed in the biphasic system enriched with Sn–1/3 MG while no hydrolysis was detected in the presence of the Sn–2 isomer, indicating that the Sn–2 MG exerts a control of the lipolytic activity. Assessment of the adsorption behaviour at the interface of polar lipids generated during lipolysis of TG using the pendant drop technique, found Sn–2 MG to be the most interfacial active with interfacial tension values lower than that of lipase (209). Hence, from an interfacial energy viewpoint it would be reasonable to assume that Sn–2 MG can expel Sn–1, 3 regiospecific lipase from the oil–water interface (209). Both experimental work and theoretical modelling of the properties of

lipolytic products from TG confirm, that Sn–2 MG rapidly take over the interface and expel FFA, DG and TG as well. The lipase appears to form a sublayer in the water phase, located just beneath the MG–covered interface. Since the TG constitutes the apolar phase of the system, enzyme removal from the reaction zone may be a self–regulatory mechanism that controls the extent of fat digestion. This self–regulation mechanism was also shown to be valid in an *in vitro* model of the gastro–intestinal system, where lipolysis of TG was decreased by up to 90% when a test meal was enriched with Sn–2 MG (210). Evidently, accumulation of this non–cleavable amphiphile, Sn–2 MG at the oil–water interface decreases the TG accessibility to lipase, leading to reduced fat digestion. The remaining *in vivo* fat digestion can then be attributed to the capacity of bile salts to solubilise Sn–2 MG into mixed micelles and transport them to the brush border cells of the upper gastro–intestinal tract. Provided that this transport mechanism fails or is slow, an accumulation of lipolytic products will occur at the oil–water interface leading to the postulated self–regulation of fat digestion.

2.4.5 Lipolysis of frying oils

Few studies have reported lipolysis of frying oils and little is known about the selectivity of pancreatic lipase toward the oxidized and polymeric compounds present in used frying fats and oils (211–213). A comparison of pancreatic lipase action *in vitro* on complex glycerides, obtained from olive oil heated at 180 °C for 150 h with unheated olive oil and 1:1 blend of heated and unheated olive oil, found the extent of hydrolysis to increase substantially with time but decrease in proportion to the degree of oil alteration (212). A much higher degree of lipase

hydrolysis was reported for TG dimers than for TG polymers in the heated olive oil (212). A study involving *in vitro* lipase hydrolysis of frying oils reported high hydrolysis rate of oxidized TG monomers and a significant discrimination against TG dimers and TG polymers in particular (213). Also, hydrolysis of intact TG monomer was shown to be affected by the presence of dimers and polymers in abused frying oil (213). The discrimination against high molecular weight lipid oxidation products may be explained due to the reported pancreatic lipase fatty acyl specificities for chain length, position and geometry of double bonds and attributed to steric hindrance for the formation of activated substrate–enzyme complex (214). Also, factors such as availability and orientation of the molecules at the water–oil interface cannot be ruled out. The lack of appreciable enzymatic hydrolysis of polymeric compounds obtained from the oxidized oils may also be attributed to their complex nature involving intra and intermolecular C–C linkages and higher polarity causing enzyme inactivation (215). Duration of exposure to lipolysis has no impact on the order of relative ease of hydrolysis with oxidized TG monomers being the fastest and TG polymers the slowest, again highlighting the influence of MW on the action of pancreatic lipase. High hydrolysis rates, comparable to that for unoxidized TG monomers, have also been reported for TG mono hydroperoxides, which again has been attributed to the similarities in their molecular structure to unoxidized TG monomers and hydrophilic properties. Under thermoxidation conditions, however, unlike oxidized TG monomers, TG mono hydroperoxides are rather unstable (216). The high hydrolysis rate of oxidized TG monomers is of particular concern since the resulting oxidized fatty

acids can be found at levels as high as 6.6% in used frying oils (217). These oxidized fatty acids are readily absorbed and have potentially negative physiological implications (218–221).

Studies comparing *in vivo* digestibility of olive oil unheated and heated at 180 °C for 50 h in the presence of air with a polar content of 46%, in young wistar rats reported a 24% decrease in the digestibility coefficient for the heated oil in comparison to the unheated oil. The inhibitory role of altered compounds, such as oligomers, on digestibility and absorption of unoxidized TG has also been observed in other studies involving heated fats (222). True digestibility of different thermoxidized compounds, quantified using silica gel column and HPSEC, was almost 80% in the case of fresh oil when compared to 30–40% from heated oil. Also, hydrolysis of unoxidized TG monomer was reported to be negatively affected by the presence of large amounts of thermoxidized compounds (223).

Fish oil studies (224) have concluded that the high molecular weight TG polymers in oxidized fish oils can be hydrolyzed by pancreatic lipase *in vitro*. In oils with less than 4% of TG polymers, both TG monomers and TG polymers were almost completely hydrolyzed by pancreatic lipase *in vitro* after 1 h. However in the case of highly oxidized oils, containing 20 or 30% of TG polymers, some TG monomers remained unhydrolyzed (224). Hydrolytic degradation of the TG polymers of highly oxidized fish oils by pancreatic lipase can produce fatty acid dimers containing more than one type of linkages, which may include a peroxy, ether or carbon linkage (224). Release of fatty acid dimers from thermally

oxidized olive oil when acted upon by pancreatic lipase, has also been reported (224).

Evidence from *in vitro* (225) *and in vivo* (226) studies suggests that long–chain polyunsaturated fatty acids (PUFA) in fish oils are liberated more slowly from TG molecules than shorter chain, less unsaturated fatty acids. Possible bases for the discrimination of pancreatic lipase against PUFA include chain length, degree of unsaturation and their distribution between the primary and secondary positions of the TG molecule. Peroxidised PUFA, however, do not appear to be discriminated against during the hydrolysis of TG by pancreatic lipase (227).

With respect to the hydrolysis of TG polymers by pancreatic lipase, findings based on thermally oxidized plant oils may not apply to air–oxidized fish oils. Apart from the obvious differences in fatty acid composition between plant oils and fish oils, the type of linkages in TG polymers may have an influence on the susceptibility of these compounds to lipase hydrolysis. Oxidation induced at the high temperatures to produce thermally oxidized oils likely generates a higher proportion of C–C linkages than autoxidation brought about by the passage of air or oxygen at moderate temperatures, a situation, which favors the formation of peroxy linkages between PUFA. Thus, the results of studies using thermally–oxidized plant oils may differ considerably from those obtained with air–oxidized fish oils (228).

In fish oil lipolysis studies (38), MG accounted for around 33% of the final lipid product in incubations of original oils and oils oxidized for 4 days, which is consistent with the known substrate specificity of lipase hydrolyzing the fatty

acids from the 1– and 3–position, but not utilizing the resultant 2–MG. Also, the reported high content of monoenoic acids in the FFA fraction of all lipase–digested fish oils is consistent with the preferential esterification of monoenes in Sn–3 position of fish oil TG and the high proportions of 22:6 ω–3 PUFA in Sn–2 MG is consistent with its stereospecific distribution in fish oil TG (229).

Another study on the specificity of hydrolysis of TG in menhaden oil by pancreatic lipase *in vitro* showed clearly that the TG remaining after 70% hydrolysis of the oil were enriched in C20 and C22 fatty acids and PUFA were most resistant to hydrolysis (225). Given that 22:6 ω–3 is heavily oxidized during autoxidation of fish oils, the Sn–2 position of fish oil TG may contain a higher proportion of autoxidized fatty acids than positions Sn –1 and Sn –3 simply on account of the high level of 22:6 ω–3 located in that position (229). Nevertheless, the highly unsaturated nature of the Sn–2 position of fish oil TG may suggest that this position is heavily involved in the formation of TG polymers by intermolecular condensation of peroxy radicals (229).

The presence of natural tensioactive compounds present in the oil and surfactants formed during frying may influence the kinetic behaviour of pancreatic lipase towards thermally oxidized oils. This process appears to be Michaelian, in which the apparent k_m and the apparent V_{max} of the enzymatic process depend more on the type of oil tested than on the degree of alteration (230). Also, the role of minor components like sterols and polyphenols, on the hydrolytic behaviour of digestive enzymes, needs further investigation (230). Thermally oxidized oils contain a complex mixture of non–volatile oxidation products such as oxidized TG

monomers, TG dimers, and TG polymers, which are mainly associated with changes in the physico–chemical properties of fats. These may not only impact the oil/water interface but could also influence the lipolytic activity of the lipase (231). In order to assess the influence of altered fatty acids resulting from the hydrolysis of complex compounds, TG dimers and TG polymers, on the activity of pancreatic lipase, velocity and substrate concentration relationships were measured and the corresponding kinetic parameters were calculated (230), v = initial velocity in µmol/min.mg of enzyme; S = the substrate concentration in mM. Palm olein and sunflower oil, with the same level of polar content (18%) were hydrolyzed by pancreatic lipase following Michaelis–Menten saturation kinetic behaviour and the data were fitted to the Michaelis–Menten equation by nonlinear regression (230). Estimated values for apparent V_{max} and apparent K_m for the two oils revealed that the degree of alteration of the oils was not the only factor impacting lipase activity. Pancreatic lipase activity was found to be greater in palm olein in comparison to sunflower oil, despite the comparable level of alteration in the two oils. The difference in the kinetic behaviour of the enzyme was attributed to the different fatty acid composition of the two oils, with a higher content of palmitic and oleic acids but a lower content of linoleic acid in palm olein. Oleic acid is known to be released by pancreatic lipase with the highest relative speed (225). Also, higher levels of tocotrienols in palm olein and tocopherol in sunflower oil in addition to the level of polyphenols and other tensioactive compounds in the two oils may impact the enzyme activity and thus the reaction rate (232, 233).

In conclusion, both *in vitro* (213) and *in vivo* (230) studies have confirmed that digestibility coefficients of altered oils are significantly lower than those of unused oils. The efficiency of lipase hydrolysis is much higher for TG monomers both unoxidized and oxidized in comparison to that for higher molecular weight lipid oxidation products like TG dimers and TG polymers. Also, hydrolysis of unoxidized TG monomers has been shown to be negatively affected in the presence of large amounts of thermally oxidized compounds.

Lack of appreciable enzymatic hydrolysis of polymeric compounds obtained from oxidized oils may be attributed to their complex nature involving intra and intermolecular C–C, C–O–C linkages and a higher polarity causing enzyme inactivation. Also, substrate availability and suitable orientation at the interface and conformational change in the lipase upon adsorption at the lipid/water interface are factors that may influence lipase catalyzed reactions. Discrimination against high molecular weight lipid oxidation products may also be attributed to the reported pancreatic lipase fatty acyl specificities for chain length, position and geometry of double bonds which may sterically hinder formation of an activated substrate–enzyme complex. Steric hindrance and hydrophobic interactions are believed to be the two primary forces that determine lipase selectivity.

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Chapter 3 Sitosterol as an antioxidant in frying oils[1]

3.1 Introduction

Phytosterols are cholesterol like molecules, found in all plants with the highest concentration occurring in vegetable oils (1). They make up a major portion of the unsaponifiable content of vegetable oils either as free sterols or esterified to fatty acids. The major plant sterols present in vegetable oil include sitosterol, campesterol, stigmasterol, brassicasterol, and avenasterol (2). Despite, their widespread occurrence, there are relatively few published reports on the effect of plant sterols on vegetable oil stability at frying temperatures, as described below. Gertz, Klostermann and Kochhar (3) have suggested that the stability of vegetable oils at frying temperature may be a function of more than just the fatty acid composition and have reported a co–relationship between the unsaponifiable matter content and oxidative stability at elevated temperatures (OSET), which is the inverse percentage of triglyceride (TG) polymers that form when oil is heated at 170 ^{o}C in the presence of water–conditioned acid silica gel for 2 h. The addition of phytosterols in refined rapeseed and sunflower oils at levels of 0.25% (w/w) was found to significantly increase the OSET value, implying a decrease in the formation of TG polymers (3).

Gordon and Magos (4) hypothesized that sterols with an ethylidene containing side chain may function as antioxidants by enabling lipid free radicals to react with the unhindered allylic protons on the side chain, with the resulting radical

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rearranging to a relatively more stable tertiary free radical. They found antioxidant activity for Δ–5 avenasterol and fucosterol, each of which possess an ethylidene side chain, in a TG mixture similar in composition to olive oil, heated at 180 °C for 72 h. Sims, Fioriti and Kanuk (5) have also reported antioxidant activity for vernosterol, Δ–7 avenasterol and fucosterol, in safflower oil heated to 180 ^{o}C but found sterols with no ethylidene side chain, ergosterol, lanosterol, β–sitosterol, stigmasterol and cholesterol, ineffective as antioxidants.

Recent reports on the impact of phytosterols on the thermal and oxidative stability of frying oils have been mixed. A phytosterol blend, which included, 3.8% brassicasterol, 26.9% campesterol, 0.6%, campestanol, 17.2% stigmasterol, 48.2% β–sitosterol, 1.1% sitostanol, 1.3% Δ5–avenasterol, and 0.8% Δ7–stigmastenol, was found to significantly decrease polymerization in stripped soybean oil heated over a period of 8 h, at a concentration of 1.0 % and 2.5%. However, the same phytosterol blend at a concentration of 2.5% was found to significantly increase polymerization of stripped high–oleic sunflower oil over a 12 h heating period.

A study assessing the anti–polymerization activity of oat sterols found significantly lower amounts of polar compounds in soybean and cottonseed oil samples heated at 180 ^{o}C for 10 days in the presence of an oat extract, in comparison to oils containing tertiary butyl hydroquinone (TBHQ), dimethyl polysiloxane (DMS) and oils with no additives (7). White and Armstrong (8) found soybean oil samples heated at 180 ^{o}C to deteriorate more slowly in the presence of oat–sterol fractions containing Δ5–avenasterol when compared to samples containing pure β–sitosterol, which altered at a rate similar to that of the

controls. Another study evaluating the effect of soy sterols consisting of β–sitosterol (45.7%), campesterol (27.3%), stigmasterol (15.3%), and brassicasterol (4.4%) on soybean oil and distilled soybean oil methyl esters found no improvement in the oxidative stability index (OSI) of the oil substrates (9). Commercially used antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and TBHQ, have been reported to be heat–sensitive and volatile, hence, lack antioxidant activity at frying temperatures. Buck (10) analyzed the volatility of BHA, BHT and TBHQ in soybean oil and reported a 70% reduction in concentration of all additives after 4 h of heating at 180 ^{o}C. Dimethyl polysiloxane (DMS) has been known to be effective in extending frying life of oils by functioning as an antifoaming agent and providing a protective inert surface that inhibits oxidation (11). However, synthetic additives in edible oil are not desirable and so it is useful to study the anti–polymerization effect of natural vegetable oil sterols at frying temperature. Also, refining of vegetable oils is known to result in the removal of considerable amounts of unsaponifiables including sterols and endogenous antioxidants, thus lowering their natural oxidative stability at frying temperatures in comparison to the non–refined vegetable oils (12).

The objective of this study was to assess the ability of sterols naturally found in vegetable oils to limit TG polymer formation in refined vegetable oils and triolein heated to a frying temperature of 180 ^{o}C for up to 72 h. A mechanistic explanation based on the structural features of β–sitosterol and campesterol is proposed, which is indicative of their potential importance as antioxidants at frying temperatures.

Table 3.1.1 Canola oil sterol composition (12)

sterols	% w/w of total sterols
β–Sitosterol	52.3
Campesterol	27.6
Avenasterol	3.0
Stigmasterol	2.8
Brassicasterol	13.8
minor sterols	0.5

3.2 Materials and methods

Refined canola and flaxseed oil samples were obtained from Richardson Oilseed Company, Lethbridge, Alberta, Canada and high oleic sunflower oil was obtained from Adams Vegetable Oils Inc, Arbuckle, California, USA. Triolein (99% pure) was obtained from Nu– check Prep Inc., Elysian, Minnesota, USA. A sterol blend, comprising of β–sitosterol, β–sitostanol and campesterol which together account for around 75–80 % of sterols commonly found in vegetable oils, (Table 3.1.1) was used in the experiment. Test samples with 1%, 2% and 5% additional sterol were prepared by adding 20 mg, 40 mg and 100 mg of β–sitosterol (assay ~ 60%, remainder being campesterol and β– sitostanol) obtained from Fluka Analytical, Buch, Switzerland, Europe, to 2 g of each oil, canola, high oleic sunflower, flaxseed and triolein contained in 7 ml open mouth glass vials, followed by gentle mixing by vortex. The samples and controls with no additional sterol were then

placed in a Stabil–Therm gravity oven (Illinois, USA) set at 180 ^{o}C for durations that ranged from 12 h to 72 h. Small aliquots, drawn in duplicate from each vial at time intervals of 12, 24, 36, 48, 60, and 72 h, were dissolved in tetrahydrofuran (THF) (HPLC–grade; Fischer scientific, Toronto, Ontario, Canada) to a concentration of 5 mg/ml and analyzed twice by High Pressure Size Exclusion Chromatography (HPSEC). The final result was an average of the four measurements.

The HPSEC system included an Agilent 1200 series binary pump (G1312 A) and a degasser (G1379 B), Agilent 1200 series (G13229A) auto sampler and Agilent 1200 series evaporative light scattering detector (ELSD) obtained from Agilent Inc. (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). The ELSD was set at 70 ^{o}C with a N_2 flow rate of 3.0 l/min under pressure of 3.5 bars. Two size exclusion columns were used in series, a Waters (Waters Limited, Mississauga, Ontario, Canada) styragel HR 100 A^{o} (4.6 x 300 mm) and a Phenomenex (Phenomenex, Torrance, California, USA) phenogel 500 A^{o} (4.6 x 300 mm). THF was used as the mobile phase at a flow rate of 0.3 ml/min. Percentage peak area, obtained by drawing vertical lines connecting the starting and ending retention points of a peak to the base line using the Chemstation software, served as the basis to follow changes in TG polymer formation, level of intact TG monomers and the extent of TG ester hydrolysis.

In order to demonstrate the possible conversion of sitosterol to sitostadiene at frying temperatures, sitosterol was heated at 180 ^{o}C for 48 h and HPSEC chromatograms were obtained using both ELSD and UV detectors. For

comparison, cholestadiene, a commercially available steradiene obtained from Sigma Aldrich, Oakville, Ontario, Canada was used as a standard.

In order to further corroborate the role played by the conjugated diene system in limiting TG polymer formation, triolein samples were heated at 180 ^{o}C for 72 h in the presence of 5% levels of sitosterol and sitostanol. Sitostanol was obtained from Sigma Aldrich, Oakville, Ontario, Canada

Statistical Analysis: Treatment effects on TG polymer formation, level of TG monomers and the extent of TG ester hydrolysis in triolein, canola, high oleic sunflower and flaxseed oils were compared by repeated measures analysis of variance using Sigma Plot 11 (Systat Software Inc., San Jose, CA). The mean peak areas for TG polymer, TG monomers and TG ester hydrolysis products for different treatments at each time point were compared using Tukey's multiple comparisons test, where $p \leq 0.05$ was deemed statistically different.

3.3 Results and Discussion

HPSEC chromatogram of triolein before heating with a single TG monomer peak at retention time ~17.7 min is shown in Figure 3.3.1a. A typical chromatogram of triolein after heating at 180 ^{o}C for 72 h, included peaks corresponding to the TG polymer at a retention time ~12.8 min, TG monomer at ~17.7 min and TG ester hydrolysis products at~18.4 min (Figure 3.3.1b). As expected, the level of unsaturation was found to influence the rate of TG polymerization, as shown by the appearance and growth of the TG polymer peak at a retention time of ~12.8 min in the HPSEC chromatogram for the vegetable oil and triolein samples heated at 180 ^{o}C for 48 h. (Figure 3.3.2). In triolein, canola and sunflower oil samples,

the TG polymer peak first appeared after 48 h of heating at 180 ^{o}C. On the other hand, flaxseed oil, with a linolenic acid content of about 53% required only 24 h of heating for TG polymer formation and with the exception of samples with the highest level of additional sterol (5%) all flaxseed oil samples were solid after 24 h of heating. Figure 3.3.3 represents the appearance and growth of the TG polymer peak in triolein over a 72 h heating period.

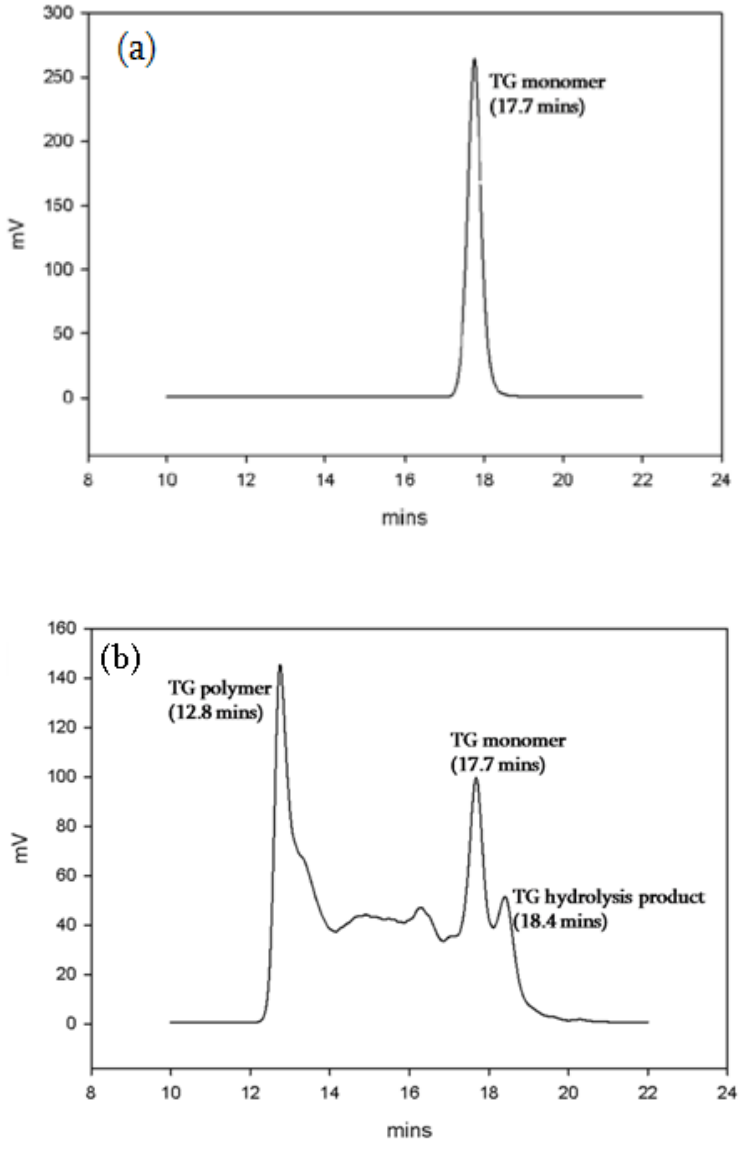


Figure 3.3.1 Triolein (a) before and (b) after heating at 180 ^{o}C for 72 h

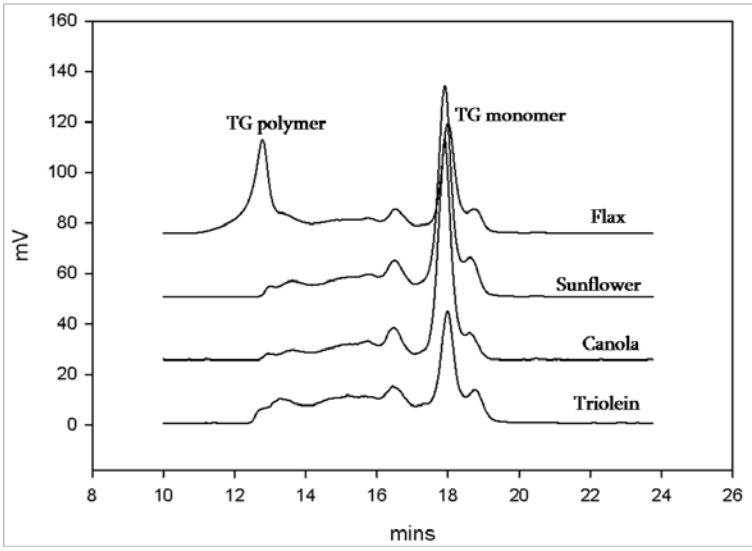


Figure 3.3.2 HPSEC chromatograms of triolein, canola, sunflower and flaxseed oils with 5% sterol heated at 180 ^{o}C for a period of 48 h

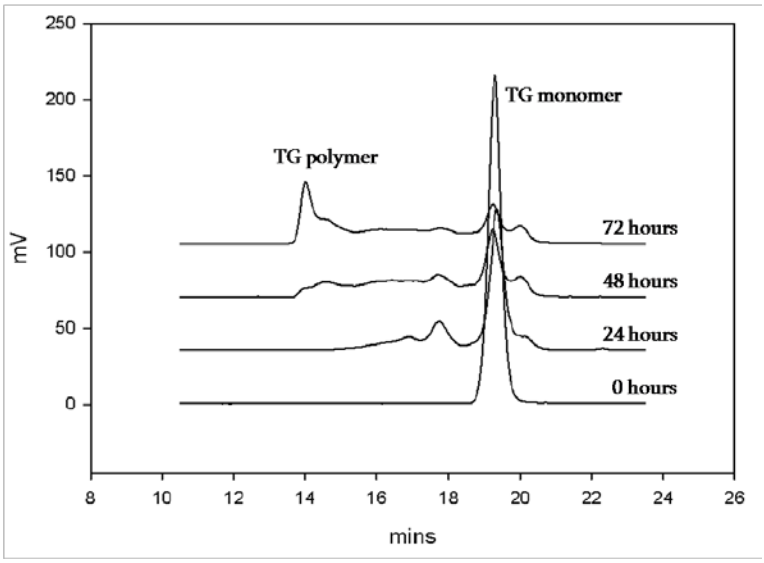


Figure 3.3.3 HPSEC chromatograms of triolein with 5% sterol heated at 180 ^{o}C for 24, 48, 60 and 72 h

The effect of sterol addition on the formation of TG polymer in triolein heated for 72 h at 180 ^{o}C is shown in Figure 3.3.4a and plotted in Figure 3.3.4b. (Table 3.3.1) Similar data for canola, high oleic sunflower and flaxseed oils is presented

in Tables 3.3.3 –3.3.5 and Figures 3.3.6 – 3.3.8. In triolein, about 40% decrease in TG polymer formation was observed in the presence of 5% sterol after 72 h of heating at frying temperature. Similarly for canola after 72 h of heating in the presence of 5% additional sterol, a 21% reduction in TG polymer formation was seen compared to samples with no additional sterol. In sunflower oil, 5% additional sterol reduced TG polymer formation by about 50% after 72 h of heating. The extent of TG polymer formation in flaxseed oil after 24 h of heating was about 54% less in samples with 5% additional sterol compared to samples without any additional sterol. Overall, the addition of sterols led to a substantial decrease in the extent of TG polymer formation in triolein, canola, high oleic sunflower and flaxseed oils heated at frying temperature.

The presence of additional sterol was also seen to impact the amount of intact TG monomer remaining in oils after heating at 180 ^{o}C for 72 h. This is illustrated by the HPSEC peak areas at retention time of 17.7 min for TG monomer in triolein samples with different levels of sterol. (Figures 3.3.4a and 3.3.4c)

The impact of sterol addition on the relative increase in % peak area for TG monomer remaining after heating at 180 ^{o}C was found to be substantial during the 48–72 h period, corresponding with the appearance and growth of the TG polymer peak, in triolein samples with 5% additional sterol (Table 3.3.2).

Table 3.3.1 Peak area measurements at 12 h intervals for (a) TG polymer, (b) TG monomer, (c) TG ester hydrolysis products in triolein samples heated at 180^{o} C for a 72 h duration

(a)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	0.00	0.00	1.45^{ab}	8.06^{a}	28.47^{ab}	38.63^{a}
1	0.00	0.00	1.38^{ab}	7.73^{ab}	26.28^{ab}	36.59^{ab}
2	0.00	0.00	2.08^{a}	5.05^{ab}	33.30^{a}	32.92^{ab}
5	0.00	0.00	1.01^{b}	2.40^{b}	14.29^{b}	22.99^{b}

(b)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	81.78^{a}	63.66^{a}	42.87^{ab}	32.34^{ab}	22.24^{ab}	17.41^{a}
1	82.38^{ab}	63.67^{a}	43.43^{ab}	31.48^{a}	22.32^{ab}	17.65^{a}
2	81.04^{a}	63.77^{a}	38.71^{a}	32.04^{ab}	18.85^{a}	18.67^{ab}
5	83.33^{b}	64.99^{a}	46.12^{b}	35.00^{b}	25.58^{b}	21.78^{b}

(c)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	2.34^{a}	3.74^{a}	4.06^{a}	4.84^{a}	4.55^{a}	4.46^{a}
1	3.09^{a}	3.57^{a}	4.84^{ab}	5.13^{ab}	5.07^{ab}	5.00^{ab}
2	3.08^{a}	3.46^{a}	5.71^{ab}	6.15^{ab}	5.13^{ab}	5.58^{ab}
5	3.47^{a}	4.42^{a}	7.29^{b}	8.73^{b}	7.89^{b}	7.33^{b}

a–b Values in the same column with different superscript letters are significantly different ($P < 0.05$)

Table 3.3.2 Effect of 5% additional sterol on the % peak area of TG monomer in triolein remaining after heating at 180 ^{o}C for a duration of 72 h

Heating duration (h)	TG monomer remaining after heating (0% sterol) (% peak area)	TG monomer remaining after heating (5% sterol) (% peak area)	TG monomer remaining after heating relative increase (% peak area)
12	81.78 ± 1.70	83.33 ± 1.37	1.9
24	63.66 ± 1.51	64.99 ± 0.78	2.1
36	42.87 ± 0.52	46.12 ± 0.75	7.6
48	32.34 ± 0.36	35.00 ± 0.41	8.2
60	22.24 ± 0.29	25.58 ± 0.63	15.0
72	17.41 ± 0.27	21.78 ± 0.73	25.1

Based on the retention time of 18.4 min for a diolein standard under identical conditions, it was concluded that the peak at retention time of 18.4 min in the heated triolein HPSEC chromatogram (Figure 3.3.1b) was indicative of the extent of TG ester hydrolysis that would occur during frying. The addition of sterol appeared to have a significant impact on the extent of TG ester hydrolysis in each of the vegetable oil samples and triolein. The effect of additional sterol on the extent of TG ester hydrolysis in triolein is shown in Figure 3.3.4a and plotted in Figure 3.3.4d. In the case of triolein, addition of 5% sterol resulted in an average increase in TG ester hydrolysis of about 19% during the first 24 h of heating compared to samples without any additional sterol. In contrast, during the 48–72 h heating period, the average increase in TG ester hydrolysis in triolein samples was found to be 74%. In canola, the percentage increase in the extent of TG ester

hydrolysis for samples with 5% additional sterol was 55% during the first 24 h, and 96% in the times between 48 and 72 h. With sunflower oil, the average increase in TG ester hydrolysis in samples with additional 5% sterol was found to be about 63 % in the first 24 h and 94% in the following 48–72 h period. In flaxseed oil, in the 24 h heating period the extent of TG ester hydrolysis was found to increase by almost 33% in the presence of additional 5% sterol.

The increase in TG ester hydrolysis that occurred during the initial 48 h of heating for all samples, both with and without sterol addition, may be due to the interaction of TG esters with hydronium ions resulting from atmospheric moisture at the air–oil interface. Once the TG ester hydrolysis attained equilibrium after ~48 h, the effects due to higher levels of sterol became more prominent. (Figure 3.3.5) This can be explained by the higher proton affinity of TG esters in comparison to the sterol OH group. The greater extent of TG ester hydrolysis during the 48–72 h heating period, seen for all vegetable oil and triolein samples with additional 5% sterol in comparison to samples with no additional sterol, may be attributed to the water released in the system during the conversion of Δ5–sterol to Δ3, 5–steradiene by the 1, 2–elimination reaction. Conversion of sterol to steradiene by the elimination of water has also been reported during the bleaching and deodorization steps of vegetable oil refining (14).

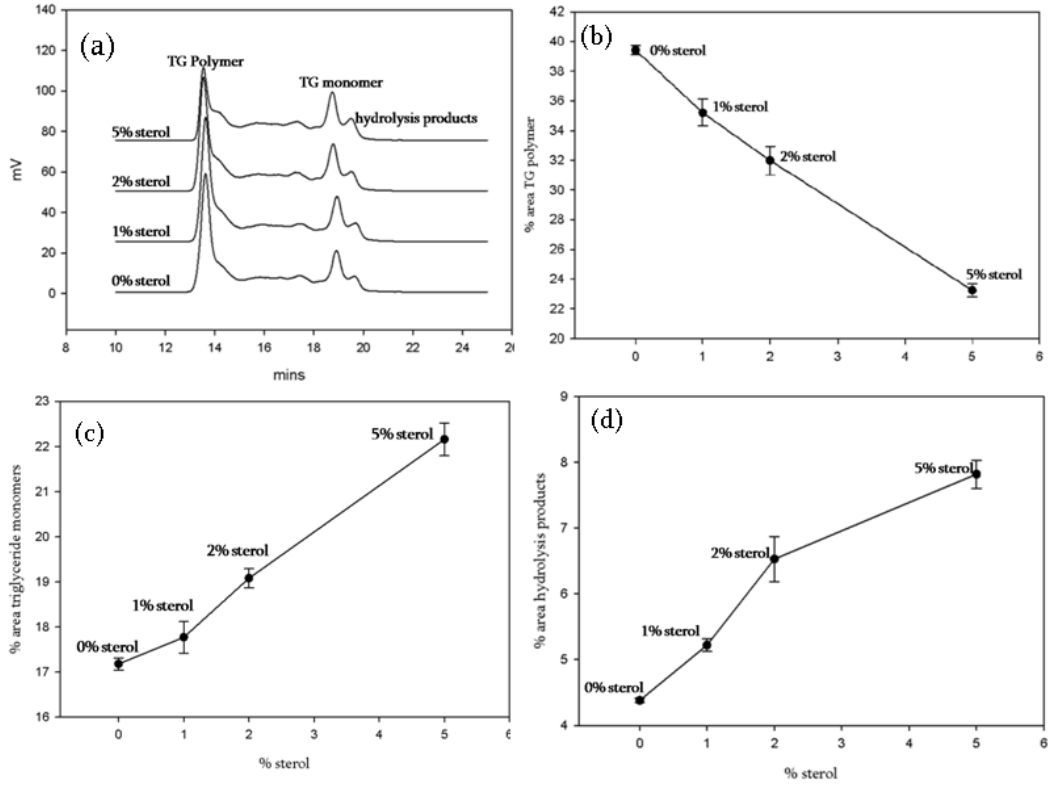


Figure 3.3.4 Triolein heated at 180 ^{o}C for 72 h in the presence of different levels of sterols (a) HPSEC chromatograms (b) effect on TG polymer formation (c) effect on level of TG monomer (d) effect on TG ester hydrolysis

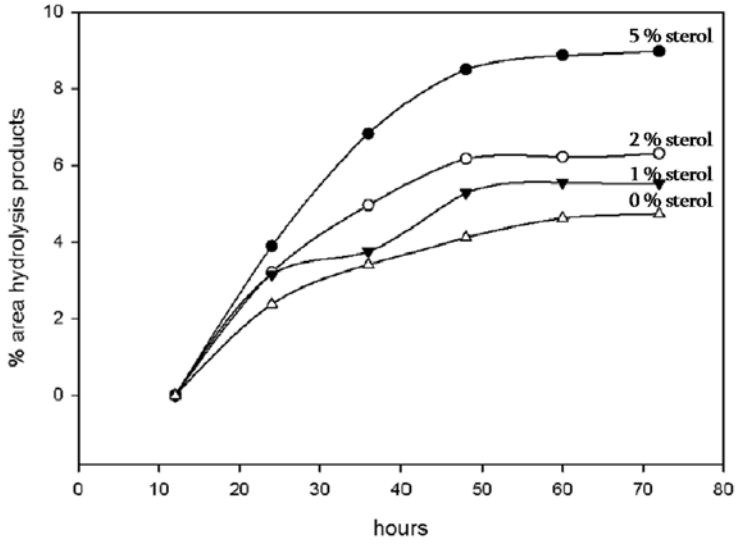


Figure 3.3.5 Progression of the extent of TG ester hydrolysis with time in triolein heated at 180 ^{o}C over a 72 h period

While the effect of additional 5% sterol in triolein samples after heating at 180 ^{o}C for 72 h resulted in a distinct drop in TG polymer formation accompanied with an increase in the remaining intact TG monomer and the extent of TG hydrolysis, the observations in canola, high oleic sunflower and flaxseed oils were not as sharp. The extent of TG ester hydrolysis in each of the oils was found to increase significantly ($p<0.05$) in the presence of 5% additional sterol over the heating duration of 72 h, but the impact on the extent of TG polymer formation and the remaining unaltered TG monomer was not the same. (Tables 3.3.3 – 3.3.5 and Figures 3.3.6 – 3.3.8)

In canola oil samples, the drop in TG polymer formation was significant ($p<0.05$) at 1% additional sterol level but not at 2% and 5% levels. In sunflower oil samples, the drop in TG polymer formation was found to be significant ($p<0.05$) at 5% additional sterol level and similar was the case with flaxseed oil samples. The level of intact TG monomer remaining after the heating duration of 72 h was found to significantly ($p<0.05$) increase at 1% additional sterol level in canola oil samples but not at 2% and 5% levels, whereas in the case of sunflower and flaxseed oil samples the increase in the level of intact TG monomer was found to be significant ($p<0.05$) at 5% additional sterol levels.

A similar increase in the extent of TG ester hydrolysis in each of the oils in the presence of additional 5% sterol levels is indicative of the fact that the extent of release of protons by the dehydration of sterols was comparable in the three oil samples. However, only sunflower and flaxseed oils indicated a corresponding decrease in TG polymer formation accompanied with an increase in unaltered TG

monomer levels. In comparison to triolein, edible oils represent a much more complex system with the presence of different TG with varying fatty acid compositions. In addition, the level of minor constituents such as phospholipids, chlorophyll, carotenoids, phenolic compounds, tocopherols and tocotrienol also tend to differ in different oils. Diversion of the protons released by the dehydration of sterols to quench radicals other than those from the lipid oxidation chains such as tocopheroxy and carotenoids may be a possible explanation for the inability of additional sterols at 5% level in canola oil samples to limit TG polymer formation to the same extent as observed in sunflower and flaxseed oil samples. Hence, a more realistic comparison of different edible oils samples would be possible if the oil samples were to be stripped of the minor constituents before testing.

HPSEC chromatograms obtained at 12 h intervals, (Figure 3.3.9) representing triolein, canola oil, sunflower oil and flaxseed oil samples with no additional sterol heated at 180 ^{o}C over a 72 h period, indicate a higher resistance to TG polymer formation in canola oil samples in comparison to sunflower oil and triolein which may be attributed to the presence of higher levels of natural antioxidants such as tocopherols, tocotrienols and carotenoids in canola oil samples. Flaxseed oil samples as indicated turned solid after 48 h of heating which is expected given the high level of linolenic acid in flaxseed oil.

Table 3.3.3 Peak area measurements at 12 h intervals for (a) TG polymer, (b) TG monomer, (c) TG ester hydrolysis products in canola oil samples heated at 180^{o} C for a 72 h duration

(a)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	0.00	0.00	0.00	1.82^{ab}	3.23^{ab}	19.78^{a}
1	0.00	0.00	0.00	1.44^{b}	3.10^{ab}	15.05^{b}
2	0.00	0.00	0.00	2.29^{a}	2.58^{b}	18.34^{ab}
5	0.00	0.00	0.00	1.92^{ab}	6.58^{a}	15.54^{ab}

(b)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	92.40^{a}	79.60^{a}	70.88^{b}	59.63^{a}	53.99^{b}	41.79^{a}
1	92.26^{a}	79.42^{a}	67.72^{a}	60.37^{a}	51.90^{ab}	43.49^{b}
2	91.80^{a}	80.40^{a}	68.96^{ab}	59.33^{a}	53.66^{b}	42.73^{ab}
5	92.29^{a}	80.03^{a}	67.58^{a}	60.06^{a}	49.39^{a}	42.65^{ab}

(c)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	0.00	1.81^{a}	2.23^{a}	3.46^{a}	3.34^{a}	3.47^{a}
1	0.00	1.93^{ab}	2.67^{ab}	3.55^{a}	4.03^{ab}	4.43^{ab}
2	0.00	1.95^{ab}	2.71^{ab}	4.33^{ab}	4.38^{ab}	5.05^{bc}
5	0.00	2.80^{b}	4.40^{b}	6.05^{b}	6.57^{b}	7.51^{c}

a–c Values in the same column with different superscript letters are significantly different ($P < 0.05$)

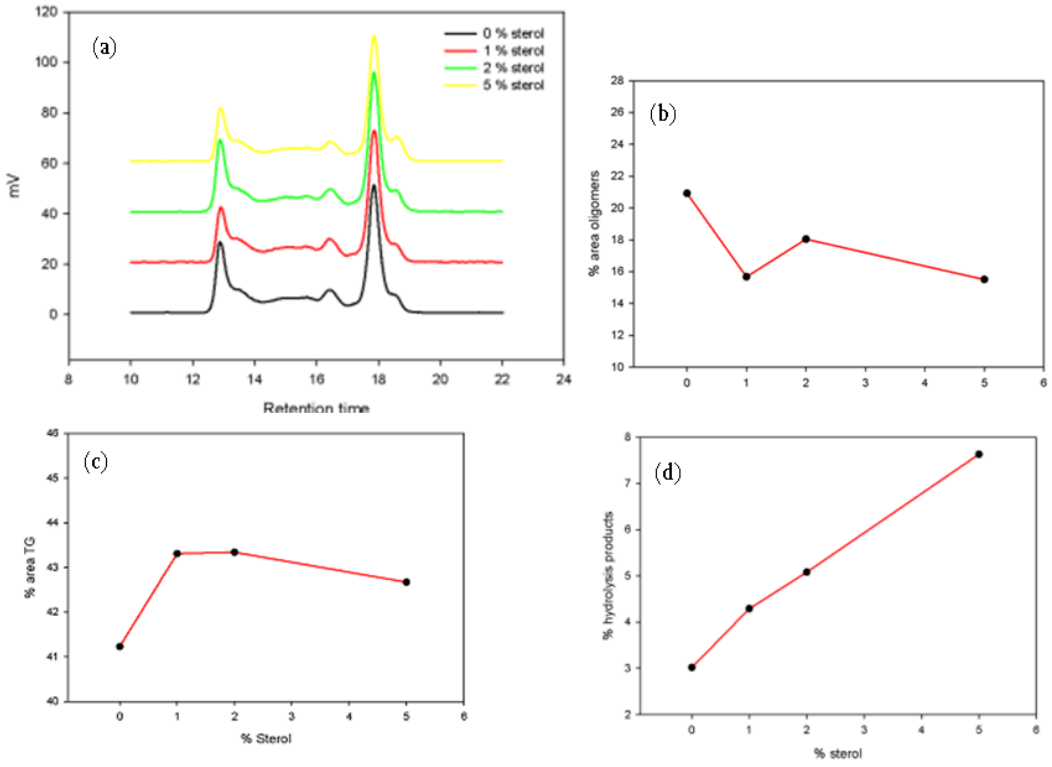


Figure 3.3.6 Canola oil samples heated at 180 ^{o}C for 72 h in the presence of different levels of sterols (a) HPSEC chromatograms (b) effect on TG polymer formation (c) effect on level of TG monomer (d) effect on TG ester hydrolysis

Table 3.3.4 Peak area measurements at 12 h intervals for (a) TG polymer, (b) TG monomer, (c) TG ester hydrolysis products in sunflower oil samples heated at 180^{o} C for a 72 h duration

(a)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	0.00	0.00	0.00	2.22^{a}	7.24^{a}	17.31^{a}
1	0.00	0.00	0.00	1.97^{ab}	5.05^{ab}	14.42^{ab}
2	0.00	0.00	0.00	1.71^{ab}	5.22^{ab}	12.59^{ab}
5	0.00	0.00	0.00	1.41^{b}	2.69^{b}	8.53^{b}

(b)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	91.29^{a}	77.85^{a}	68.23^{b}	59.32^{b}	43.25^{ab}	41.02^{a}
1	91.12^{a}	75.94^{a}	66.93^{ab}	52.90^{ab}	44.04^{ab}	42.71^{b}
2	91.44^{a}	75.73^{a}	65.19^{a}	52.13^{a}	42.83^{a}	42.31^{ab}
5	90.59^{a}	76.94^{a}	65.45^{ab}	53.50^{ab}	44.69^{b}	44.26^{c}

(c)

% sterol	12 h	24 h	36 h	48 h	60 h	72 h
0	0.00	2.38^{a}	3.41^{a}	4.12^{a}	4.62^{a}	4.92^{a}
1	0.00	3.15^{ab}	3.75^{a}	5.28^{ab}	5.55^{ab}	6.12^{ab}
2	0.00	3.20^{ab}	4.96^{ab}	6.17^{ab}	6.22^{ab}	6.79^{bc}
5	0.00	3.89^{b}	6.83^{b}	8.50^{b}	8.87^{b}	8.71^{c}

a–c Values in the same column with different superscript letters are significantly different ($P < 0.05$)

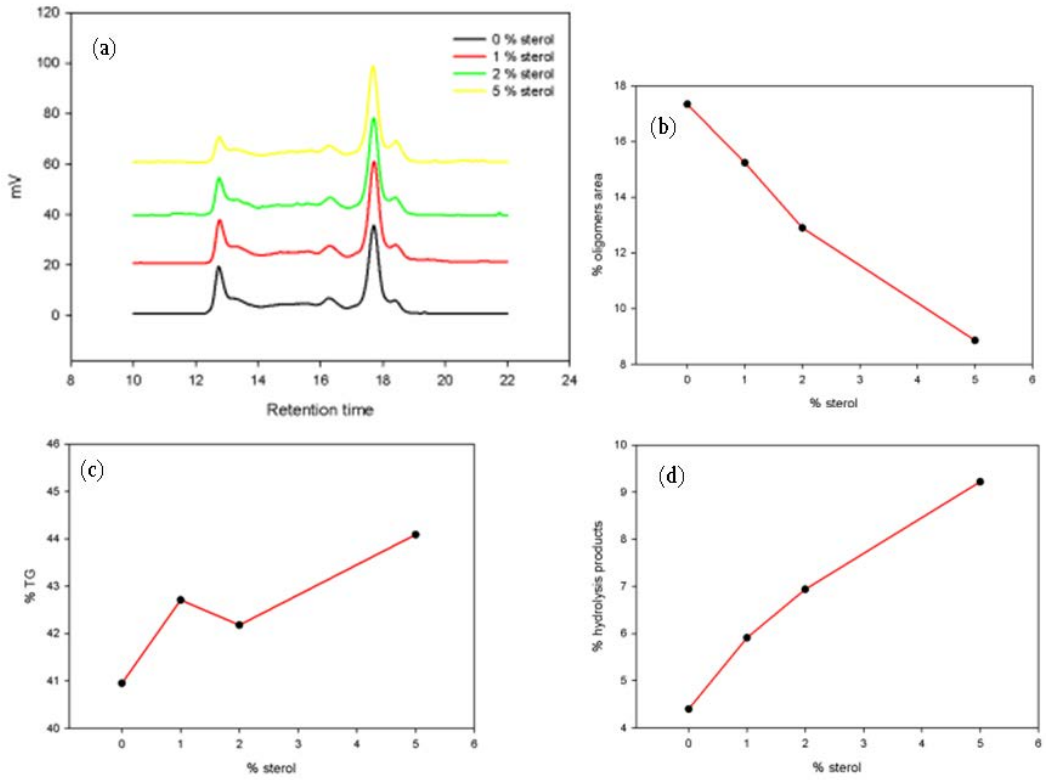


Figure 3.3.7 Sunflower oil samples heated at 180 ^{o}C for 72 h in the presence of different levels of sterols (a) HPSEC chromatograms (b) effect on TG polymer formation (c) effect on level of TG monomer (d) effect on TG ester hydrolysis

Table 3.3.5 Peak area measurements at 12h intervals for (a) TG polymer, (b) TG monomer, (c) TG ester hydrolysis products in flaxseed oil samples heated at 180^{o} C for a 72 h duration

(a)

% sterol	12 h	24 h	36 h	48 h
0	0.00	19.45^{ab}	na	na
1	0.00	20.32^{a}	na	na
2	0.00	17.15^{ab}	na	na
5	0.00	8.99^{b}	30.28	33.00

(b)

% sterol	12 h	24 h	36 h	48 h
0	82.80^{a}	47.47^{ab}	na	na
1	80.36^{b}	45.76^{a}	na	na
2	80.41^{b}	46.61^{ab}	na	na
5	82.27^{ab}	49.69^{b}	32.65	31.20

(c)

% sterol	12 h	24 h	36 h	48 h
0	2.62^{a}	2.98^{a}	na	na
1	2.77^{ab}	3.36^{ab}	na	na
2	2.84^{ab}	3.44^{ab}	na	na
5	3.52^{b}	3.91^{b}	4.57	5.97

a–b Values in the same column with different superscript letters are significantly different ($P < 0.05$)

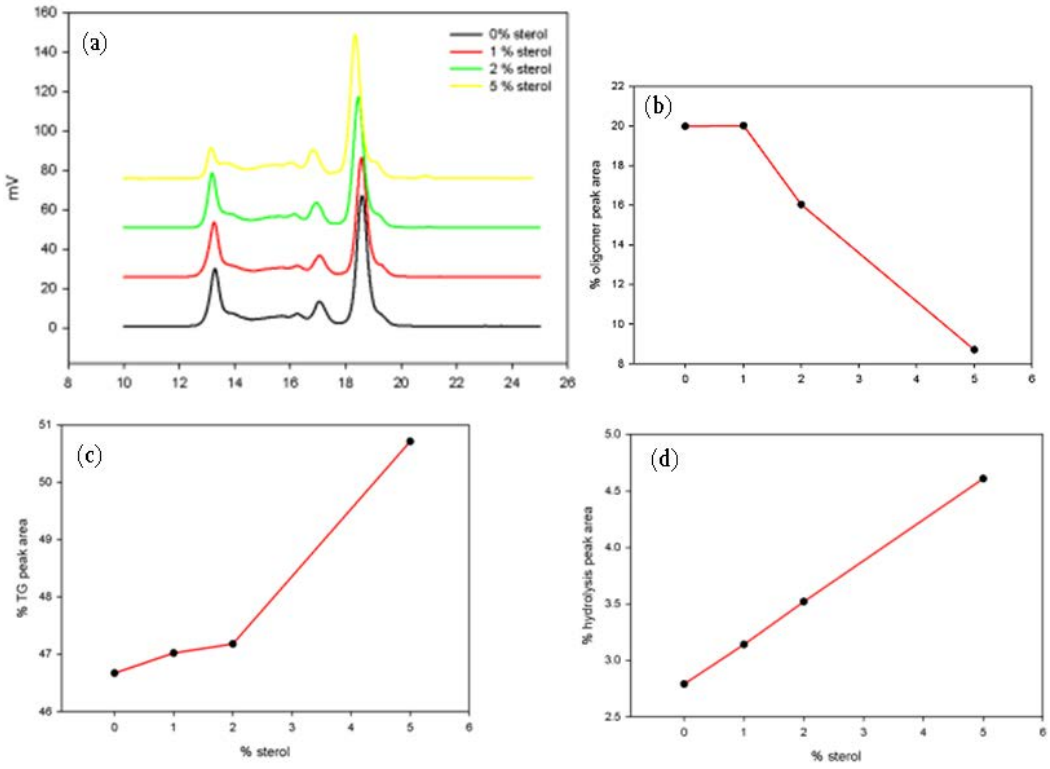


Figure 3.3.8 Flaxseed oil samples heated at 180 ^{o}C for 72 h in the presence of different levels of sterols (a) HPSEC chromatograms (b) effect on TG polymer formation (c) effect on level of TG monomer (d) effect on TG ester hydrolysis

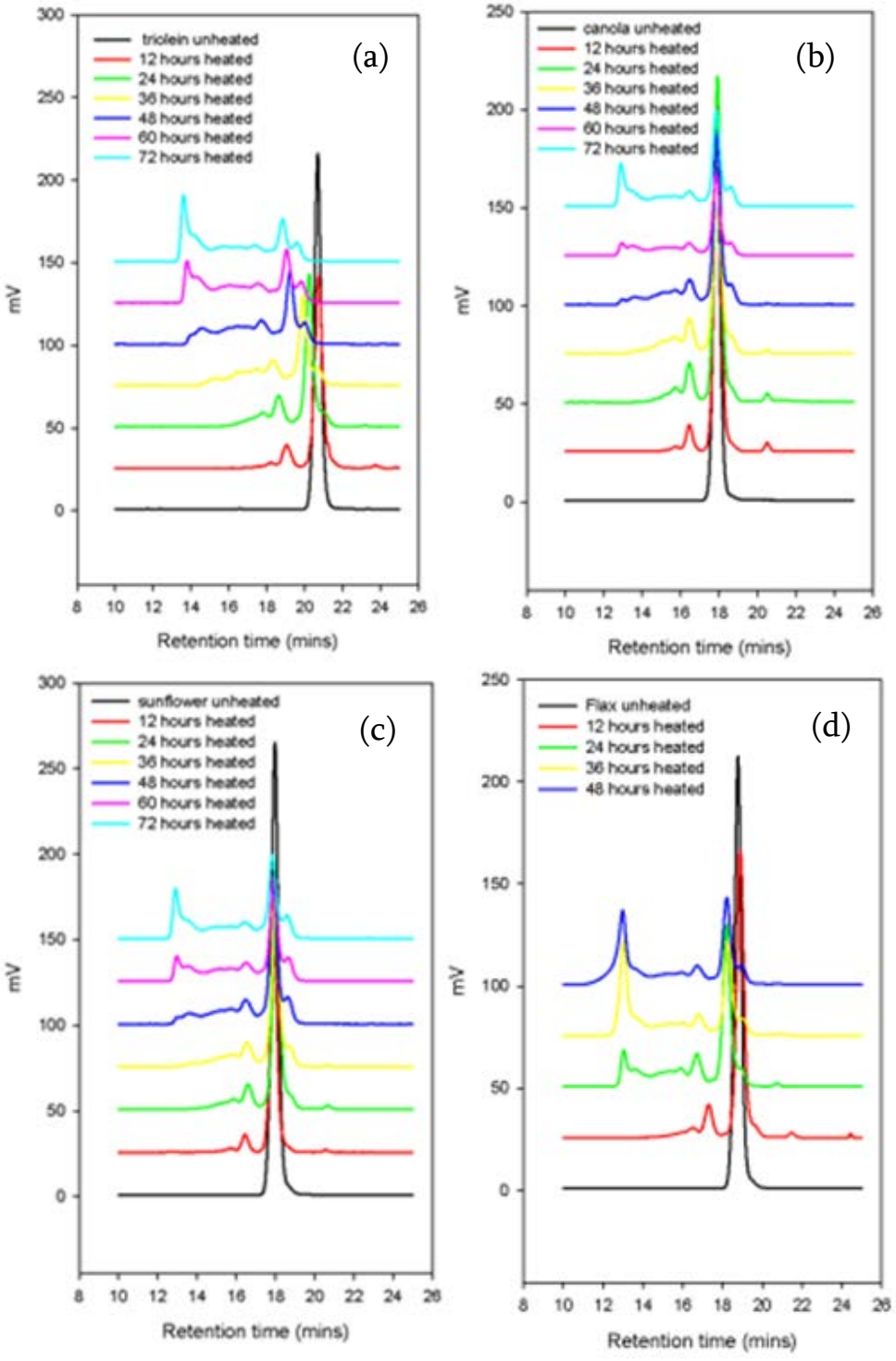


Figure 3.3.9 HPSEC chromatograms at 12 h intervals representing (a) triolein (b) canola oil (c) sunflower oil (d) flaxseed oil samples heated at 180 ^{o}C over a 72 h period

Despite the absence of structural features of avenasterol and vernosterol, known to be responsible for their antioxidative effect, (4,5) the sitosterol blend used in this experiment was found to be effective in limiting TG polymer formation in triolein, canola, high oleic sunflower and flaxseed oils heated at frying temperature.

In the case avenasterol (refer chapter 2) it was hypothesized that the presence of an ethylidene side chain, with unhindered allylic hydrogen atoms, promotes release of hydrogen for the termination of lipid oxidation chains. The resulting radical then isomerizes into a more stable tertiary free radical. (Figure 3.3.10)

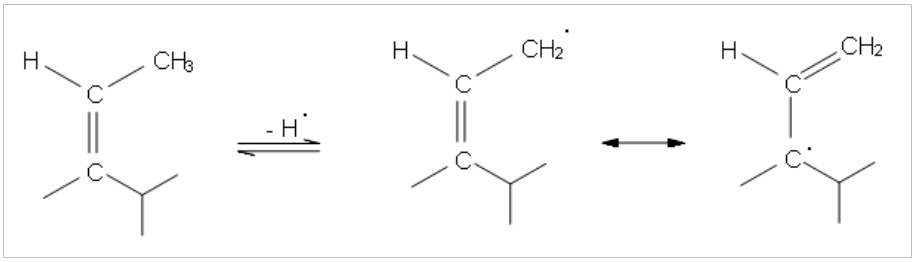
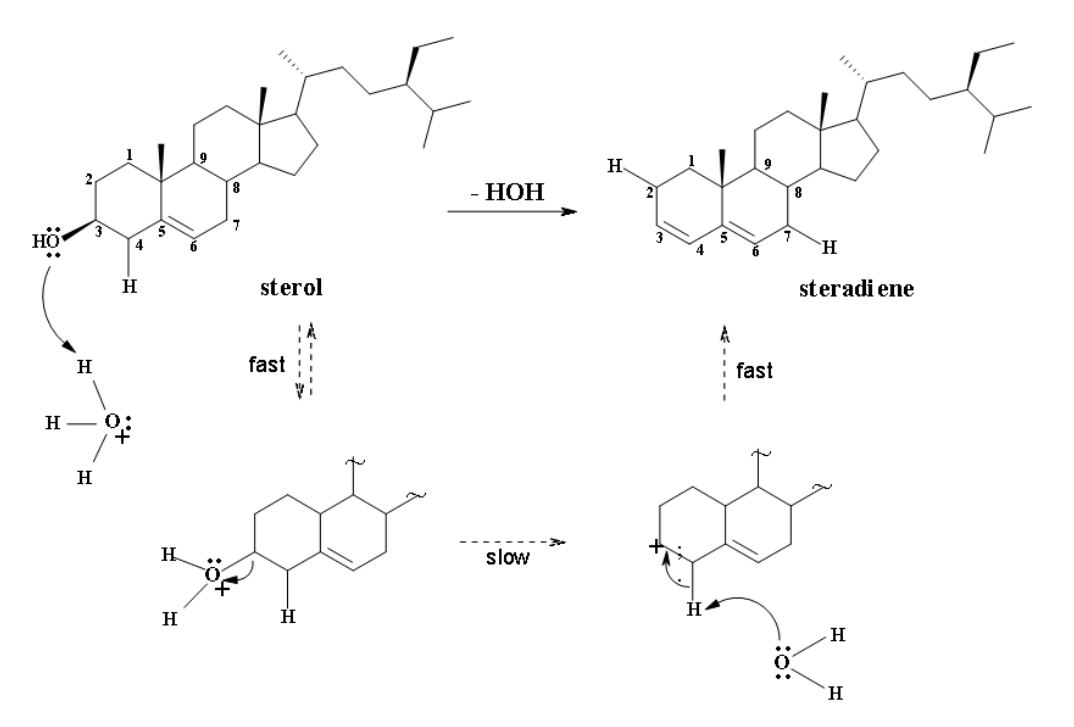


Figure 3.3.10 Loss of proton in the ethylidene side chain followed by radical stabilization by delocalization

The higher antioxidative effect in vernosterol (refer chapter 2) is then attributed to the presence of two endocyclic double bonds at the C_8 and C_{14} positions in addition to the ethylidene side chain. This makes available two further allyl hydrogens at C_{11} and C_{16} to interact with and terminate lipid oxidation chains, with the resulting radicals being stabilized by delocalization over the conjugated diene system.

The structural features in sitosterol and campesterol include an OH group at C_3 position and one endocyclic double bond at the C_5 position. Based on physical organic chemistry principles (15), the presence of a double bond at the C_5 position with its ability to stabilize through conjugation a developing double bond at the C_3 position, would strongly favor a 1, 2–elimination reaction involving the OH group at C_3 and the allylic H at C_4 carbon atoms. The loss of water involving the OH group at C_3 and the allylic H at C_4 would convert sterol to steradiene generating a conjugated diene system comprising of C_3–C_4–C_5–C_6 atoms. This would make the allylic H at C_2 and C_7 more labile and hence available to interact with and terminate lipid oxidation chains, with the resulting radicals being stabilized by delocalization over the conjugated diene system. (Scheme 3.3.1)



Scheme 3.3.1 1, 2–elimination of water from sitosterol to generate sitostadiene

Hydronium ions resulting from atmospheric moisture at the air–oil interface may catalyze the elimination reaction resulting in the conversion of sterol to steradiene. Hydronium ions with a known proton affinity of 166.4 kcal/mol would readily protonate the OH group at C_3 position in sterol, which would be comparatively more basic, based on the known proton affinities for CH_3OH (181.0 kcal/mol) and CH_3CH_2OH (186.8 kcal/mol) (16).

Elimination E1 reactions are normally accompanied by S_N1 substitution as both proceed through a common carbocation intermediate. However, at higher temperatures, elimination would be favored over substitution, which may be attributed to the fact that elimination is accompanied with an increase in the number of particles in comparison to substitution, resulting in a more positive entropy term (ΔS). This would result in a lower free energy of activation ($\Delta G^{\neq}$), given by the equation, $\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$ as at higher temperatures the $T\Delta S^{\neq}$ term would increasingly outweigh a less favorable $\Delta H^{\neq}$ term. Also, given the large size of the attacking sterol nucleophile, steric effects would play a role in restricting substitution and ensuring a higher proportion of elimination. In addition, sp^2 hybridized carbon atom in the carbocation would remain sp^2 hybridized on elimination but would have to undergo a change in hybridization to sp^3 on substitution, which would be another factor favoring elimination.

In Figure 3.3.11, trace 1 represents the ELSD chromatogram for sitosterol heated at frying temperature with the single peak at retention time of 20.3 min representing sitosterol. Trace 2 represents the UV absorbance of heated sitosterol at wavelength 232 nm, which is the absorbance maximum for cholestadiene.

Trace 3 represents the ELSD chromatogram for heated sitosterol and cholestadiene, used as a retention time standard and shown by the peak at 21.1 min. Trace 4 is part of the ELSD chromatogram for heated sitosterol, covering a retention time range of 20.3–22.0 min. The single peak with a retention time of 20.9 min in trace 4 may represent the sitostadiene peak as it closely matches the cholestadiene retention time of 21.1 min. The slight shift (0.2 min) to the left for sitostadiene in comparison with cholestadiene may be attributed to the comparatively bigger size of sitostadiene due to the presence of an additional ethyl group in the side chain at the C–24 position. In addition, appearance of a peak at the retention time of 20.9 min in trace 2, representing the UV absorbance of heated sitosterol at wavelength 232 nm, may also indicate the likely presence of sitostadiene in sitosterol heated to frying temperature.

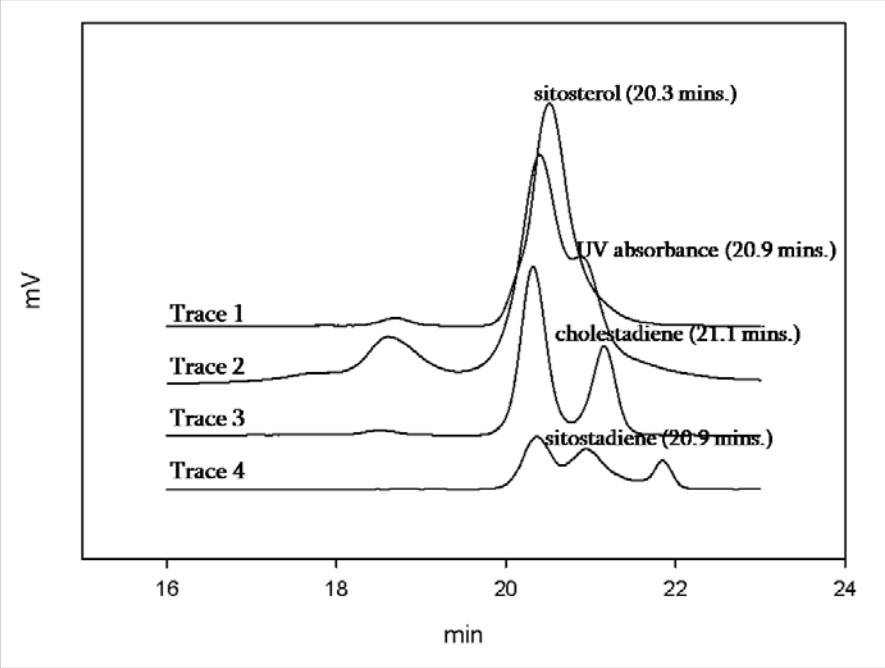


Figure 3.3.11 HPSEC chromatograms with ELSD (trace 1, 3, 4) and UV (trace 2) detection for sitosterol heated at180 ^{o}C for 48h

Corroboration of the fact that conjugated diene system of steradiene played a role in limiting TG polymer formation in frying oils was obtained by comparing the effects of the presence of sitosterol and sitostanol on TG polymer formation in triolein heated at 180 ^{o}C. Sitostanol, due to the absence of a double bond at the C_5 position would not generate a conjugated diene system that would readily form in sitosterol upon the 1, 2–elimination of water involving the OH group at C_3 and the allylic H at C_4. Figure 3.3.12 represents HPSEC chromatograms for Triolein, Triolein + 5% sitostanol and Triolein + 5% sitosterol samples heated at 180 ^{o}C for a period of 72 h.

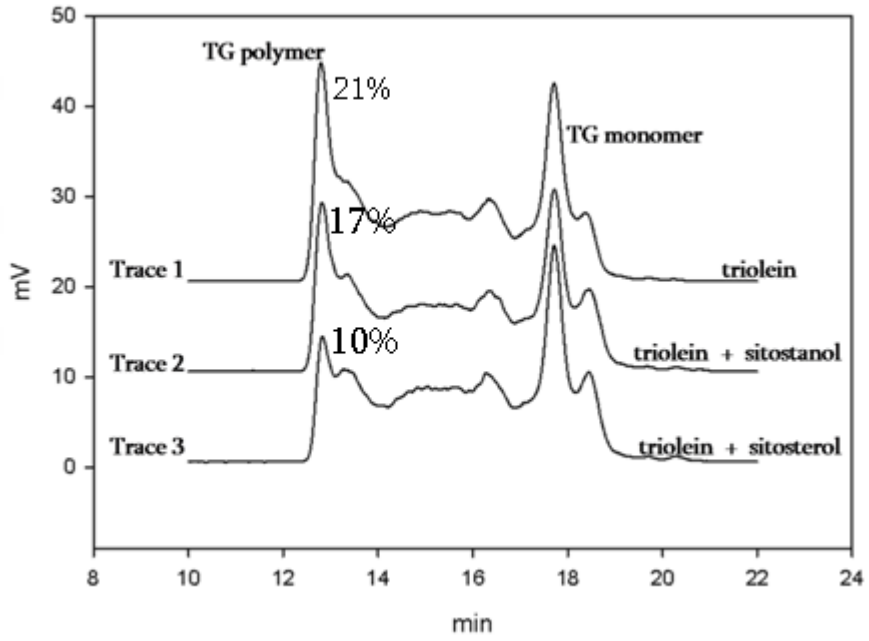


Figure 3.3.12 Effect of the presence of sitosterol and sitostanol on TG polymer formation in triolein heated at 180 ^{o}C for 72 h

In Figure 3.3.12, Trace 1 represents triolein with no sitosterol or sitostanol addition, heated at 180 ^{o}C for 72 h, giving a relative TG polymer peak area of 21%. Trace 2 represents triolein heated in the presence of 5% sitostanol showing a reduced TG polymer peak area of 17%. Trace 3 indicating a comparatively low TG polymer peak area of 10%, represents a triolein sample heated in the presence of 5% sitosterol. The inability of sitostanol to greatly limit TG polymer formation in triolein samples heated to frying temperatures can be attributed to the fact that unlike sitosterol, sitostanol cannot generate a conjugated diene system. Hence this observation is further evidence that formation of a conjugated diene system in sitosterol plays an important role in limiting the extent of TG polymerization in frying oils.

Recent findings have established that unrefined oils have higher stability at frying temperature compared to the refined oils which tend to lose some of the natural components including sterols at various stages of refining, particularly during neutralization and deodorization. The unsaponifiable fraction in the soap stock generated from the neutralization process has been found to contain on average 70% sterols (17) while the concentration of sterols in the deodorizer distillate has been reported to range from 2 to 20% (18). Overall, sterol losses during the complete refining of vegetable oils, has been reported to range between 10 and 70% (19). Hence, given the effectiveness of sterols, naturally found in vegetable oils, in limiting TG polymer formation at frying temperature, ensuring presence of an optimum level of sterols in refined oils is of critical importance.

3.4 Conclusion

Presence of additional sitosterol was found to limit TG polymer formation in triolein, refined canola, high oleic sunflower and flaxseed oils heated at frying temperature of 180 ^{o}C. The anti–polymerization effect of sitosterol has been attributed to the conversion of sterol to steradiene by the 1, 2–elimination of water. This fact was further corroborated by the significant increase in TG ester hydrolysis during the 48–72 h period, corresponding with the appearance and growth of the TG polymer peak.

Also, triolein and vegetable oil samples with 5% additional sterol were found to have the lowest level of TG polymer formation, in comparison to control and samples with 1% and 2% additional sterol, after heating at 180 ^{o}C for 72 h. Hence, vegetable oils enriched with sterols, at levels higher than those naturally present, may be considered as a means of further enhancing oxidative stability at frying temperatures. This would also ensure higher levels of sterols in fried foods, which may be desirable given their known anti–cholesterolemic effects (20). Interest in foods enriched with sterols has soared in recent times with products, including cooking oils, diglyceride, spreads, salad dressings, mayonnaise, chocolate, yogurt, bread and breakfast cereal being marketed globally (21).

3.5 References

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Chapter 4 Molecular weight estimation of heated triolein fractions using polystyrene standards

4.1 Introduction

While frying foods has been linked to flavor enhancement and improved acceptability, there is growing concern over the detrimental health effects of ingesting non–volatile lipid oxidation products along with fried foods (1). Hence, characterization of non–volatile components in frying oils is essential for the evaluation of quality and oxidative stability of frying oils. Adsorption chromatography techniques have been routinely used to determine the extent of deterioration in frying oils (2, 3). Also, High Performance Size Exclusion Chromatography (HPSEC) in combination with an evaporative light scattering detector (ELSD) or a refractive index (RI) detector has been used to analyze non–volatile lipid oxidation products (4–7). However, not much effort has been directed towards the estimation of molecular weight (MW) of non–volatile components in frying oils. Currently, available methods include use of a calibration curve, obtained using a set of standards with known MW (8, 9). However, these methods do not provide absolute MW information on the non–volatile components in frying oils.

For the determination of MW distribution by HPSEC using concentration detection, the separation system must be calibrated with a set of polymers, chemically and structurally identical with the polymers to be analyzed and covering the molecular weight range of the potential analytes. However, as in the case of frying oil fractions, an identical set of analyte polymers may not be available, hence the separation system has to be calibrated with a different

polymer, usually commercial polystyrene standards. MW thus obtained do not represent true molecular weights but values more appropriately referred to as polystyrene equivalent molecular weights or more generally, standard–equivalent molecular weight. These standards may have the same hydrodynamic volume as a molecule of the analyte and elute from the separation system at the same elution volume. However, the standard –equivalent molecular weight and the true molecular weight of the analyte are not the same (10).

The universal–calibration concept postulates that the separation in SEC is based on the hydrodynamic volume, which is the product of intrinsic viscosity and molecular weight (11). Molecular weight dependence of intrinsic viscosity is given by the Mark–Houwink–Kuhn–Sakurada (MHKS) equation,

$$[\eta] = KM^{\alpha}$$

where K and α are parameters depending on the polymer, solvent and temperature. The universal–calibration concept is applicable for polymers assuming in solution a conformation of flexible coils, characterized by MHKS exponent value of $0.5 < \alpha < 0.8$ (12), which may include linear molecules as well as molecules with various types and degrees of branching (13, 14). However, for polymers with stiff chains characterized by $\alpha > 0.8$; the universal calibration concept fails (15, 16).

The objective of this project was to characterize the various fractions present in a triolein sample heated at 180 ^{o}C for 72 h by estimating their molecular weights, making use of a calibration curve based on retention times of polystyrene standards with known MW, using HPSEC with ELSD detection.

4.2 Materials and methods

Polystyrene standards (Shodex–Waters) were obtained from Waters Limited, Mississauga, Ontario, Canada. A 25 µl injection volume of 10 mg/ml concentration solution for each standard with molecular weights 780,1650, 2340, 3950, 6180, 10200, 13000 and 20000 was used to generate the calibration curve (Figure 4.3.1) using the Agilent GPC software and HPSEC with ELSD detection (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada). An average of four measurements obtained by duplicate 25 µl injections of two separate 10 mg/ml concentration solutions of each standard was used for computing the calibration curve.

HPSEC / ELSD: Two size exclusion columns, a Waters (Waters Limited, Mississauga, Ontario, Canada) styragel HR 100 A^o (4.6 x 300 mm) and Phenomenex (Phenomenex, Torrance, California, USA) phenogel 500 A^o (4.6 x 300 mm) were used in series. Tetrahydrofuran (THF) (HPLC–grade; Fischer Scientific, Toronto, Ontario, Canada) was used as the mobile phase at a flow rate of 0.3 ml/min. The ELSD was set at a temperature of 70 ^oC and N_2 flow rate of 3l/min under a pressure of 3.5 bars.

Sample preparation: 2 grams of Triolein (Nuchek Inc., Elysian, Minnesota, USA) in a 7 ml glass vial was heated at 180 ^oC continuously for a period of 72 h in a Stabil–Therm gravity oven (Blue M, New Columbia, Pennsylvania, USA). Small aliquots drawn from the vial were dissolved in THF to obtain a final concentration of 10 mg / ml of which repeated 25 µl injections were made to obtain sufficient volumes of the 7 fractions that covered a retention time range from 12.8 min to

18.3 min using a fraction collector (Agilent 1200 series, G1364C). Each of the 7 fractions was then concentrated to a 10 mg/ml solution in THF. Four separate 25 µl injections of each fraction were then used to obtain the reported retention time values which represent an average of the four measurements. The average molecular weight of each fraction of heated triolein was then estimated based on the polystyrene calibration curve and the Agilent GPC software.

A typical chromatogram of triolein heated at 180 ^{o}C for 72 h included peaks corresponding to the triglyceride (TG) polymer at a retention time of ~ 12.8 min, TG monomer peak at a retention time of ~17.7 min and a peak corresponding to TG ester hydrolysis products at a retention time of ~ 18.4 min. TG oligomers (TG dimers, TG trimers etc.) appeared at retention times between the TG monomer and TG polymer peak, ~14–17 min.

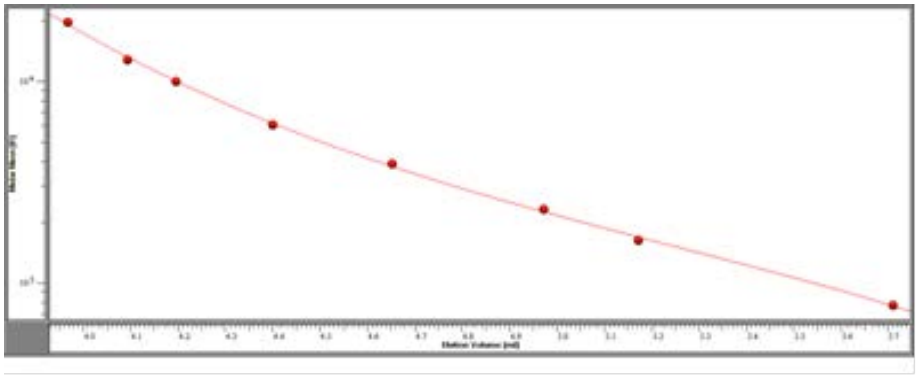

Figure 4.2.1 Polystyrene standard calibration curve used for molecular weight estimation of heated triolein fractions using Agilent GPC software (X–axis: retention time; Y–axis: molecular weight)

Statistical Analysis: Standard deviations for the measurements were computed and are reported as relative standard deviations (% RSD)

4.3 Results and Discussion

Frying oil components have been traditionally characterized by calibration with standard plots of logarithmic MW vs. HPSEC retention times using polystyrene (8, 17) or polypropylene glycol (18) standards. However, the universal calibration method has been reported to provide better molecular weight estimation by enabling determination of hydrodynamic volume of the analytes by the use of a dual HPSEC–viscometric (VIS) / refractometric (RI) detection and utilizing plots of log [$\eta \times$ MW] vs. elution volume (9).

The customary designation of non–volatile components in frying oils as monomer, dimer, trimer, tetramer, and high polymer TG derivatives may be an oversimplification, given the complexity of food frying processes resulting in degradation products from a diversity of TG in oils. No evidence of the formation of well–defined non–volatile components in the form of distinct increments of TG monomer such as TG dimer, TG trimer and higher TG polymers, was found. Also, no systematic trend of variations in individual component MW of TG derivatives with frying times was observed (9).

MW distribution of the various fractions of refined, bleached and deodorized (RBD) soybean oil (300g), heated in 500–ml pyrex beaker to a frying temperature of 182 ^{o}C intermittently for a total of 56 h spread over a period of 8 days, was obtained based on a comparison of the retention volumes of the frying oil fractions and polystyrene standards, using HPSEC with variable wavelength UV/VIS detector. The results were found to be as follows: TG monomers and fatty acid trimers (1000 g/mole); TG dimers (2000 g/mole); TG tetramers (4000

g/mole) and fraction corresponding to larger than tetramers (4000 – 6000 g/mole) (8).

Similar estimates for HOSUN (High Oleic Sunflower Oil) used for intermittent frying of french–fried potatoes for a total of 30 h spread over a period of 4 days at 190 °C, using HPSEC with dual viscometric / refractometric detection, resulted in the following values for the four separated fractions; high mol. wt. fraction 1 (6700 g/mole); intermediate fraction 2 (5271 g/mole); intermediate fraction 3 (3369 g/mole); low mol. wt. fraction (1355 g/mole) (9). Oil sample weighing 5000 g was heated in a 14 litre fryer and the stock was replenished with 300g of fresh oil each day (9). The overall range of molecular weight distribution ~ 1000 – 6000, for different frying oil fractions obtained by the two methods (8, 9) appears comparable.

In this work, 2 grams of triolein in a 7 ml vial was heated continuously in an oven set at 180 ^{o}C for a period of 72 h and the samples were analyzed using HPSEC with ELSD detection. A total of 7 fractions were obtained with a molecular weight distribution that ranged from 1144 –25159 (g/mole). The MW distribution for the first 5 fractions from the lower end of 1144–7624 (g/mole), which seemed comparable to the MW values for frying oil fractions reported earlier (8, 9). Also, taking into consideration the sample size, surface to volume ratio and the duration of continuous heating, which were significantly different from conditions used in the reported studies, two additional high molecular weight fractions were obtained with estimated molecular weights of 15321 and 25159 (g/mole).

A striking feature of the eight polystyrene standards used for generating the calibration curve was the response factor that was found comparable for the entire range of standards with molecular weights 800–20000 (Table 4.3.1). However, the response factor for the various fractions obtained from heated triolein appeared to vary significantly within a range of 2037–4570 (Table 4.3.2). This again is indicative of the fact that designation of various fractions as increments of TG monomer may indeed be an oversimplification. Even within the same increment, the nature of the bond connecting the TG monomers may differ. A C–C, C–O–C or a C–O–O–C linkage may impact the flexibility of the resulting polymer and result in varying α value in the MHKS equation.

Table 4.3.1 lists the retention times and the response factors for polystyrene standards with different molecular weights. The response factor for polystyrene standards ranged from 1.9 e^4 – 2.2 e^4. The retention times ranged from 13.2 min for the standard with the highest molecular weight of 20000 to 19.0 min for the standard with the lowest molecular weight of 780 (Figure 4.3.1).

Table: 4.3.1 Molecular weight and response factor values for polystyrene standards with different molecular weights

Standard	Retention time	Molecular weight	Response area	concentration mg/ml (25 µl)
1	13.2	20000	$1.90454e^4$	10
2	13.6	13000	$1.98716e^4$	10
3	14.0	10200	$2.10620e^4$	10
4	14.7	6180	$2.12701e^4$	10
5	15.5	3950	$2.20982e^4$	10
6	16.6	2340	$2.19206e^4$	10
7	17.2	1650	$2.18734e^4$	10
8	19.0	780	$2.14082e^4$	10

RSD% = (SD/mean) × 100, *n* = 4: 2 – 5

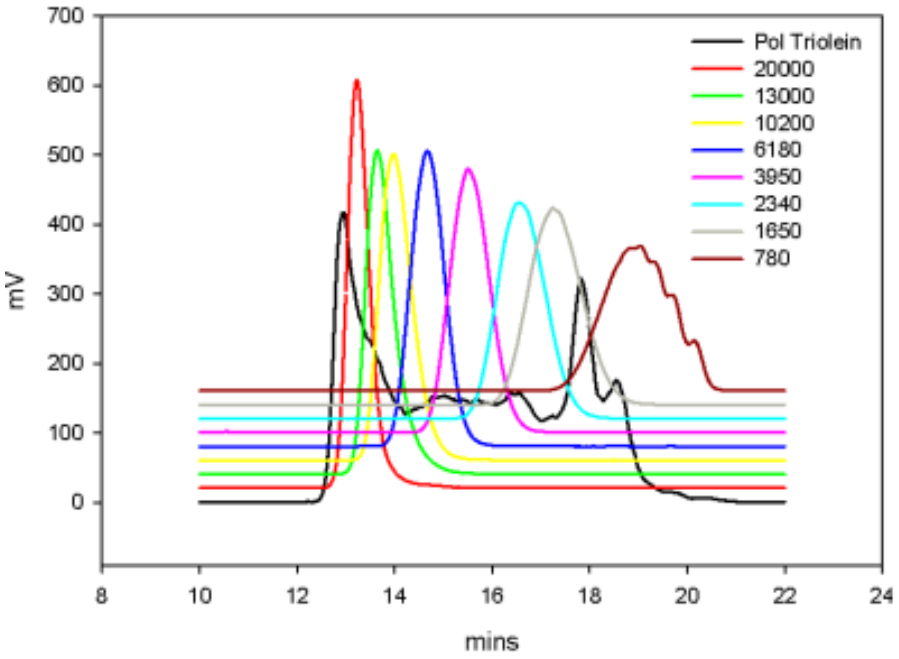

Figure 4.3.1 HPSEC chromatograms for polystyrene standards with molecular weights ranging from780–20000

Table: 4.3.2 Molecular weight and response factor values for different fractions of triolein heated at 180 ^{o}C for 72 h

Fraction	Retention time	Molecular weight	Response area	concentration mg/ml (25 µl)
1	12.8	25159	3724	10
2	13.6	15321	2522	10
3	14.9	7624	2794	10
4	15.8	4445	3145	10
5	16.5	2527	2870	10
6	17.6	1532	4570	10
7	18.3	1144	2037	10

RSD% = (SD/mean) × 100, *n* = 4: 2 – 7

Table 4.4.2 lists the estimated molecular weights for different fractions of heated triolein. The retention times for the various fractions ranged from 12.8 min for the highest molecular weight fraction to 18.3 min for the lowest molecular weight fraction. The peak response area for the various fractions was found to range between 2037 and 4570. Estimated molecular weights for the fractions of heated triolein ranged between 25159 for the fraction with retention time 12.8 min and 1144 for fraction with retention time 18.3 min.

Figure 4.3.2 represents HPSEC chromatograms for fractions of triolein heated at180 ^{o}C for 72 h with a chromatogram of triolein heated at 180 ^{o}C for duration of 72 h, superimposed. Fraction 1, with a retention time of 12.8 min, corresponds to TG polymer with an estimated average molecular weight of 25159. Fraction 7 with a retention time of 18.3 min may include hydrolysis products, TG monomers and TG dimers with an estimated average molecular weight of 1144. Fraction 6

with a retention time of 17.6 with an estimated molecular weight of 1532 may include TG monomers and TG dimers. Fraction 5 with a retention time 16.5 and an estimated average molecular weight of 2527 may include TG dimers, TG trimers and TG tetramers. Fraction 4, 3 and 2 with retention times 15.8, 14.9 and 13.6 represent higher TG oligomers with estimated molecular weights of 4445, 7624 and 15321 respectively.

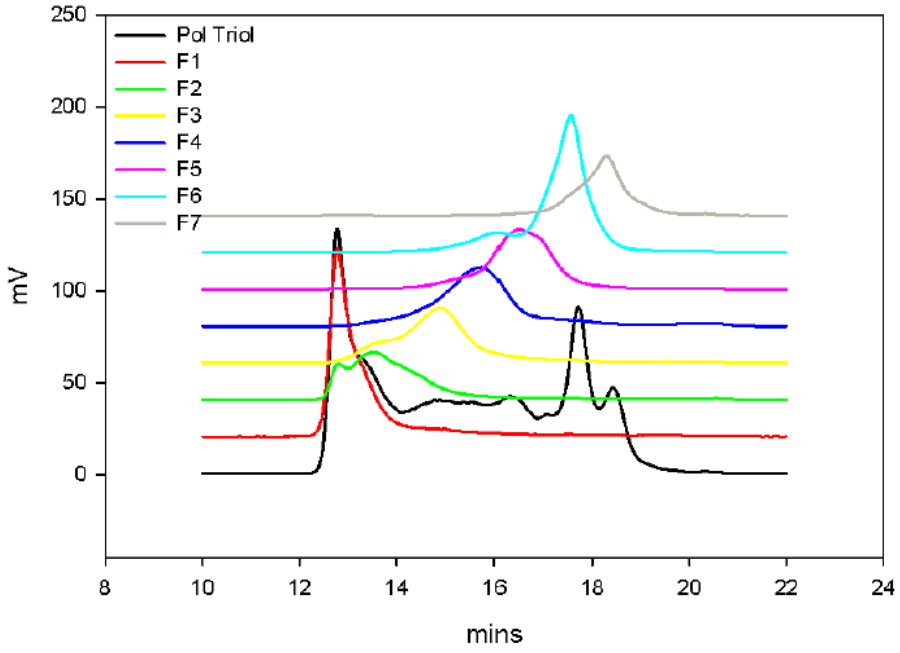

Figure 4.3.2 HPSEC chromatograms for fractions of triolein heated at 180 ^{o}C for 72 h

Further confirmation using methods like, High Performance Size exclusion chromatography – Multiple Angle Laser Light Scattering (HPSEC–MALS), Static Light Scattering (SLS), Matrix–Assisted Laser Desorption / Ionisation – Time of Flight Mass spectrometry (MALDI–TOFMS) and Proton Nuclear Magnetic Resonance (^{1}H NMR) may be needed before any structural predictions could be made on high molecular weight frying oil fractions. Average MW measured by

HPSEC–MALS and SLS have been reported to agree well with certified values of all polystyrene standards except for lower MW. Average MW estimates using MALDI–TOFMS have also been reported to be in good agreement with smaller polydispersity predictions. ^{1}H NMR has also been reported to be a powerful technique for determining number–average molecular weight accurately (19).

4.4 Conclusion

MW distribution values for frying oil fractions obtained using polystyrene standard calibration and HPSEC with ELSD detection appeared comparable to those obtained using dual viscometer/RI detection. However, a calibration curve resulting from use of actual nonvolatile lipid oxidation product standards, obtained using preparative HPSEC, may provide a more reliable MW estimate of frying oil fractions. Also, designation of various fractions of nonvolatile component in frying oils as increments of TG monomer may be an oversimplification given the complexity of degradation products that result during frying.

4.5 References

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Chapter 5 Use of C30 stationary phase for the analysis of frying oils by HPLC

5.1 Introduction

The separation and characterization of compounds that result from heating vegetable oils at frying temperatures, is quite an analytical challenge. Options available currently for the analysis of frying oils include reverse phase C18 and size exclusion chromatography. In liquid chromatography, stationary phase properties such as monomeric vs. polymeric surface modification (1), bonding density (2), and alkyl chain length (3) are known to influence selectivity, retention and hence separation of compounds with subtle differences in molecular structure. Improved separation of carotenoid isomers has been reported for C30 columns with densely bonded phases, as the greater thickness of C30 stationary phases in comparison with C18 stationary phases is believed to provide more extensive interactions with large molecules like carotene. Also, longer alkyl chain length of the stationary phase has been shown to improve separation of isomeric compounds (4). Polymeric C18 columns prepared with trifunctional silanes have been shown to provide better separations of complex polycyclic aromatic hydrocarbon isomer mixtures compared to monomeric C18 columns that are prepared with monofunctional silanes (5).

Separation efficiency of monomeric, polymeric C18 columns, and polymeric C30 columns was compared using polar and nonpolar carotenoid standards (6). Nonpolar hydrocarbon carotenoids were better resolved on polymeric C18 column when compared to monomeric C18 column, but the *cis*/*trans* isomers of β–carotene remained unresolved. Using the same mobile phase gradient, near

baseline resolution of the components was achieved with the polymeric C30 column (6). Higher resolution separation of mixtures of geometric isomers of carotenoids obtained by iodine–catalyzed photo isomerization, have also been demonstrated using a polymeric C30 capillary column operated in the electro–chromatography model (7). On–line peak identification of β–carotene isomers using coupled C30 LC–NMR has enabled identification of two di–cis isomers (13, 15–di–cis– and 9, 13–di–cis–β–carotene) (8). In each case, better separations of the isomer mixtures were achieved with polymeric C30 columns compared with C18 columns.

Given the complexity of the lipid oxidation products formed during the frying process, liquid chromatography is believed to be the most appropriate technique for the qualitative and quantitative analyses of new compounds formed during deep fat frying. These compounds, which vary substantially in size and polarity, include volatile compounds with molecular weights lower than those of the triglyceride (TG) molecule, monoglycerides (MG), unoxidized and oxidized diglycerides (DG), unoxidized and oxidized TG monomers with additional oxygen in the form of hydroxy, keto or epoxy groups on the fatty acyl chains (9), unoxidized and oxidized TG dimers (10) and TG oligomers (11).

Autoxidation reaction with a free radical mechanism is believed to be mainly responsible for the formation of a variety of new compounds differing in polarity and molecular weight during the frying process (12). This involves formation of alkyl radicals ($R^{\cdot}$) in the initiation step, alkyl peroxy radicals ($ROO^{\cdot}$) by the addition of oxygen and alkoxy radicals ($RO^{\cdot}$) during hydroperoxide

decomposition (13). Oxidized TG monomers result from the decomposition of hydroperoxides (14). Non polar TG dimers are formed through C–C linkages, mainly from reactions between alkyl radicals (15). Polar dimers are generated by the oxidation of nonpolar TG dimers or by the dimerization of two oxidized TG monomers (16). TG oligomers include TG dimers and TG trimers joined through C–C and C–O–C linkages (17).

In recent years, concerns have grown over the potential biological effects of oxidized lipids as there is increasing evidence of their detrimental effects on health, particularly in connection with diseases related to oxidative stress, including atherosclerosis, cancer, heart attacks and liver damage (18). While a number of volatile decomposition products formed in frying oils have been identified, detailed information on the nonvolatile compounds is unavailable due to the complexities of this fraction and the limitations of the analytical methodologies used for isolation and quantitation of such compounds.

High Performance Size Exclusion Chromatography (HPSEC) using Refractive Index (RI) or Evaporative Light Scattering Detector (ELSD) detection has been used for the separation and identification of high molecular weight, nonvolatile lipid oxidation products (19, 20). While, HPSEC provides a quantitative estimate of the various groups of lipid oxidation products formed, no specific structural information is available. Reverse phase C18 chromatography has been used for the separation and study of TG hydroperoxides (21) and more recently combined with mass spectrometry in the study of high molecular weight (MW) TG oxidation products (22). Atmospheric pressure chemical ionization (APCI–MS)

and electrospray ionization (ESI–MS) mass spectrometry have been shown to be very effective for the analysis of normal triacylglycerides as well as mixtures of high molecular weight TG oxidation products by allowing direct identification and characterization (23, 24). The objective of this project was to improve the separation and identification of different lipid oxidation products that form in frying oils by developing a new High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS) method combining the enhanced selectivity of reverse phase C30 columns with electrospray ionization–mass spectrometry (ESI–MS) and utilizing retention times of similar lipid oxidation products obtained using HPSEC.

5.2 Materials and methods

Triolein (99% pure) obtained from Nucheck Inc. Elysian, Minnesota, USA, was used for sample preparation. 2 g of triolein was placed in a 7 ml open mouth glass vial and heated without stirring in a Stabil–Therm gravity oven (Blue M, New Columbia, Pennsylvania, USA) set at 180 ^{o}C for 24 h. After gentle mixing by vortex, small aliquots drawn from the vial was dissolved in THF to a concentration of 10 mg/ml and analyzed using C30 RPHPLC. The HPLC system included an Agilent 1200 series binary pump (G1312 A) and a degasser (G1379 B), Agilent 1200 series (G13229A) auto sampler and Agilent 1200 series evaporative light scattering detector (ELSD). The ELSD was set at 70 ^{o}C with a N_2 flow rate of 3.0 l/min under pressure of 3.5 bars. For RPHPLC, a sample volume of 20 µl was injected on to the C30 carotenoid column (2.0 x 250 mm) obtained from YMC America Inc., Allentown, Pennsylvania, USA. The solvent

system included methanol and tert– butyl methyl ether (TBME) (HPLC–grade; Fischer Scientific, Toronto, Ontario, Canada) and the following gradient program was used at a flow rate of 0.275 ml/min, methanol/TBME (80:20 v/v) linear to (65:35 v/v) at 15 min; linear to (55:45 v/v) at 20 min; linear to (60:40 v/v) at 30 min; linear to starting conditions (80:20 v/v) at 40 min. Agilent 1200 series fraction collector was used to collect the four fractions separated using the C30 column. Each of the four fractions was injected separately and analyzed by HPSEC using two size exclusion columns in series, a Waters (Waters Limited, Mississauga, Ontario, Canada) styragel HR 100 A^o (4.6 x 300 mm) and a Phenomenex (Phenomenex, Torrance, California, USA) phenogel 500 A^o (4.6 x 300 mm). Tetrahydrofuran (THF) (HPLC–grade; Fischer Scientific, Toronto, Ontario, Canada), was used as the mobile phase at a flow rate of 0.3 ml/min. HPLC–ESI method: The Agilent 1200 series HPLC system was coupled to QSTAR Elite mass spectrometer with turbo spray ion source (AB SCIEX, Concord, ON, Canada). Analyst QS 2.0 software was used for data acquisition and analyses. The mass spectrometer was tuned by infusing porcine renin substrate tetradeca peptide (*m*/*z* 879.9723, doubly charged ion) at a resolution of 13000 full width at half–maximum (FWHM) under positive mode and calibrated using an electrospray ionization (ESI) low concentration tuning mix (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Mass spectrometric analysis was performed using positive ion ESI in information dependent acquisition (IDA) mode. The mass range recorded for both full–scan mass spectra was from *m*/*z* 50 to 4000. Nitrogen was used as curtain gas, nebulizing gas and

drying gas. All other instrumental parameters used were as follows, curtain gas at 25 arbitrary units; gas 1 at 20; gas 2 at 60; ion spray voltage at 5500 V for positive mode and ion source temperature at 400 °C. The declustering potential (DP), focus potential (FP), and DP2 were 75 V, 280 V, and 15 V respectively in positive mode.

5.3 Results and Discussion

Reverse Phase High Performance Liquid Chromatography (RPHPLC) chromatograms using C30 stationary phase for triolein samples before and after heating at 180 ^{o}C for 24 h are shown in Figure 5.3.1. As described in the experimental procedure, a 40 min long gradient separation was employed with methanol and tert– butyl methyl ether as the solvent system that resulted in the separation of heated triolein into four main fractions. The fractions were collected separately using a fraction collector and the identity of each fraction was established using size exclusion chromatography and confirmed using ESI mass spectrometry. The HPSEC chromatogram for each of the separated fractions is shown in Figure 5.3.2a. HPSEC chromatogram for the heated triolein sample is shown in Figure 5.3.2b.

Based on the known retention times for similar lipid oxidation products using HPSEC, fraction 1 in the C30 column chromatogram was found to contain free fatty acid (FFA), MG, oxidized DG and oxidized TG monomers. Fraction 2 included DG and TG monomer oxidized to a lesser extent compared to the one in fraction 1. The retention time for TG monomer before heating, shown in Figure 5.3.1a confirmed the presence of an unoxidized TG monomer in fraction 3 along

with TG dimers. TG oligomers including TG trimers and TG tetramers were present in fraction 4.

Comparing the retention times of fraction 4 separated using C30 column (Figure 5.3.2a) with the HPSEC chromatogram for heated triolein (Figure 5.3.2b), bulk of fraction 4 appears to represent TG oligomers. Separation of TG oligomers as a separate block is not possible with methods currently in use. Hence, use of C30RPHPLC may provide a measure of TG oligomer content in frying oils that could serve as a quality indicator in frying oils.

Figure 5.3.3a represents the TG monomer fraction obtained using HPSEC. Figure 5.3.3b represents the RPC30 chromatographic separation of the same TG monomer fraction obtained using HPSEC. The single TG monomer peak from HPSEC was separated into a series of peaks representing TG monomers oxidized to different extents.

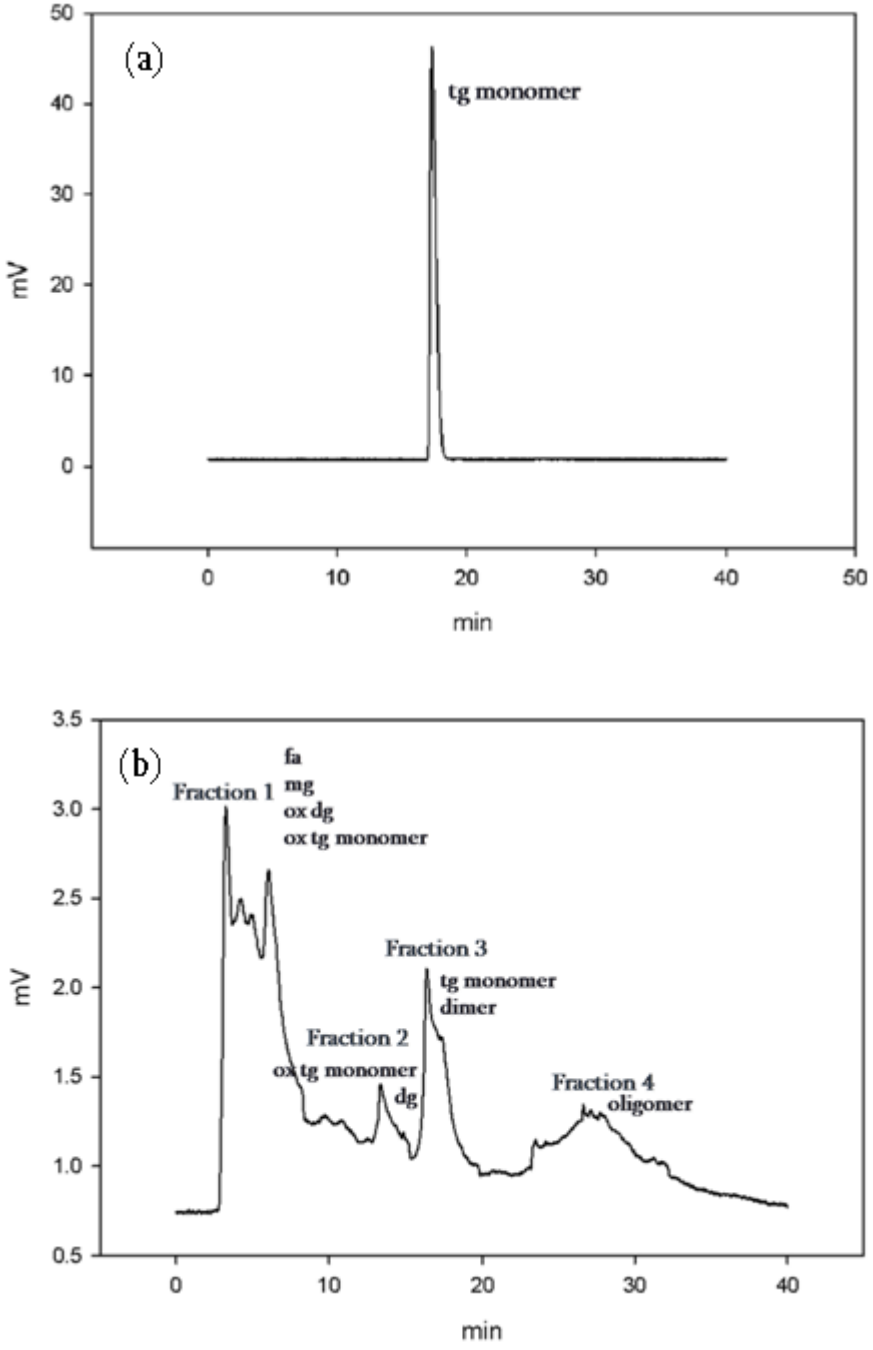

Figure 5.3.1 RPC30 chromatogram of triolein (a) before and (b) after heating at 180 ^{o}C for 24 h

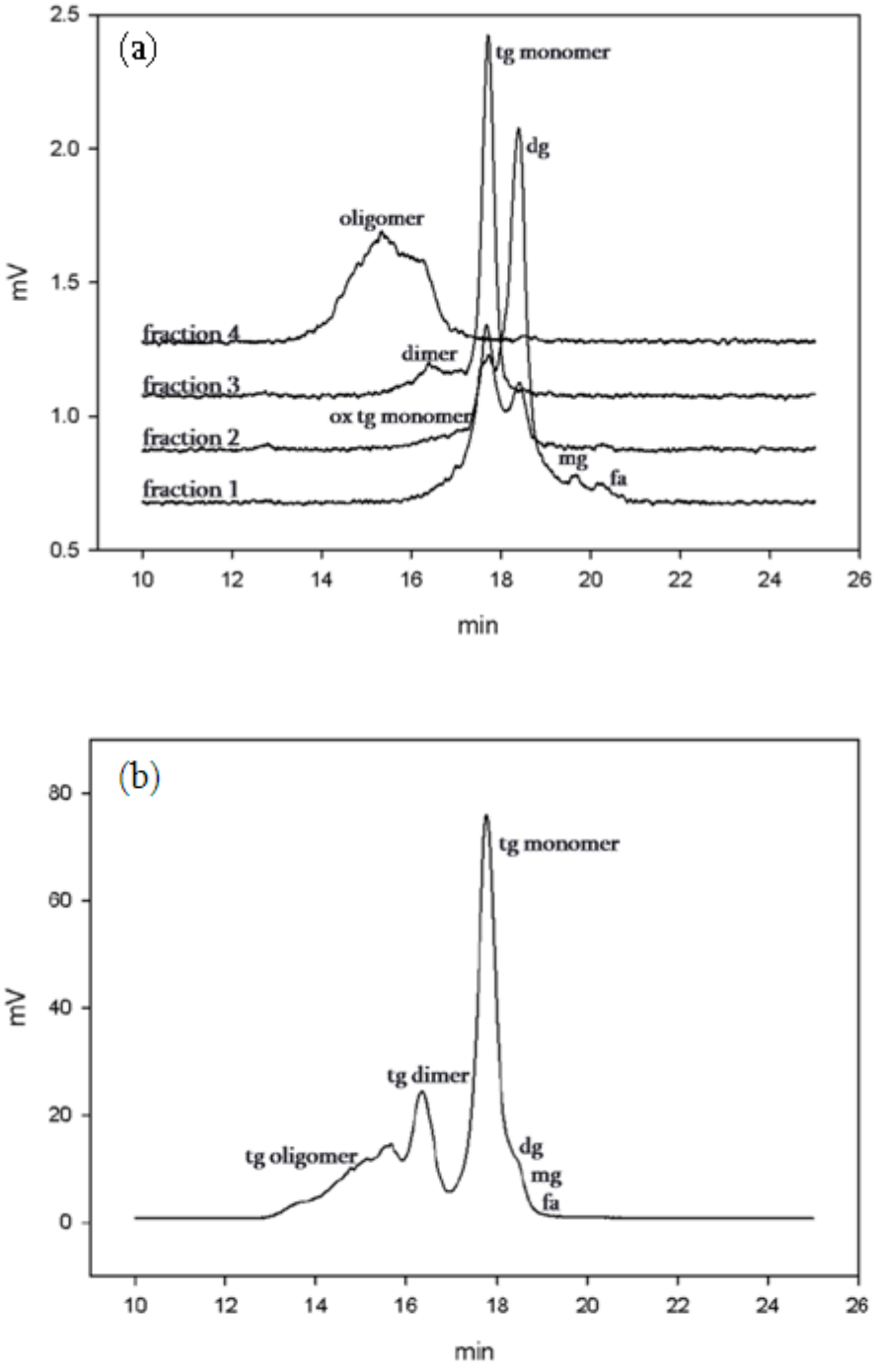

Figure 5.3.2 HPSEC chromatogram of (a) RPC30 column separated fractions of triolein heated at 180 ^{o}C for 24 h (b) triolein heated at 180 ^{o}C for 24 h

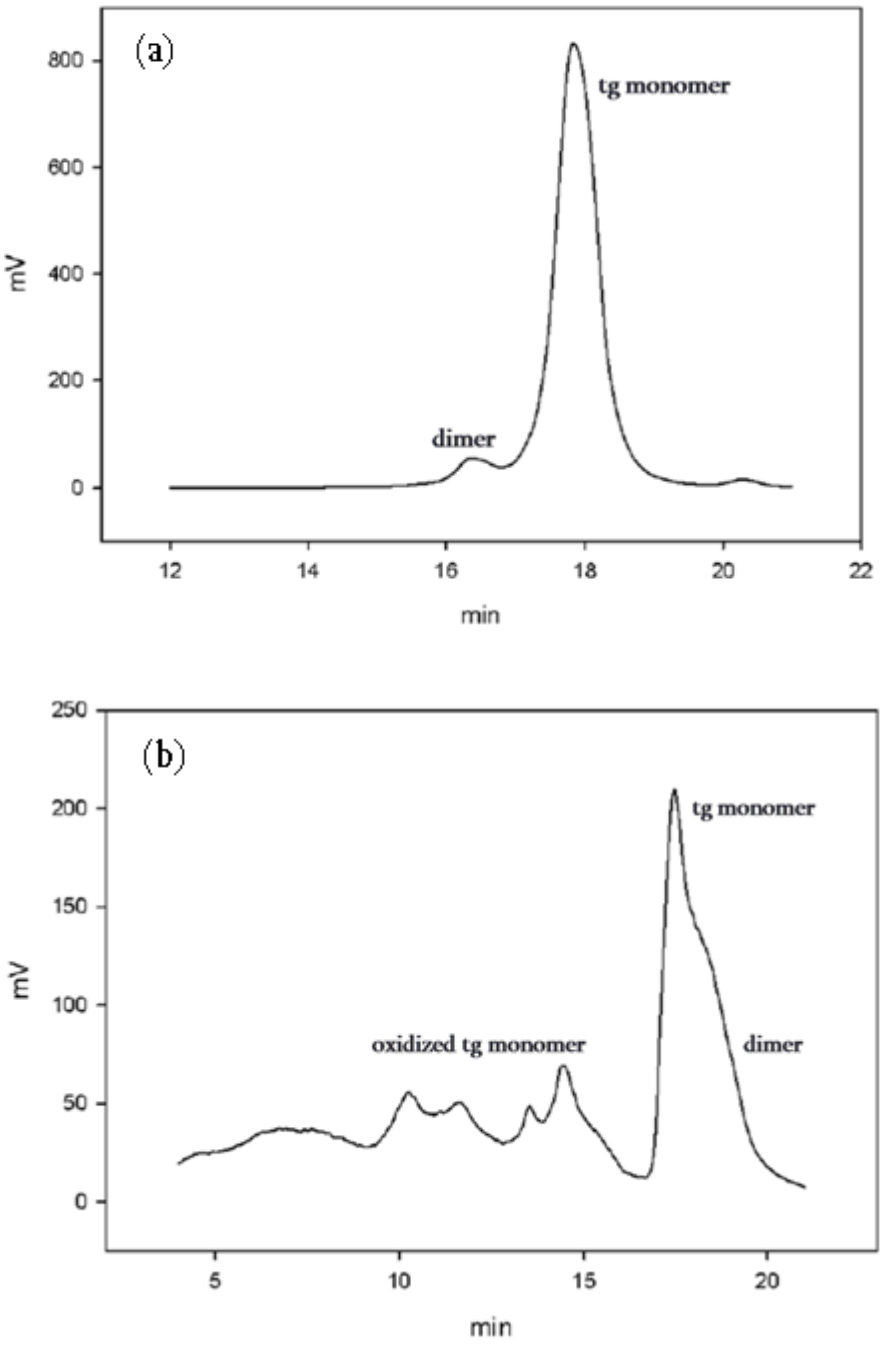

Figure 5.3.3 TG monomer fraction (a) obtained using HPSEC (b) same fraction subjected to RPC30

ESI–Mass Spectrometry: Mass confirmation for each of the fractions identified in Figure 5.3.1b was obtained by coupling the RPC30 column to QSTAR Elite mass spectrometer through an electrospray ion source. The extracted ion chromatogram (XIC) for ammoniated triolein TG monomer, at *m/z* 902.8 is shown in Figure 5.3.4a ($[OOO + NH_4]^+ = C_{57}H_{104}O_6 + NH_4 = [(57 \times 12.000000 + 104 \times 1.007825 + 6 \times 15.9994) + (14.003074 + (4 \times 1.007825)] = 884.78 + 18.03 = 902.8$) The TG monomer was found to elute between18.0 and 22.0 min with the chromatographic peak located at 20.1 min. (Figure 5.3.4a) Expanded view of the mass spectrum of this chromatographic peak is shown in Figure 5.3.4b

Oxidized TG monomers include additional oxygen atoms in the fatty acyl chain of the TG molecule, which may be in the form of functionalities such as hydroperoxy, hydroxy, keto or epoxy. The extracted ion chromatogram (XIC) for ammoniated oxidized TG monomer, at *m/z* 918.8 with one additional oxygen atom is shown in Figure 5.3.5a and the corresponding mass spectrum for the chromatographic peak at retention time of 17.45 min is shown in Figure 5.3.5b. The retention time for oxidized TG monomers was found to range between 8.0 and 18.0 min with the TG monomers oxidized to a greater extent eluting first. The XIC for the m/z range of 902.8 to 980.8 which included unoxidized and oxidized TG monomers is shown in Figure 5.3.6a. The expanded view of the mass spectrum over a retention time range of 9.3 to 9.6 min is shown in Figure 5.3.6b indicating the presence of oxidized TG monomers with one, two, three, four or five additional oxygen, with and without additional unsaturation with m/z 918.8, 934.8, 948.8, 962.8 and 978.8.

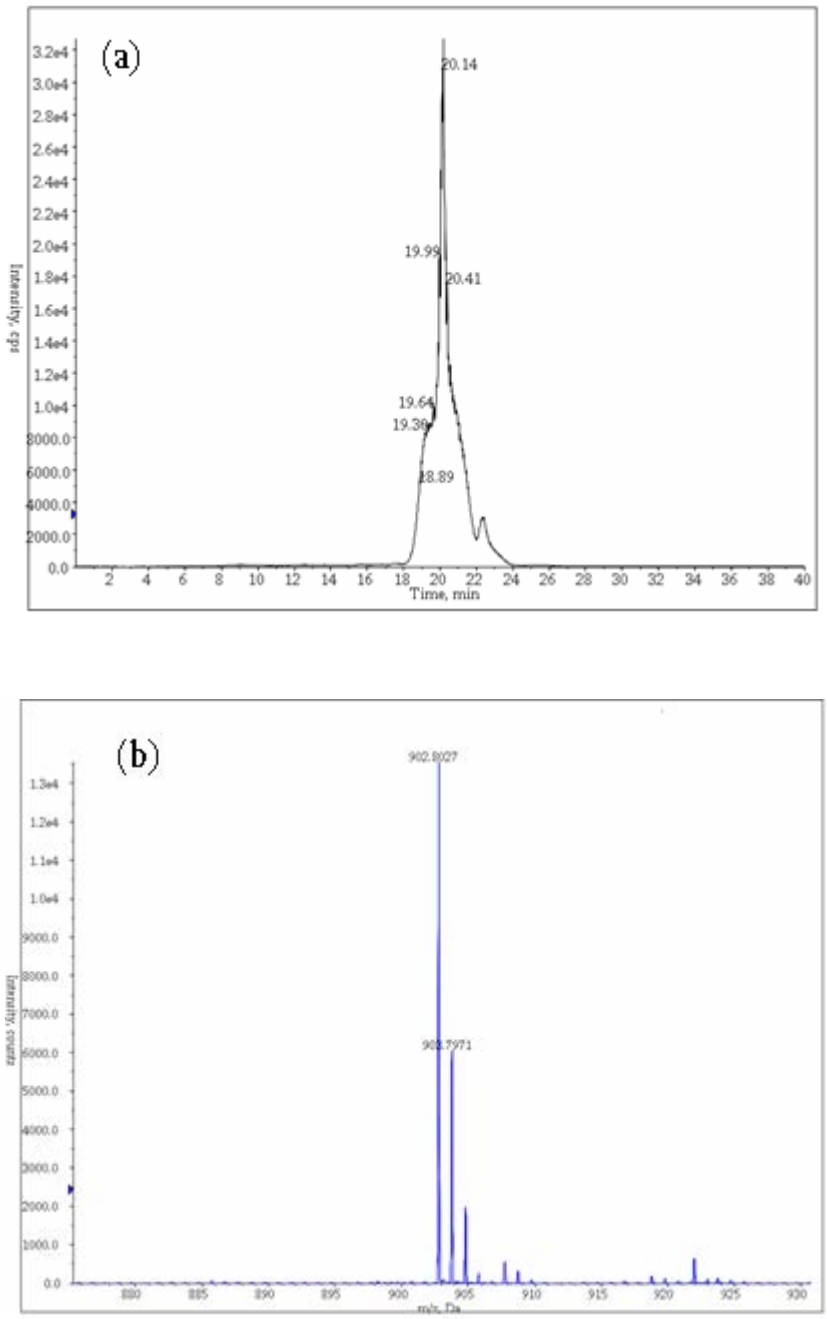

Figure 5.3.4 Extracted ion chromatogram and mass spectra for TG monomer present in heated triolein (a) XIC of +TOF m/z 902.8 (b) +TOF MS: 20.14 to 20.26 min; m/z 902.8

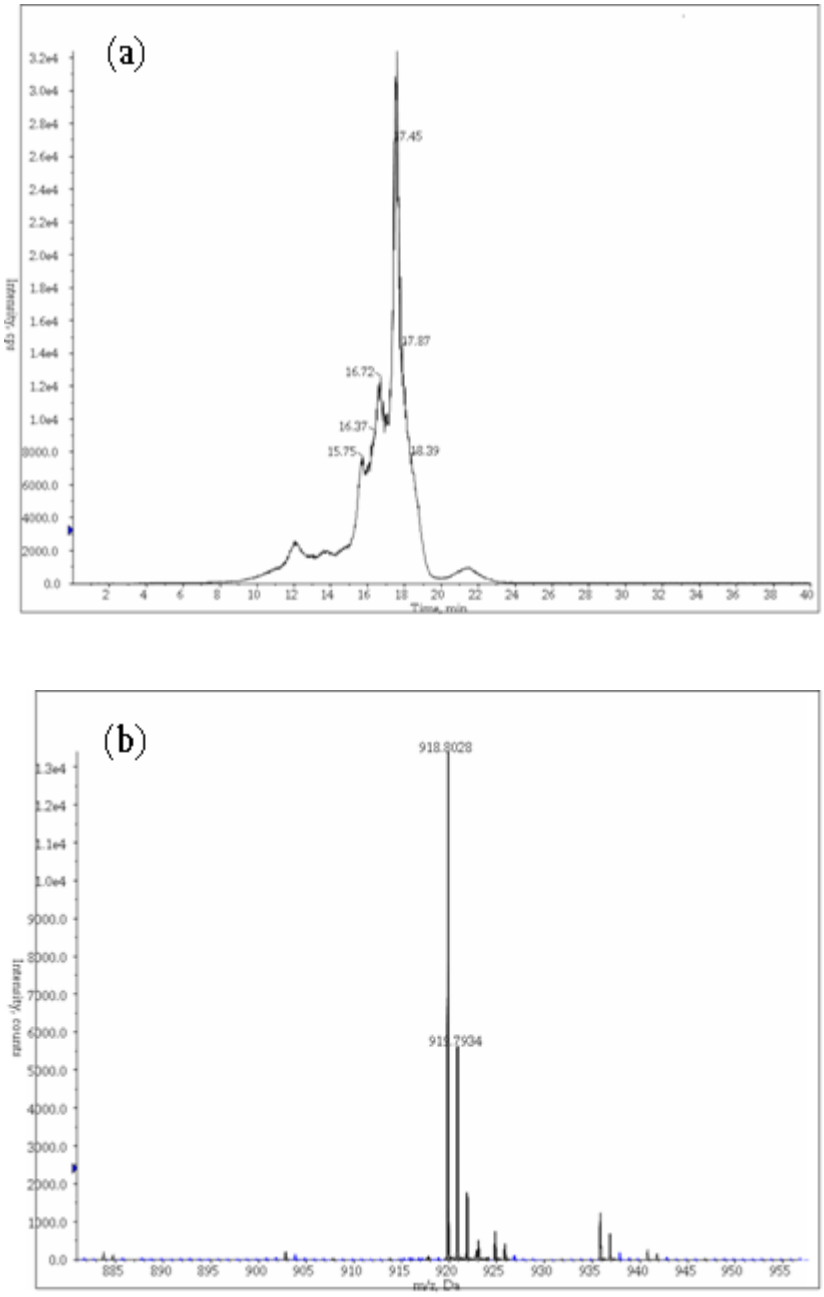

Figure 5.3.5 Extracted ion chromatogram and mass spectra for oxidized TG monomer with one additional oxygen present in heated triolein (a) XIC of +TOF m/z 918.8 (b) +TOF MS: 17.41 to 17.49 min; m/z 918.8

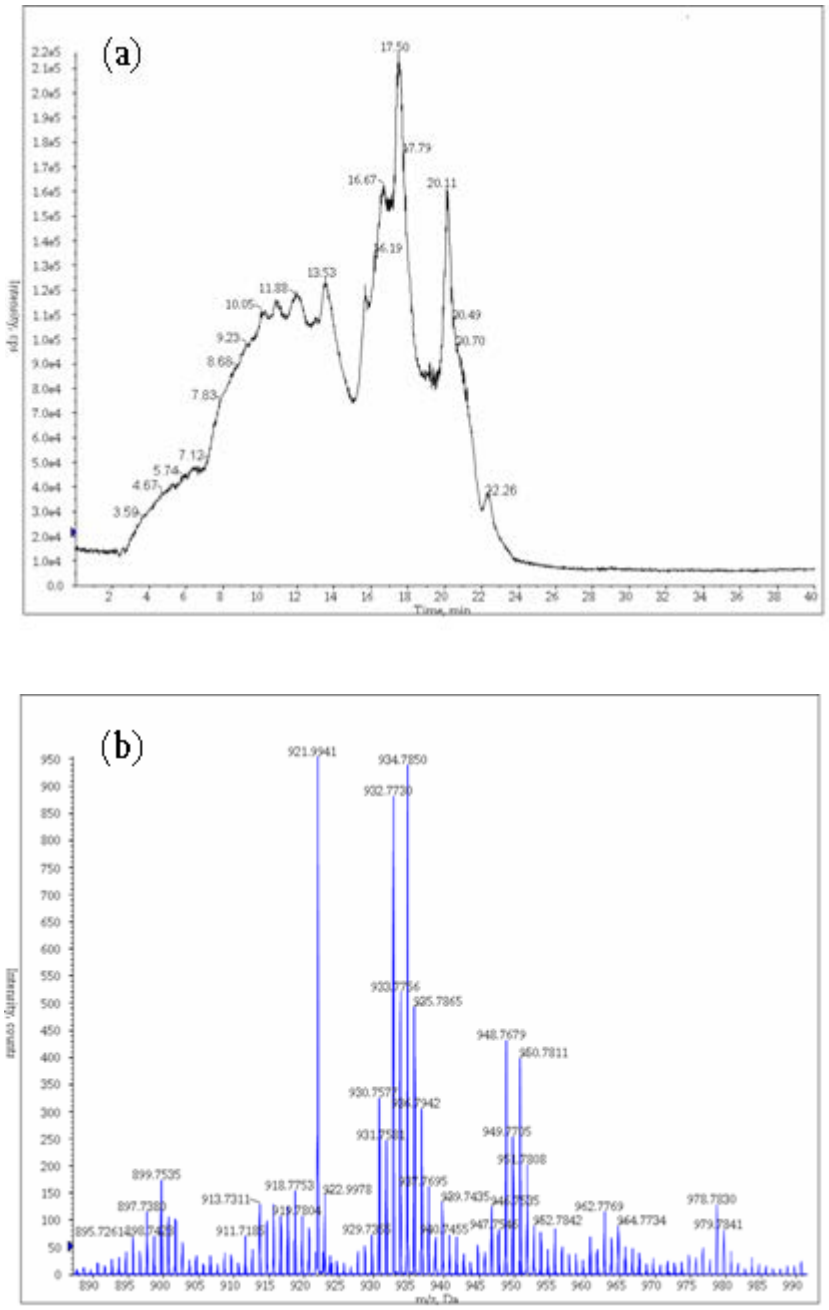

Figure 5.3.6 Extracted ion chromatogram and mass spectra for oxidized TG monomer with one, two, three, four or five additional oxygen present in heated triolein (a) XIC of +TOF MS m/z 902.8– m/z 980.8 (b) +TOF MS: 9.35 to 9.58 min; m/z 902.8, 918.8, 934.8, 950.8, 962.8, 978.8

Figure 5.3.7a and Figure 5.3.8a represent the XIC for MG and DG respectively. Expanded view of the corresponding mass spectra is shown in Figure 5.3.7b and 5.3.8b. Detection of m/z 638.5, m/z 654.5 and m/z 670.5 in the mass spectra for DG over a retention time range of 5.47 to 5.74 min may confirm the presence of oxidized DG with one, two, or three additional oxygen atoms. Presence of m/z 391.3 in the mass spectra for MG over the retention time range 2.54 to 2.72 min may indicate existence of an ammoniated MG with an additional oxygen atom. The XIC for TG dimers covering a range of m/z 1785.0 –1788.0 is shown in Figure 5.3.9a. The expanded view of the mass spectra for the chromatographic peak located at 20.16 min is shown in Figure 5.3.9b indicating the presence of TG dimer with no net loss of H atom with *m/z* 1787.6 The XIC for oxidized TG dimers is shown in Figure 5.3.10a and covers a range of m/z 1799.0–1834.0 Figure 5.3.10b represents the corresponding mass spectra covering a retention time range of 13.43 to 13.60 min. The oxidized TG dimers with two and three additional oxygen with m/z 1817.5 and m/z 1833.5 were detected.

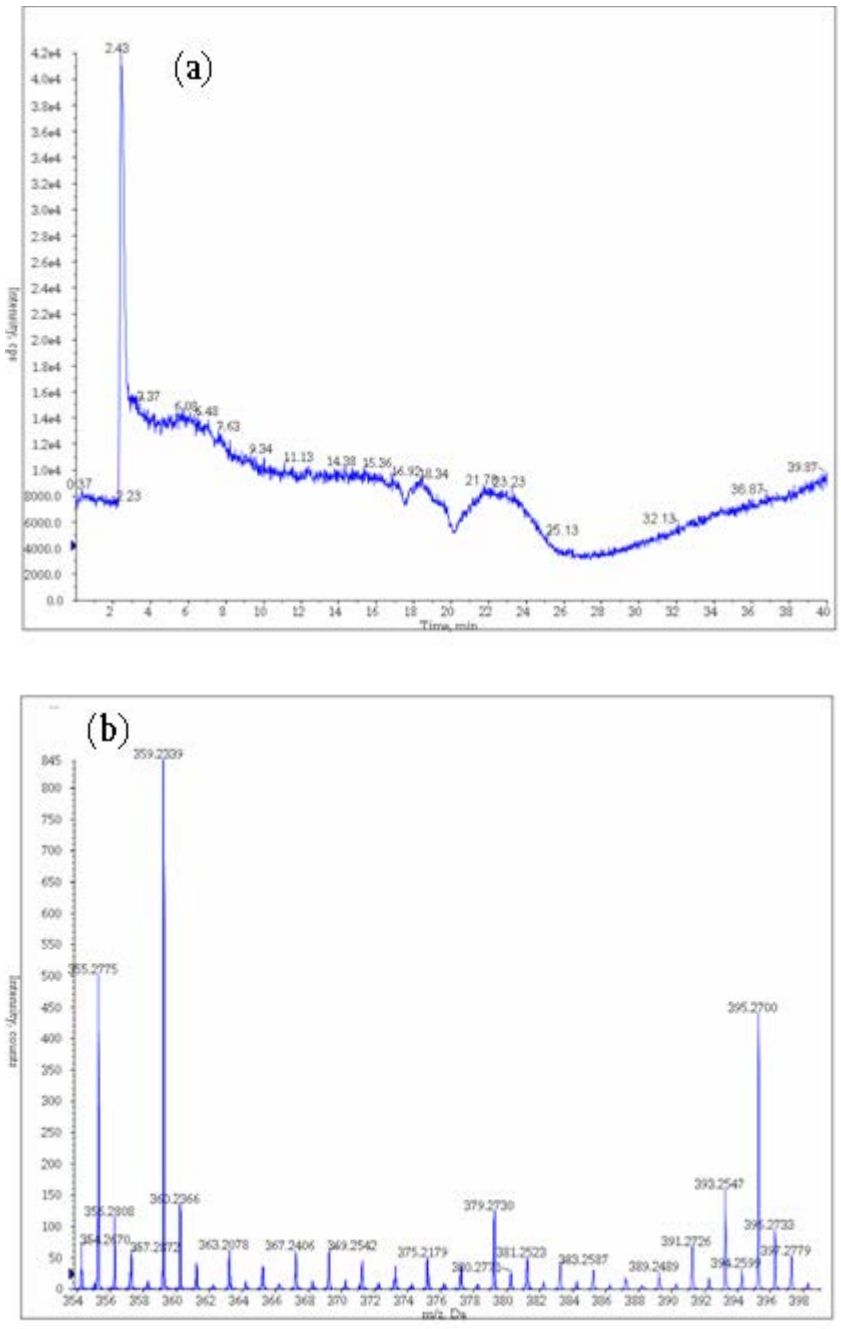

Figure 5.3.7 Extracted ion chromatogram and mass spectra for oxidized and unoxidized MG present in heated triolein (a) XIC of +TOF MS: 356 to 399 Da (b) +TOF MS: 2.537 to 2.720 min m/z 391.3

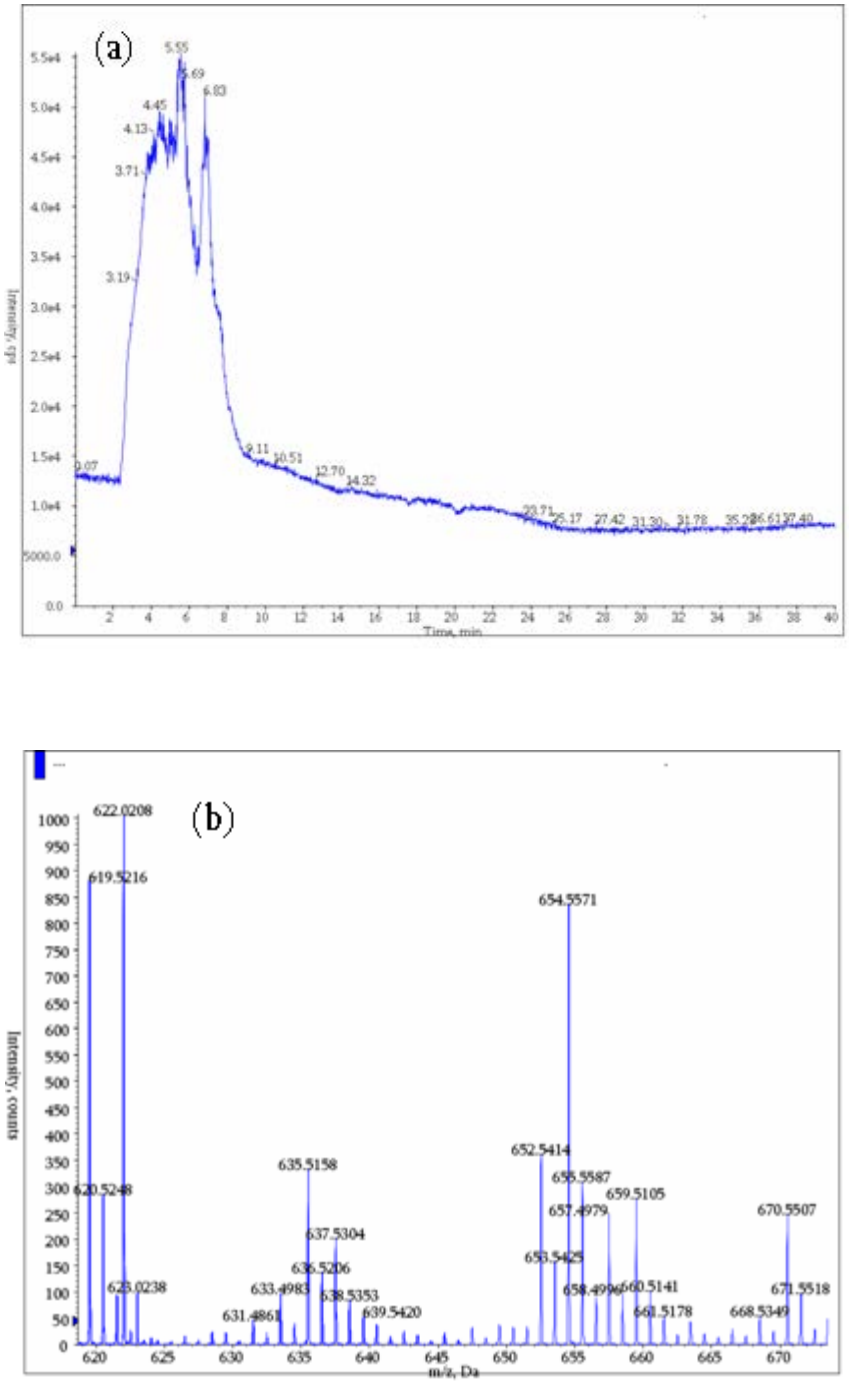

Figure 5.3.8 Extracted ion chromatogram and mass spectra for oxidized and unoxidized DG present in heated triolein (a) XIC of +TOF MS: 621 to 671Da (b) +TOF MS: 4.473 to 4.740 min; m/z 638.5, 654.5, 670.5

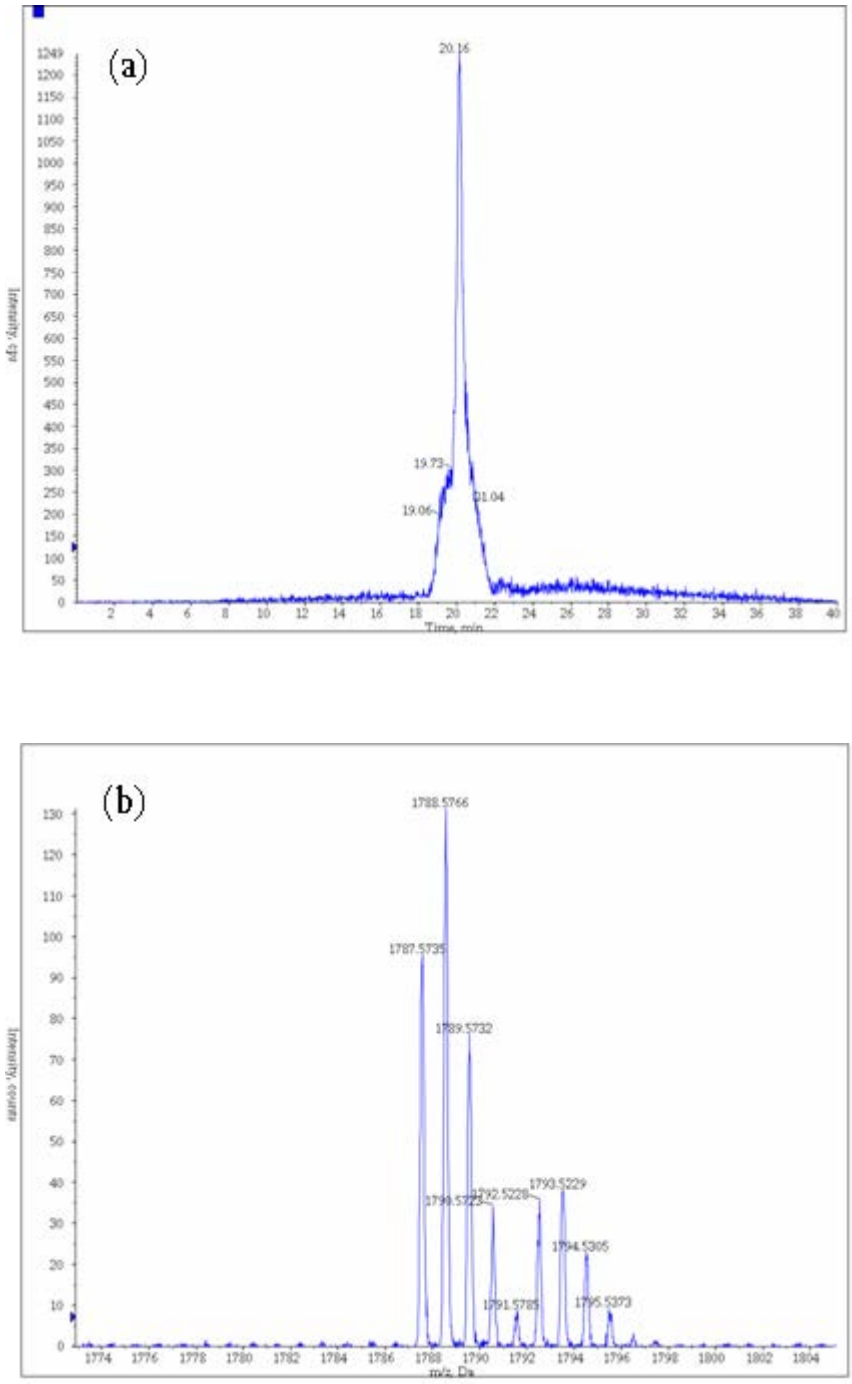

Figure 5.3.9 Extracted ion chromatogram and mass spectra for TG dimer present in heated triolein (a) XIC of +TOF MS: 1785 to 1788 Da (b) +TOF MS: 20.127 to 20.210 min; m/z 1787.6

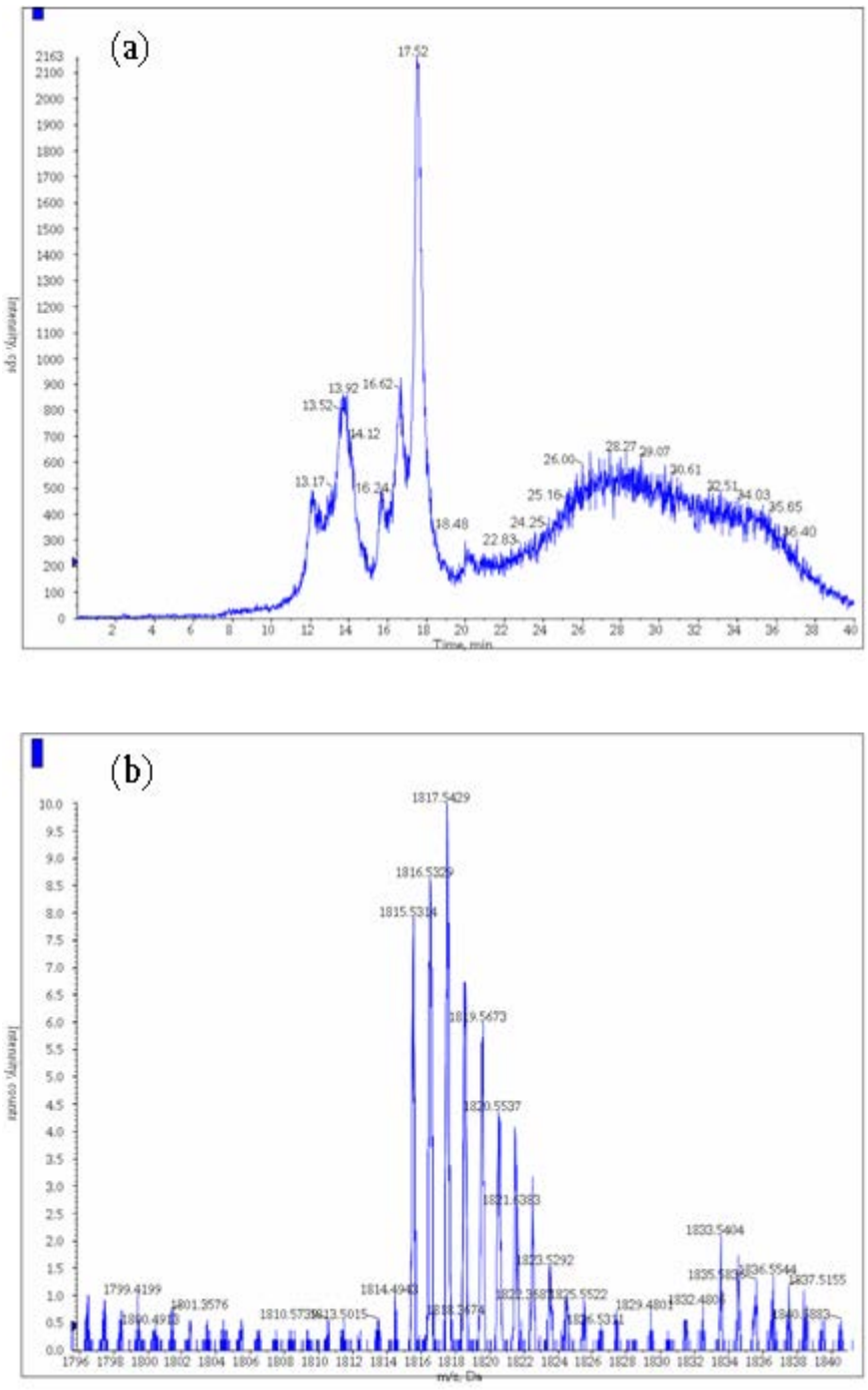

Figure 5.5.10 Extracted ion chromatogram and mass spectra for oxidized TG dimer present in heated triolein (a) XIC of +TOF MS: 1799 to 1834 Da (b) +TOF MS: 13.43 to 13.60 min; m/z 1817.5, 1833.5

TG dimers resulting by the joining of two triolein molecules through a C– C linkage may form by no net loss of hydrogen atoms or by the loss of 2 or 4 hydrogen atoms resulting in m/z values of 1787.6 / 1785.6 / 1783.6 [(OOO x 2) – 2 H + NH_4+ = 2 × 884.78 – 2.0 + 18.0 = 1785.6]. The oxidized TG dimers can take a variety of forms which may include two triolein molecules linked by an oxygen atom through a C–O–C linkage with an epoxy or a hydroxy group on a fatty acyl chain or a carbon linked dimer with a hydroperoxide group on a fatty acyl chain. The oxidized TG dimer peak of m/z 1817.6 would have contributions from at least two molecular species represented by [(OOO x 2) – 4H + 2O + NH_4^+] and [(OOO x 2) – 2H + 2O + NH_4^+] one involving a loss of 4 H atoms and the other a loss of 2 H atoms (22).

5.4 Conclusion

Separation and characterization of components in heated triolein or frying oils is a novel application for reverse phase C30 columns, which have been widely used in the study of carotenoids, retenoids, and tocopherols. Longer alkyl chain length coupled with thicker and densely bonded phases are believed to enable C30 columns to provide more extensive interactions with molecules resulting in enhanced selectivity, retention and hence better separation when compared with the C18 stationary phase.

RPHPLC of heated triolein samples with C30 stationary phase resulted in the separation of four main fractions based on differences in polarity and size. Low molecular weight components including oxidized and unoxidized MG, DG and TG monomers were resolved efficiently with the extent of oxidation dictating the

order of elution. TG monomers unoxidized and oxidized to different extents with up to one, two, three, four or five additional oxygen atoms were found to elute in the order of decreasing polarity with the unoxidized TG monomer being the most retained.

A similar trend was observed for TG dimers, with the more polar oxidized dimer eluting at shorter retention time. High molecular weight TG oligomers, including TG trimers and TG tetramers, eluted as a separate block between retention times of 22.0 and 38.0 min. This may enable separate quantification of the TG oligomer level and potentially serve as a quality indicator in frying oils. In comparison, size exclusion chromatography was useful in the identification of fractions separated using C30 column based on size but the oxidized and unoxidized fractions of DG, TG monomers and TG dimers remained unresolved and eluted at the same retention time.

RPHPLC with C18 column has been used for the separation and tentative identification of products of oxidized TG monomers such as hydroperoxide, diepoxide and hydroxide based on relative retention times of standards and the estimated elution factors and locations of functional groups (25). Effective separation of TG monomers oxidized to different extents with up to five additional oxygens has been made possible with RPC30 column in the study. With the known ability of C30 columns to enhance the resolution of carotenoid isomers, use of C30 stationary phase may be extended to study isomers of the oxidized TG monomers with different functionalities that form when vegetable oil is heated to frying temperature.

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Chapter 6 A new adsorption chromatography method for assessing the quality of frying oils

6.1 Introduction

Majority of the new alteration compounds that result from frying have higher polarity than their parent triglycerides (TG). Hence, adsorption chromatography is considered to be the most appropriate technique to determine the alteration level in used frying oils by means of isolation and quantification of the total polar compounds. These include oxidized TG monomers, with at least one of the three fatty acyl chains bearing an oxygenated functionality, and TG dimers, TG oligomers and TG polymers. Also included are free fatty acids (FFA), monoglycerides (MG) and diglycerides (DG) released through hydrolytic reactions (1).

The level of polar content in frying oils is considered to be a good indicator of the extent of oxidative degradation. A polar content value provides a direct measure of the degradation produced by the different variables involved in the frying process, which include high temperature, oxygen and moisture. Also, polar content determination is independent of the type of oil used in frying since the initial level of polar compounds is similar in unused oils. Most of the present regulations limiting the degradation of used frying fats and oils for human consumption have established a maximum level of polar compounds of around 25% (2).

Adsorption chromatography employing silica cartridges with organic solvents hexane and ether is normally used for the measurement of polar content in frying oils. The polar content level of 25% currently being used globally as the limit

beyond which frying oil is considered unfit for human consumption is easily reached after a frying duration of about 24 h and it is not uncommon to find oil samples with a level of polar content higher than 25%. Hence, it is important to improve quantification by developing rapid and simple methods to evaluate the quality of frying oils in fried food outlets (3).

In addition to the determination of polar compounds, measurement of polymer content, which includes TG dimers and higher TG oligomers, is another recommended analytical technique for the quality control of used frying fats and oils. The standard method for polymer content determination includes use of High Performance Size Exclusion Chromatography (HPSEC) with refractive index (RI) or evaporative light scattering detector (ELSD) detection. A maximum polymer content of 12% has been suggested as the limit for discarding used frying oils (4).

In this study, the objective was to develop an alternative adsorption chromatography method for the quantification of polar compounds as a measure of the extent of deterioration in frying oils. A solvent blend of 90% acetonitrile and 10% dichloromethane was used for the elution of nonpolar fraction which was followed by the elution of polar fraction using 100% THF.

6.2 Materials and methods

Hydrated silica gel was obtained by adding 5% (w/w) water to silica gel (63–100 µm; 70 – 230 mesh) that was heated in an oven at 160 ^{o}C overnight. The flask containing silica gel was then sealed and shaken vigorously to ensure disappearance of any lumps and then allowed to stand overnight for equal distribution of water.

Silica cartridge was prepared by adding 1.0 gram of 5% hydrated silica gel directly into a 15 cm long, 10 ml plastic pipette tip. The conical part of the pipette tip was plugged with cotton wool extracted with hexane for a duration of 4 h. Another cotton wool plug was placed on top of silica to compact and even the surface.

Sample preparation: Test samples was prepared by weighing 1000 ± 0.01mg of canola oil samples, obtained from Richardson Oilseed Company, Lethbridge, Alberta, Canada, unheated and heated for durations, 6, 12, 18, 24 h, in a 10 ml volumetric flask and diluting with toluene to obtain 100 mg of oil sample in 1.0 ml solution. Toluene, Acetonitrile (ACN), Dichloromethane (DCM) and tert–butyl methyl ether (TBME) solvents were obtained from Sigma Aldrich Oakville, Ontario, Canada.

Chromatography: Canola oil sample (1.0 ml solution in toluene) was applied to the top of the cartridge. The cartridge was soaked for a couple of min with 1.0 ml eluent. The non–polar fraction was then eluted with 2 x 4.0 ml portions of eluent, a combination of hexane:TBME (85:15 v/v) for the method currently in use and ACN:DCM (90:10 v/v) for the new method. The polar fraction was eluted with 2

x 4.0 ml portions of 100% tert–butyl methyl ether for the Hex:TBME method and 100% tetrahydrofuran (THF) (HPLC–grade; Fischer Scientific, Toronto, Ontario, Canada) for the new ACN:DCM method. The eluate from the cartridge was collected in 20 ml glass vials and the solvent removed by heating on a hot plate at 80 ^{o}C in the presence of a stream of pure air. The glass vials were then weighed on a gravimetric balance to determine the grams of non–polar and polar fractions eluted. Percentage polar content was reported as the average of the values obtained by the two equations given below:

% polar content = [(grams of sample – grams of non–polar fraction) /grams of sample] x 100

% polar content = [grams of polar fraction)/grams of sample] x 100

The same oil samples were also analyzed using HPSEC to determine the polymer content values, which corresponded to the percentage peak area to the left of the TG monomer peak in the HPSEC chromatogram. The reported polar content values for the two gravimetric methods and the polymer content values using HPSEC represent an average of 12 measurements. HPSEC chromatograms were also obtained for the polar and nonpolar fractions by injecting 10 µl volume for the ACN:DCM method and 25 µl volume for the Hex:TBME method of a 5 mg/ml concentration of each fraction in THF.

HPSEC: The HPSEC system included an Agilent 1200 series binary pump (G1312 A) and a degasser (G1379 B), Agilent 1200 series (G13229A) auto sampler and Agilent 1200 series evaporative light scattering detector (ELSD). The ELSD was set at temperature of 70 ^{o}C with a N_2 gas flow of 3l/min at pressure of 3.5 bars. Two size exclusion columns were used in series, a Waters (Waters

Limited, Mississauga, Ontario, Canada) styragel HR 100 A^o (4.6 x 300 mm) and a Phenomenex (Phenomenex, Torrance, California, USA) phenogel 500 A^o (4.6 x 300 mm). THF was used as the mobile phase at a flow rate of 0.3 ml/min.

Statistical analysis: Standard deviations of the polar content and polymer content measurements were computed and the reported values represent an average of 12 measurements. Linear regression analysis between HPSEC and the cartridge methods was performed by using Sigma Plot 11 (Systat Software Inc., San Jose, CA).

6.3 Results and Discussion

A typical chromatogram representing the polar fraction of canola oil heated at 180 ^oC for 24 h included peaks corresponding to the TG polymer at retention time ~12.9 min, TG monomer peak at retention time ~17.9 min and a peak corresponding to TG ester hydrolysis products at a retention time of ~18.4 min. TG oligomers (TG dimers, TG trimers etc.) appeared at retention times between the TG monomer and TG polymer peak, ~14–17 min.

Figures 6.3.1–6.3.2 represent the HPSEC chromatograms for the polar and nonpolar fractions of canola oil samples heated for durations that ranged from 0, 12, and 24 h obtained by the ACN:DCM and Hex:TBME methods. Injection volumes of 10 µl and 25 µl of a 5 mg /ml solution of the two fractions in THF were used for the ACN:DCM and Hex :TBME methods respectively.

The peak at retention time 18.4 min representing the high polarity low molecular weight (MW) TG hydrolysis products such as DG and fatty acids was missing in the polar fractions obtained by the ACN:DCM method as indicated by the

HPSEC chromatograms in Figure 6.3.1whereas the chromatograms representing polar fraction obtained by the Hex–TBME method (Figure 6.3.2) clearly indicate the presence of hydrolysis products at retention time 18.4 min. This may be indicative of the fact that the low MW high polarity functionalities like hydroxyl in DG and carboxylates in fatty acids (Table 6.3.1) get eluted along with the ACN:DCM (v/v 90:10) solvent blend used for eluting the nonpolar fraction. This may not be unexpected, given the higher dielectric constant value of acetonitrile (Table 6.3.2).

While the presence of high polarity functionalities like hydroxyl cannot be ruled out in oxidized TG monomer, oxidized TG dimers and higher TG oligomers, the increasing hydrophobicity in these molecules may keep them from getting eluted along with the ACN:DCM solvent blend and hence oxidized monomer, oxidized dimers and higher oligomers appear in the polar fraction eluted using THF. The balance between the hydrophobicity and polar strength of the functionality present in the molecule appears to be the determining factor in the elution pattern of the analytes and may provide an explanation for the lower polar content values for the ACN:DCM method in comparison to the Hex:TBME method. With the ability of the ACN:DCM method to isolate only the oxidized TG monomer, TG dimers and higher TG polymers in the polar fraction, it may be used for the quantification of only those components in polar compounds considered physiologically harmful (5).

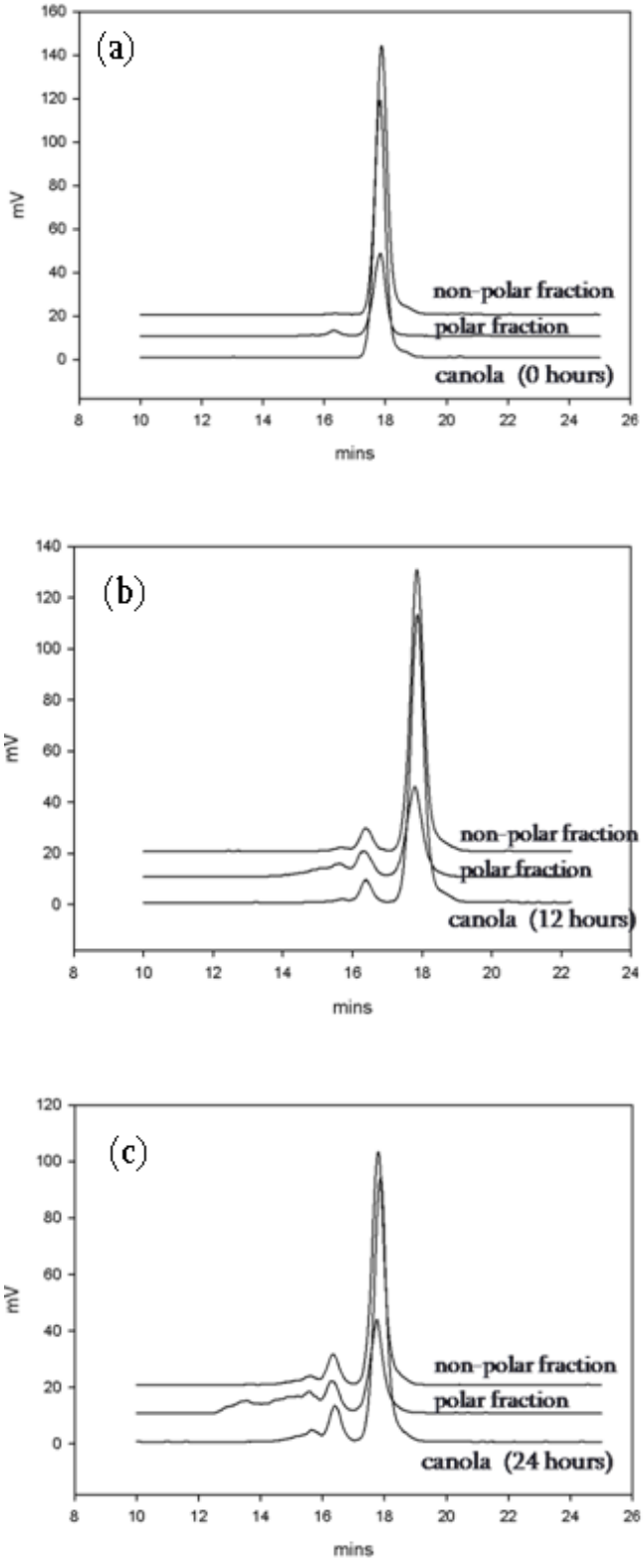

Figure 6.3.1 Canola oil samples heated at 180 ^{o}C for (a) 0 h (b) 12 h (c) 24 h (ACN:DCM method)

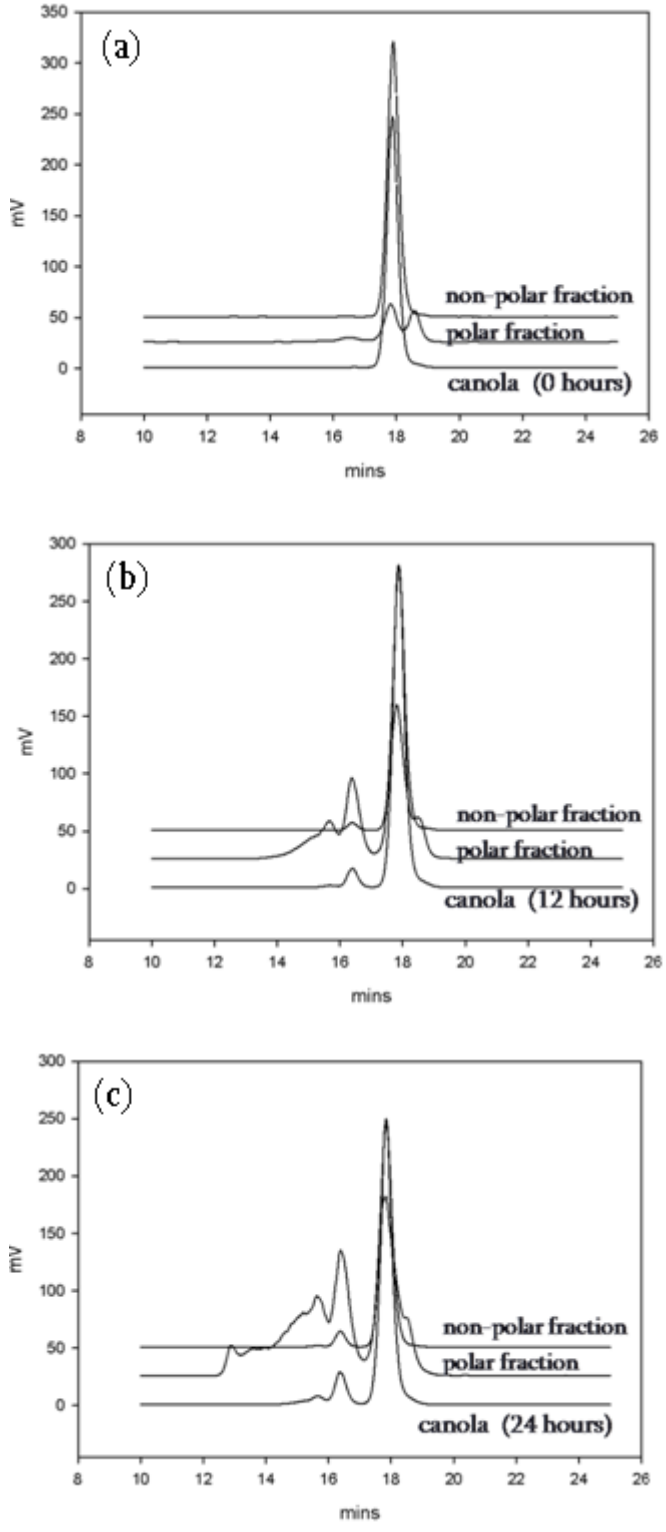

Figure 6.3.2 Canola oil samples heated at 180 ^{o}C for (a) 0 h (b) 12 h (c) 24 h (Hexane:TBME method)

Table 6.3.1 Polarity ranking of the functional groups in organic compounds (most polar first) (6)

1	Amide
2	Acid
3	Alcohol
4	Ketone ~ Aldehyde
5	Amine
6	Ester
7	Ether
8	Alkane

Table 6.3.2 Dielectric constant values for solvents used in adsorption chromatography (6)

Solvent	dielectric constant
Hexane	1.9
Ether	4.3
Acetonitrile	37.5
Dichloromethane	9.1
Tetrahydrofuran	7.5

Data for polar content measurements using adsorption chromatography and the polymer content measurements using HPSEC for canola oil samples unheated and heated for durations ranging from 6 – 24 h is shown in Table 6.3.3.

Table 6.3.3 Polar and polymer content values in canola oil samples heated for different durations (n = 12) at 180 ^{o}C

Heating duration (h)	Hexane:TBME polar content	ACN:DCM polar content	HPSEC polymer content
0	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
6	8.3 ± 0.8	2.6 ± 0.6	2.0 ± 0.3
12	11.9 ± 0.9	7.5 ± 0.6	5.7 ± 0.6
18	17.8 ± 1.0	11.7 ± 0.6	9.6 ± 0.6
24	23.7 ± 1.0	16.9 ± 1.1	14.2 ± 0.6

The polar content values obtained by the ACN:DCM method were found to correlate better (R = 0.999) with the polymer content measurements obtained using HPSEC in comparison to the polar content values obtained using the Hex:TBME method (R = 0.979). Also, the polar content values obtained by the ACN:DCM method appeared much closer to the polymer content values obtained using HPSEC for all canola oil samples. The linear correlation between the polar content measurements using the adsorption chromatography methods and the polymer content measurements for the same samples using HPSEC is shown in Figure 6.3.3 (Hex:TBME) and Figure 6.3.4 (ACN:DCM).

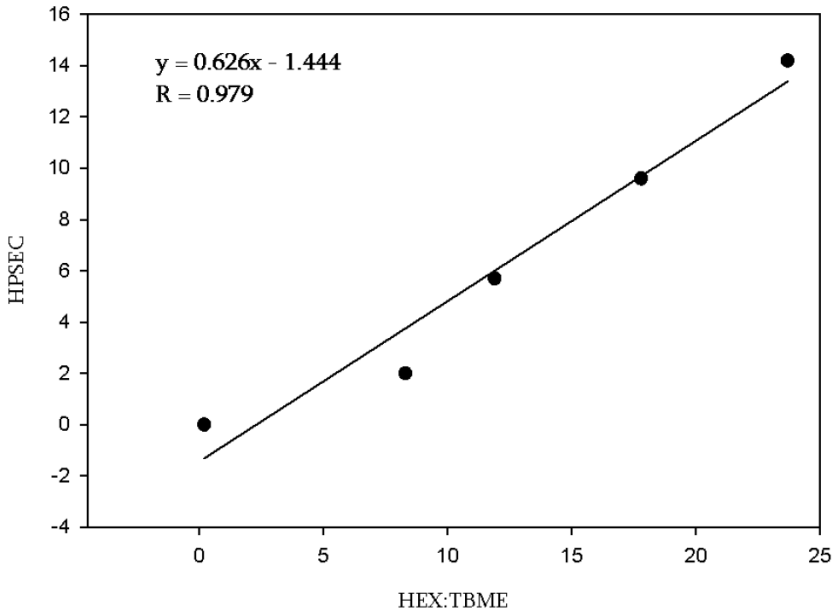

Figure 6.3.3 Linear regression between HPSEC measurements and Hex:TBME method

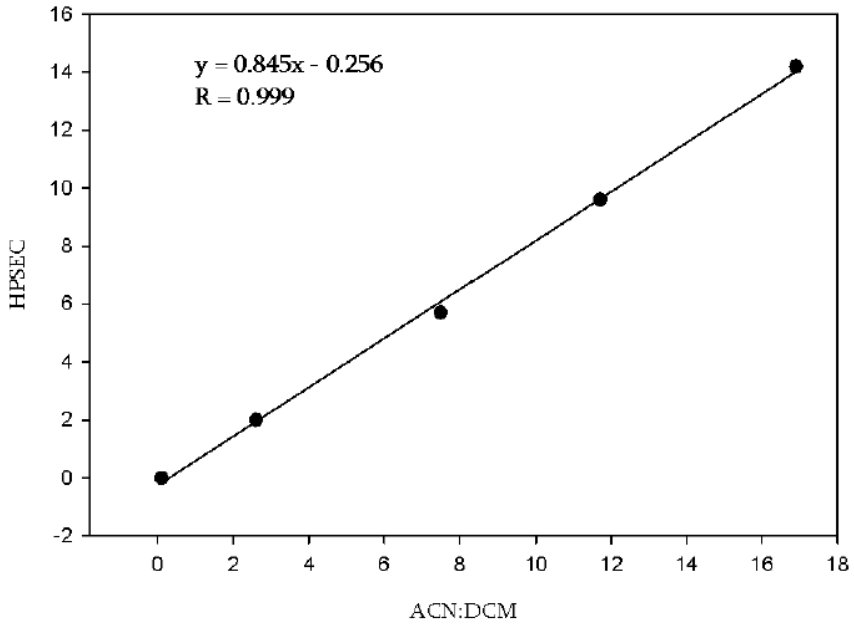

Figure 6.3.4 Linear regression between HPSEC measurements and ACN:DCM method

6.4 Conclusion

While the Hexane:TBME method is a commonly used solvent blend in adsorption chromatography methods for asssessing the extent of degradation in frying oils, introduction of the ACN:DCM method may provide an additional option. Use of the ACN:DCM method may enable quantification of components in polar compounds considered physiologically harmful. Based on linear regression equation and the identified cut off limit of 12% for the HPSEC method, a reasonable estimate for the cut off limit for discarding used frying oils using the ACN:DCM method would be ~ 15%. More work needs to be done to establish ACN:DCM method as a viable alternative with an identified cut off limit beyond which the frying oil sample can be considered unfit for human consumption.

6.5 References

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Chapter 7 Effect of polymer content on *in vitro* lipolysis in frying oils

7.1 Introduction

With increased consumption of fried foods, concerns over the ingestion of non–volatile lipid oxidation products have grown in recent years. There is increasing evidence of the detrimental effects of non–volatile lipid oxidation products on health, particularly in connection with diseases related to oxidative stress, including atherosclerosis, cancer, heart attacks and liver damage (1). Also, non–volatile lipid oxidation products that form in frying oil, tend not to hydrolyze readily and are known to impact the hydrolysis of triglyceride (TG) monomers, which is believed to significantly affect the release and hence digestibility of oxidized and unoxidized fatty acids (2).

Autoxidation reaction with a free radical mechanism is believed to be mainly responsible for the formation of a variety of new compounds differing in polarity and molecular weight (MW) during the frying process (3). These include oxidized TG monomers, nonpolar and polar TG dimers, and TG polymers. Oxidized TG monomers result from the decomposition of hydroperoxides and contain an additional oxygenated functionality that may include epoxides, ketones and alcohols (4) Non polar TG dimers with C–C linkages result from reactions between alkyl radicals (5). Polar dimers are generated by the oxidation of nonpolar TG dimers or by the dimerization of two oxidized TG monomers (6). TG polymers and higher TG oligomers represent complex structures, mainly with C–C and C–O–C linkages (7).

The level of polar content in frying oils is considered to be a good indicator of the extent of oxidative degradation. Adsorption chromatography, employing silica cartridges with organic solvents hexane and ether, is normally used for the measurement of polar content in frying oils. A polar content level of 25% is currently being used globally as the limit beyond which frying oil is considered unfit for human consumption. This level is easily reached after a frying duration of about 24 h and it is not uncommon to find oil samples with a level of polar content higher than 25% (8). Hence, it is important to understand the influence of these compounds on the lipolysis of unaltered TG monomers.

In the past, evaluation of pancreatic lipase action was based mainly on titration of free fatty acids released and hence it was difficult to quantify and compare the extent to which different components of polar compounds hydrolysed. Introduction of High Performance Size Exclusion Chromatography (HPSEC) has enabled the quantification of not only fatty acids released but also changes in the contents of diglyceride (DG), monoglyceride (MG) and the remaining TG monomers. Combination of adsorption chromatography and HPSEC allows the separation of polar compounds and comparison of the susceptibilities to lipase hydrolysis of different components of polar compounds, which may include oxidized TG monomers, TG dimers and TG polymers (9).

In this study the objective was to quantify and compare the effect of polymer content level on the extent of TG monomer hydrolysis in canola oil samples heated for different durations by combining the technique of adsorption chromatography and HPSEC.

7.2 Materials and methods

Refined canola oil was obtained from Richardson oilseed company, Lethbridge, Alberta, Canada. Test samples were prepared by heating 2 g canola oil in 7 ml open mouth glass vials placed in a Stabil–Therm gravity oven (Blue M, New Columbia, Pennsylvania, USA) set at 180 ^{o}C for durations that ranged from 0 to 36 h. Small aliquots, drawn in duplicate from each vial heated for 0, 18, and 36 h was dissolved in tetrahydrofuran (THF) (HPLC–grade;Fischer Scientific,Toronto, Ontario, Canada) to a concentration of 5 mg/ml and analyzed twice using HPSEC with the reported peak area representing an average of the four measurements. The same canola oil samples were subjected to lipase hydrolysis.

Enzymatic hydrolysis: Lipase from porcine pancreas Type II L3126, 100–400 units/mg protein (using olive oil (30 min incubation), 30–90 units/mg protein (using triacetin) obtained from Sigma Aldrich, Oakville, Ontario, Canada was used for the *in vitro* hydrolysis of canola oil samples. To a canola oil sample weighing 100 ± 0.1 mg in a 20 ml glass vial were added 2 ml of tris (hydroxymethyl) – amino methane buffer (0.1 M, pH = 8.0), 1 ml of 22% $CaCl_2$, 0.5 ml of 0.1% sodium cholate, and 30 mg pancreatic lipase. The vial was placed in a water bath maintained at 37 °C on a hot plate and the contents of the vial were stirred with the help of a magnetic stir bar for duration of 5 min.

Lipase amount of 30 mg and the incubation duration of 5 min were determined to be the optimum values under the chosen experimental conditions based on TG monomer peak area measurements resulting from subjecting unheated canola samples to lipase hydrolysis with 10 mg lipase for durations of 5, 10, 20 min and

varying lipase levels from 10mg, 20mg, 30mg, 40mg for a fixed incubation period of 5 min. After the digestion period, the reaction was quenched by the addition of 1 ml 6 M HCl, followed by the extraction of the acidified mixture with four 10 mL portions of diethyl ether in a separatory funnel. The combined ether extracts were then washed with distilled water to remove traces of HCl and the solvent removed by evaporation under vacuum. The resulting product was then dissolved in THF to a concentration of 5 mg/ml and analyzed twice using HPSEC. Canola oil samples were subjected to lipase hydrolysis in duplicate with the reported peak area representing an average of the four HPSEC measurements.

Variation in peak areas obtained by the Chemstation software was used as the basis to assess the impact of the level of polymer content on the *in vitro* hydrolysis of unaltered TG monomers in canola oil samples. The HPSEC peaks were identified based on retention times with the peak at retention time 17.9 min representing TG monomer, DG appearing at 18.4 min, MG at 19.7 min and free fatty acids (FFA) at 20.3 min. Peak at 16.4 min represented TG dimers with the region between 14 and 16 min representing higher TG oligomers. The HPSEC system including the ELSD detector described in Chapter 3 was used in this experiment.

Statistical Analysis: Standard deviations of the peak area measurements were computed and reported as relative standard deviations, RSD % = (SD/mean) × 100, $n = 4$.

7.3 Results and Discussion

The TG monomer peak areas in HPSEC chromatograms (Figure 7.3.1) are indicative of the effect of duration of incubation on the extent of TG monomer hydrolysis. An incubation period of 5 min was found to provide the maximum TG monomer peak area reduction of ~ 30%, which then leveled off at ~ 20% over incubation periods of 10 and 20 min.

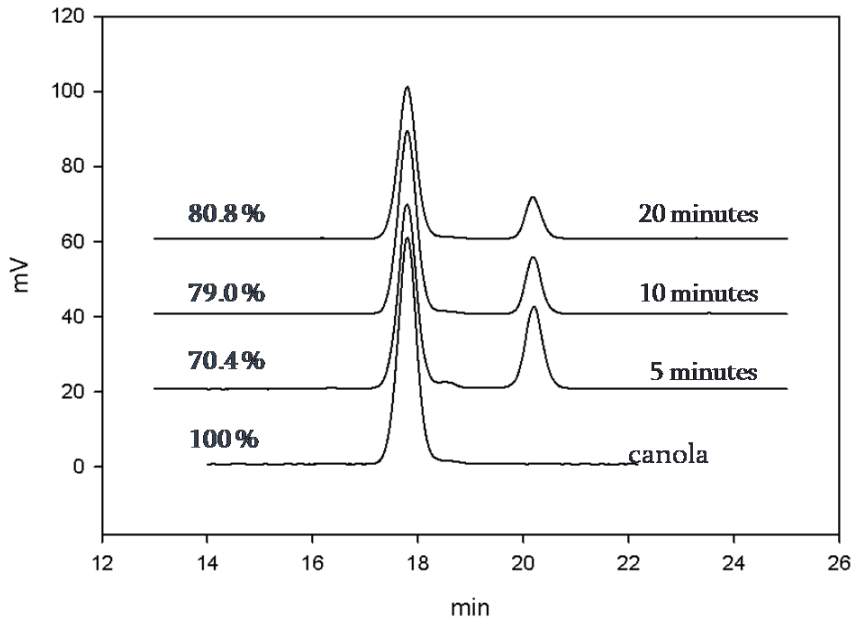

Figure 7.3.1 Canola oil hydrolyzed with 10 mg porcine lipase for different durations

The optimum level of porcine lipase to be used in the hydrolysis experiment was established by subjecting unheated canola oil samples to incubation with 10mg, 20 mg, 30mg and 40mg lipase for 5 min duration. HPSEC chromatograms (Figure 7.3.2) are indicative of the effect of amount of porcine lipase on the extent of TG monomer hydrolysis. In comparison to the unhydrolyzed canola oil sample, a ~ 60% reduction in the TG monomer peak area was observed in the presence of 30

mg of lipase. This was accompanied with the appearance of a fatty acid peak at retention time 20.3 min, a MG peak at retention time of 19.7 min and a DG peak at retention time of 18.4 min, indicated in the chromatogram corresponding to the 30 mg lipase sample.

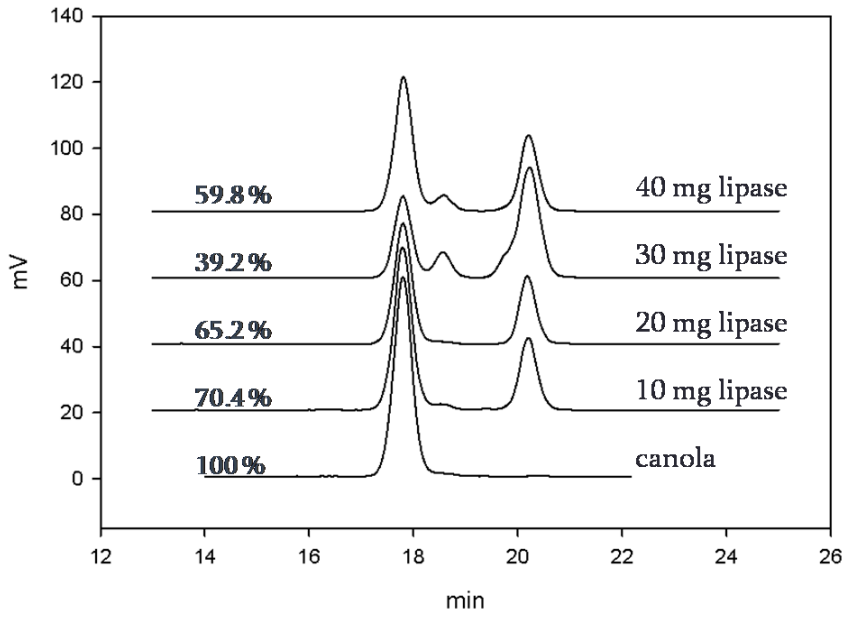

Figure 7.3.2 Canola oil hydrolyzed for 5 min with different amounts of porcine lipase

HPSEC chromatograms in Figure 7.3.3 are indicative of the effect of the level of polymer content on the extent of TG monomer hydrolysis in canola oil samples *in vitro*, highlighted by the extent of reduction in TG monomer peak area before and after lipase hydrolysis in unheated canola oil and canola oil samples heated for 36 h duration.

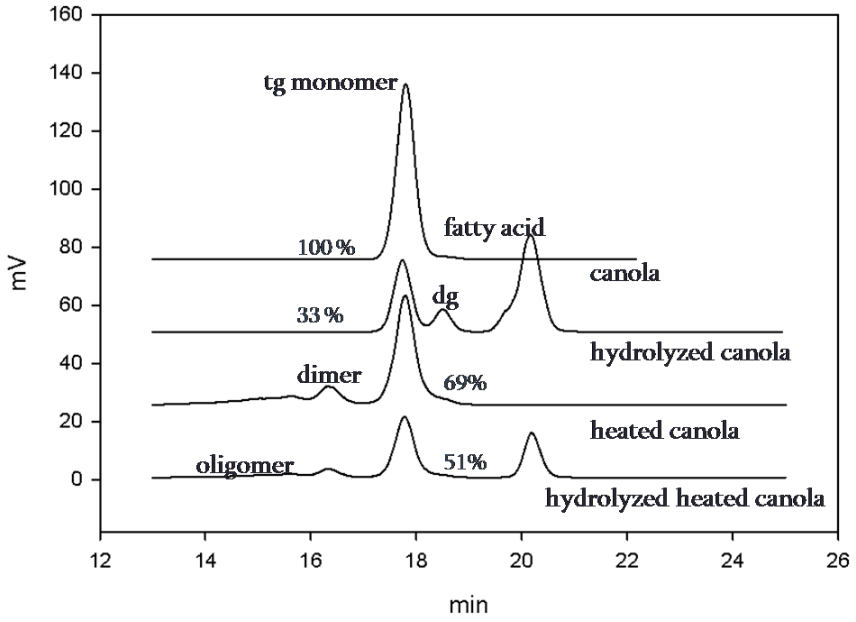

Figure 7.3.3 Effect of the level of polymer content on TG monomer hydrolysis in canola

The chromatogram with a TG monomer peak area of 100% represents unheated canola oil sample before hydrolysis. The TG monomer peak area for the same sample was found to be 33% after lipase hydrolysis, indicating a 67% reduction in TG monomer in the absence of any TG dimers or TG polymers under the chosen experimental conditions. In comparison, for canola oil samples heated at 180 ^{o}C for a period of 36 h with a 29% polymer content level and an intact TG monomer peak area of 69%, the reduction in TG monomer peak area was found to be 26% as indicated by the hydrolyzed heated canola chromatogram with a TG monomer peak area of 51%. A similar comparison for canola oil samples heated for 18 h with a 14% polymer content indicated a ~ 50% reduction in the extent of TG monomer hydrolysis. Peak area for TG monomers before and after hydrolysis in samples heated for different durations is given in Table 7.3.1.

Table 7.3.1: Effect of polymer content level on the extent of TG monomer hydrolysis

Heating duration (h)	Polymer content	%TG monomer before hydrolysis (a)	%TG monomer after hydrolysis (b)	% RSD (n = 4)	Percentage hydrolysis (a – b) / a
0	0	100	33	8.4	67
18	14	85	42	7.2	50
36	29	69	51	9.6	26

An excellent inverse correlation ($R^2 = 0.994$) was observed between the level of polymer content and the extent of TG monomer hydrolysis in canola oil samples heated for different durations. (Fig. 7.3.4)

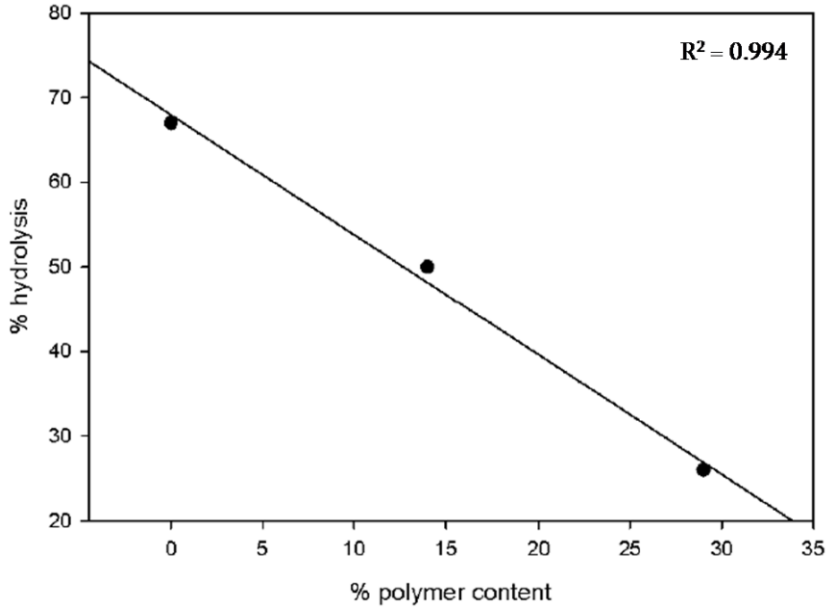

Figure 7.3.4 Correlation between the level of polymer content and the extent of TG monomer hydrolysis in canola oil heated for different duration ($p < 0.05$)

Similar effects of the presence of TG polymer and TG dimer on the hydrolysis of unaltered TG monomers by pancreatic lipase were reported in an earlier study (2) which also found an excellent correlation between the percentage of unhydrolyzed TG monomers and the global percentage of TG dimeric and TG polymeric compounds. The effects were observed regardless of the extent of alteration with samples around the limit of rejection of 25% polar compounds, established in frying–fat regulations for human consumption, also showing reduced hydrolysis of unaltered TG monomers (2). However, despite reports of pancreatic lipase activity being much greater under physiological conditions than under *in vitro* conditions, estimated to be 100–1000–fold in excess of that needed for complete hydrolysis of TG in the small intestine (12), *in vivo* studies have also confirmed reduced digestibility coefficients of altered oils in comparison to those of unused oils (13, 14).

Results clearly indicate that hydrolysis of unoxidized TG monomers by pancreatic lipase can be affected in the presence of TG dimers and TG polymers. Lipase are known to show fatty acyl specificities for chain length, position and geometry of double bonds, generally suggested to be due to steric hindrance for formation of the activated substrate–enzyme complex (10). Significant discrimination of pancreatic lipase against dimers and particularly polymers has also been reported (2), which provides further evidence of the important influence of MW on the action of pancreatic lipase. Unoxidized and oxidized TG monomers have been reported to hydrolyze at a much higher rate in comparison to TG dimers which tend to hydrolyze faster than TG polymers (2). The high hydrolysis rate of

oxidized TG monomers is a matter of concern because the oxidized fatty acids yielded therefrom can be found at levels as high as 6.6% in real used frying oils (5) that are readily absorbed (11), with potential physiological implications (15–18).

7.4 Conclusion

An increase in the level of polymer content was found to adversely impact the extent of TG monomer hydrolysis in the canola oil samples heated for different durations. An inverse correlation ($R^2 = 0.994$) was observed between the extent of hydrolysis of unaltered TG monomers and the level of TG polymer content in the oil samples. Hence, in addition to negatively impacting human health, presence of TG polymers in frying oils can also interfere with the hydrolysis of unaltered TG monomers.

7.5 References

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Chapter 8 Conclusions & Recommendations

A mechanism to explain the role of sitosterol as an antioxidant in frying oils was presented in Chapter 3. Higher levels of sitosterol were found to limit triglyceride (TG) polymerization in frying oils. The anti–polymerization effect of sitosterol is attributed to the conversion of sterol to steradiene by the 1, 2–elimination of water, catalyzed by the presence of moisture at the air–oil interface. This was further corroborated by a corresponding increase in TG ester hydrolysis, likely caused by the release of water in the system during the conversion of sterol to steradiene.

Several studies have reported a gap between the amount of initial sterol before heating and those of unaltered sterol and its oxides measured in frying oils after heating, which appeared to be much larger at frying temperatures. This gap may provide further evidence of conversion of sterols to steradienes in frying oils, since steradienes represent a highly unstable (4n) closed ring system which can easily decompose and volatilize at frying temperatures.

Unrefined oils have been reported to have a higher stability at frying temperatures in comparison to refined oils. Refined oils tend to lose some of the natural components including sterols at various stages of refining, particularly during neutralization and deodorization. Ensuring the presence of optimum levels of sterols in refined oils may be a critical future development for the fats and oils refining industry.

Future work should include optimizing the effect of sitosterol as an antioxidant under controlled humidity conditions. This may be accomplished by comparing

the extent of TG polymerization in a control triolein sample and a test triolein sample with 5% sitosterol in a controlled humidity chamber set at a constant temperature of 180 ^{o}C. Establishing a relationship between percentage humidity and the antioxidative effect of sitosterol would further reinforce the mechanistic explanation provided for the sitosterol antioxidative effect.

Chapter 4 provided estimates of molecular weights (MW) of different fractions of triolein heated at 180 ^{o}C for 72 h based on a calibration curve obtained using polystyrene standards. Average MW for the TG polymer fraction with a retention time of 12.8 min, was estimated to be ~ 25000. An average MW of 1144 was estimated for the fraction representing hydrolysis products, TG monomers and TG dimers. The fraction with an estimated MW of 1532 may include TG monomers and TG dimers. The fraction with an estimated average MW of 2527 may represent TG dimers, TG trimers and TG tetramers. However, designation of various fractions of nonvolatile components in frying oils as increments of TG monomer may be an oversimplification given the complexity of degradation products that result during frying. A calibration curve resulting from the use of actual nonvolatile lipid oxidation product standards, obtained using preparative High Performance Size Exclusion Chromatography (HPSEC), may provide a more reliable MW estimate of frying oil fractions.

Chapter 5 highlighted the use of Reverse Phase (RPC30) and Time of Flight–Mass Spectrometry (TOF–MS) for the separation and identification of heated triolein fractions. Separation of four main fractions was based on differences in polarity and size. HPSEC based retention times of known standards was used as

the basis for identifying the fractions. Low MW components including oxidized and unoxidized monoglycerides (MG), diglycerides (DG) and TG monomers were resolved efficiently with the extent of oxidation dictating the order of elution. TG monomers unoxidized and oxidized to different extents with up to one, two, three, four or five additional oxygen atoms were found to elute in the order of decreasing polarity with the unoxidized TG monomer being the most retained. A similar trend was observed for TG dimers, with the more polar oxidized dimer eluting at shorter retention time. High MW TG oligomers, including TG trimers and TG tetramers, eluted as a separate block between retention times of 22.0 and 38.0 min. This may enable separate quantification of the TG oligomer level and potentially serve as a quality indicator in frying oils. The future use of RPC30 stationary phase could be extended to study isomers of oxidized TG monomers with different functionalities that form in frying oils.

Chapter 6 introduced a new adsorption chromatography method employing silica cartridges with organic solvents acetonitrile (ACN), dichloromethane (DCM) and tetrahydrofuran (THF) for estimating polar content levels as an indicator of the extent of deterioration in frying oils. The introduction of the ACN:DCM method may provide an additional option for polar content masurement in frying oils. Based on the correlation between polymer content values obtained by the HPSEC method and the polar content values obtained by the ACN:DCM method and the known cut off limit of 12% for polymer content values, a reasonable estimate for the cut off limit for discarding used frying oils using the ACN:DCM method would be ~ 15%. Further validation of the method may be accomplished by

analyzing frying oil samples from different sources at different levels of degradation.

Chapter 7 provides an assessment of the impact of the polymer content on TG monomer hydrolysis in canola oil heated to frying temperature. Results indicate that the lipolysis of unoxidized TG monomers may be negatively affected by the presence of TG polymers and TG dimers. A correlation between the polymer content level and the extent of TG monomer hydrolysis in frying oil samples may serve as a dual quality indicator.

Phytosterols have already been widely studied for their hypocholesterolemic, anticarcinogenic, and other health benefits. Highlighting the role of sitosterol as a natural and heat–stable way to stabilize lipid oxidation should only further enhance the status of Δ5 sterols as a precious and value–added component in the food industry. The potential of sitosterol as a natural, safe, antioxidant added to frying oils or foods containing unsaturated lipids may be an important development for the food processing industry.