Study on the Characteristic Oxidative Stability of Plant and Seaweed Lipids: Application to Halal Alternative Ingredients

(植物および海藻脂質の特徴的な安定性に関する研究：ハラル食品素材の開発を目指して)

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Chapter 1: Introduction

Different chemical mechanisms are responsible for the oxidation of fats and oils during processing, storage, and cooking. Two types of oxygen, atmospheric triplet oxygen and singlet oxygen, can react with fats and oils. Triplet oxygen, having a radical character, reacts with radicals and causes autoxidation. The non-radical electrophilic singlet oxygen does not require radicals to react with; it directly reacts with the double bonds of unsaturated fats and oils with high electron densities, which is called type II photosensitized oxidation (Choe and Min 2005).

Plant leaves contain up to 7 % of lipid per dry weight. Major constituents which made up these lipids are monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG). These constituents, collectively called Glyceroglycolipids (GL), made up the photosynthetic membrane (chloroplast; in the layers of the thylakoids) of higher plants, algae and bacteria. The fatty acid composition of each of these GLs have been proven to have unusually high in polyunsaturated fatty acid (PUFA) namely; α-Linolenic acid (18:3n-3) as the main fatty acid. In seaweeds, the main fatty acid composition comprise of Stearidonic acid (SA,18:4n-3), Arachidonic acid (AA, 20:4n-6) and Eicosapentanoic acid (EPA, 20:5n-3). All these PUFA are easily oxidized as they are structured in the chloroplast. Continual exposure to oxidative stress with involving in the absorbtion of light energy would surely exhaust the fatty acid through oxidation. However, instead of it being oxidized, the opposite response was found to happen. Protective mechanisms underlining this contradictory response are still being research upon.

On the same note, oxidation decreases consumer acceptability of foods by producing low-molecular-weight off-flavor compounds, as well as by destroying essential nutrients, and it produces toxic compounds and dimers or polymers of lipids and proteins (Aruoma 1998). Oxidation of foods can be minimized by removing prooxidants such as free fatty acids, metals, and oxidized compounds, and by protecting foods from light.

Objective of the study:

1) To determine and evaluate the lipid class, fatty acids and bis-allylic content

2) To establish and compare oxidative stability between Glyceroglycolipids (GL) and Triacylglycerol (TAG)

3) To analyze volatile compounds formed throughout the oxidation process

4) To assess the efficacy of Glyceroglycolipids (GL) with other compounds as a source of potential for halal ingredients in food

Chapter 2: Literature Review

2.1 Introduction

2.1.1 Membrane Cell

The boundaries of cells are formed by biological membranes, the barriers that define the inside and the outside of a cell. These barriers prevent molecules generated inside the cell from leaking out and unwanted molecules from diffusing in; yet they also contain transport systems that allow specific molecules to be taken up and unwanted compounds to be removed from the cell. Such transport systems confer on membranes the important property of selective permeability. Membranes are dynamic structures in which proteins float in a sea of lipids. The lipid components of the membrane form the permeability barrier, and protein components act as a transport system of pumps and channels that endow the membrane with selective permeability.

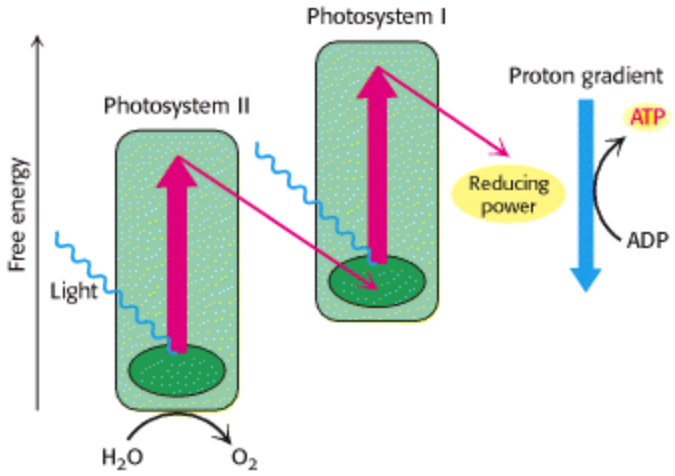
In addition to an external cell membrane (called the plasma membrane), eukaryotic cells also contain internal membranes that form the boundaries of organelles such as mitochondria, chloroplasts, peroxisomes, and lysosomes. Functional specialization in the course of evolution has been closely linked to the formation of such compartments. Specific systems have evolved to allow targeting of selected proteins into or through particular internal membranes and, hence, into specific organelles. External and internal membranes have essential features in common, and these essential features are the subject of this chapter.

Biological membranes serve several additional important functions indispensable for life, such as energy storage and information transduction, that are dictated by the proteins associated with them.

2.1.2 Photosynthesis

Photosynthesis, the means of converting light into chemical energy, is sequestered into organelles called chloroplasts, typically 5 μm long. Like a mitochondrion, a chloroplast has an outer membrane and an inner membrane, with an intervening intermembrane space. The inner membrane surrounds a stroma, which is the site of the carbon chemistry of photosynthesis. In the stroma are membranous structures called thylakoids, which are flattened sacs, or discs. The thylakoid sacs are stacked to

form a granum. Different grana are linked by regions of thylakoid membrane called stroma lamellae. The thylakoid membranes separate the thylakoid space from the stroma space. Thus, chloroplasts have three different membranes (outer, inner, and thy-lakoid membranes) and three separate spaces (intermembrane, stroma, and thylakoid spaces). In developing chloroplasts, thylakoids are believed to arise from invaginations of the inner membrane, and so they are analogous to the mitochondrial cristae. Like the mitochondrial cristae, they are the site of coupled oxidation reduction reactions that generate the proton-motive force.



2.1: Chloroplasts (left) convert light energy into chemical energy. High-energy electrons in chloroplasts are transported through two photosystems (right). During this transit, which culminates in the generation of reducing power, ATP is synthesized in a manner analogous to mitochondrial ATP synthesis. Unlike as in mitochondrial electrontransport, however, electrons in chloroplasts are energized by light. [(Left) Herb Charles Ohlmeyer/Fran Heyl Associates.]

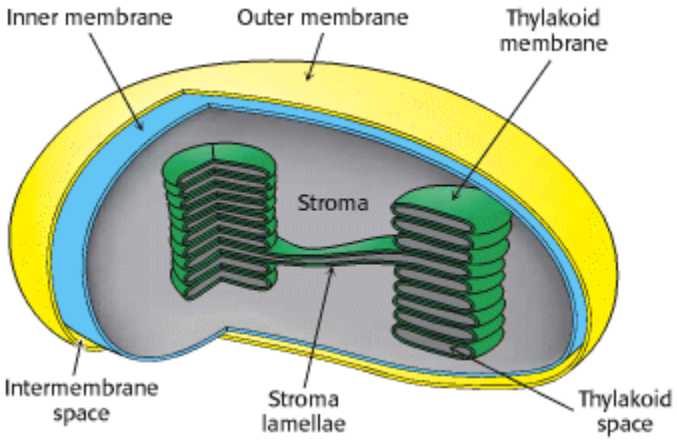
2.1.3 Thylakoids

The thylakoid membranes contain the energy-transducing machinery: light-harvesting proteins, reaction centers, electrontransport chains, and ATP synthase. They have nearly equal amounts of lipids and proteins. The lipid composition is highly distinctive: about 40 % of the total lipids are galactolipids and 4 % are sulfolipids, whereas only 10 % are phospholipids. The thylakoid membrane and the inner membrane, like the inner mitochondrial membrane, are impermeable to most molecules and ions. The outer membrane of a chloroplast, like that of a mitochondrion, is highly permeable to small molecules and ions. The stroma contains the soluble enzymes that utilize the NADPH and ATP synthesized by the thylakoids to convert CO^2 into sugar. Plant leaf

cells contain between 1 and 100 chloroplasts, depending on the species, cell type, and growth conditions.

2.1.4 Chloroplast

Chloroplasts contain their own DNA and the machinery for replicating and expressing it. However, chloroplasts are not autonomous: they also contain many proteins encoded by nuclear DNA. It is believe that, in a manner analogous to the evolution of mitochondria chloroplasts are the result of endosymbiotic events in which a photosynthetic microorganism, most likely an ancestor of a cyanobacterium, was engulfed by a eukaryotic host. Evidence suggests that chloroplasts in higher plants and green algae are derived from a single endosymbiotic event, whereas those in red and brown algae arose from at least one additional event. The chloroplast genome is smaller than that of a cyanobacterium; however, like that of a cyanobacterium, it is circular with a single start site for DNA replication, and its genes are arranged in operons sequences of functionally related genes under common control. In the course of evolution, many of the genes of the chloroplast ancestor were transferred to the plant cell's nucleus or, in some cases, lost entirely, thus establishing a fully dependent relation.



2.2: Diagram of a Chloroplast. [After S. L. Wolfe, *Biology of the Cell*, p. 130. © 1972 by Wadsworth Publishing Company, Inc. Adapted by permission of the publisher.]

2.1.5 Glyceroglycolipids (GL)

The glyceroglycolipids or glycolipids are formed when a 1,2-diacyl-*sn*-3-glycerol is linked via the *sn*-3 position to a carbohydrate molecule. The carbohydrate is usually a

mono- or a disaccharide, less commonly a tri- or tetrasaccharide. Galactose is the most common carbohydrate molecule in plant glyceroglycolipids. Structures and nomenclature for some glyceroglycolipids are shown in Figure 2.3 below. The names monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are used in common nomenclature. The standard nomenclature identifies the ring structure and bonding of the carbohydrate groups.

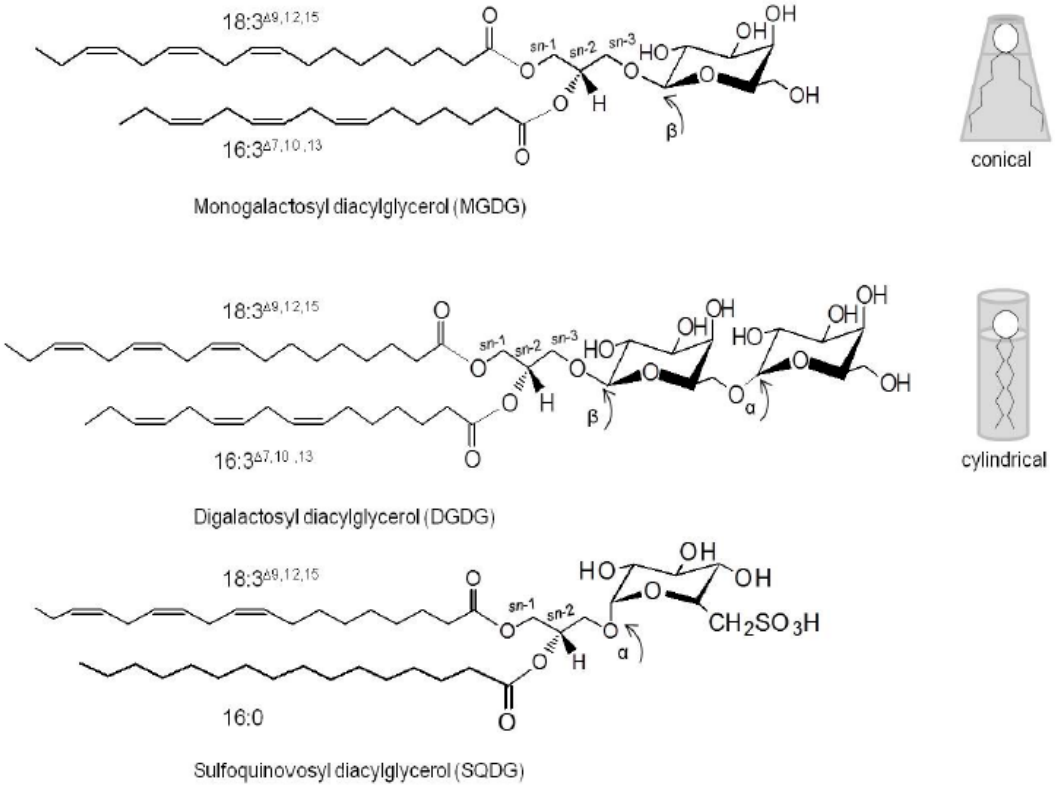


Figure 2.3: Main glycerolipid classes conserved in photosynthetic membranes of algae: MGDG, monogalactosyl diacylglycerol; DGDG, diagalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol; R1 and R2 represent fatty acyl chains. In the membrane structure of chloroplasts, MDGD tends to adapt a conical shape; DGDG and SQDG tend to adapt cylindrical shapes. Image and content adapted from de Costa et al., (2016)

2.2 Oxidation

Unsaturated fatty acids of phospholipids are susceptible to oxidation through both enzymatically controlled processes and random autoxidation processes. The mechanism of autoxidation is basically similar to the oxidative mechanism of fatty acids or esters in the bulk phase or in inert organic solvents. This mechanism is characterized by three main phases: initiation, propagation, and termination.

Initiation occurs as hydrogen is abstracted from an unsaturated fatty acid of a phospholipid, resulting in a lipid free radical. The lipid free radical in turn reacts with molecular oxygen to form a lipid peroxyl radical. While irradiation can directly abstract hydrogen from phospholipids, initiation is frequently attributed to reaction of the fatty acids with active oxygen species, such as the hydroxyl free radical and the protonated form of superoxide. These active oxygen species are produced when a metal ion, particularly iron, interacts with triplet oxygen, hydrogen peroxide, and superoxide anion.

On the other hand, enzymatic abstraction of hydrogen from an unsaturated fatty acid occurs when Fe^{3+}at the active site of lipoxygenase is reduced to Fe^{2+} While the majority of lipoxygenases require free fatty acids, there have been reports of lipoxygenase acting directly on fatty acids in phospholipids, (Kuhn, 1990; Hence, enzymatic hydrolysis may not always be required prior to lipoxygenase activity.

2.3 Global Halal Scenario

The food industry, like any other industry, responds to the needs and desires of the consumer. People all over the world are now more conscious about foods, health, and nutrition. They are interested in eating healthy foods that are low in calories, cholesterol, fat, and sodium. Many people are interested in foods that are organically produced without the use of synthetic pesticides and other non-natural chemicals. The ethnic and religious diversity in America and Europe has encouraged the food industry to prepare products which are suitable to different groups such as the Chinese, Japanese, Italian, Indian, Mexican, Seventh Day Adventist, vegetarian, Jewish, and Muslim.

Islam is the world's second largest religion, also the fastest growing, both globally and in the U.S. More than 7 million Muslims live in the U.S. (Cornell University, 2002), and the worldwide Muslim population is ca. 1.3 billion (Chaudry, 2002). The Muslim population is estimated to reach 12.2 million in 2018 in the U.S. (*USA Today*, 1999). Islam is not merely a religion of rituals — it is a way of life. Rules and manners govern the life of the individual Muslim. In Islam, eating is considered a matter of worship of God, like ritual prayers. Muslims follow the Islamic dietary code, and foods that meet that code are called halal (lawful or permitted). Muslims are supposed to make an effort to obtain halal food of good quality. It is their religious obligation to consume

only halal food. For non-Muslim consumers, halal foods often are perceived as specially selected and processed to achieve the highest standards of quality.

Between 300 and 400 million Muslims are estimated to live as minorities in different nations of the world, forming a part of many different cultures and societies. In spite of their geographic and ethnic diversity, all Muslims follow their beliefs and the religion of Islam. Halal is a very important and integral part of religious observance for all Muslims. Hence, halal constitutes a universal standard for a Muslim to live by.

By definition, halal foods are those that are free from any component that Muslims are prohibited from consuming. According to the Qur'an (the Muslim scripture), all good and clean foods are halal. Consequently, almost all foods of plant and animal origin are considered halal except those that have been specifically prohibited by the Quran and the Sunnah (the life, actions, and teachings of the Prophet Muhammad).

Until now, there has been no book available combining the religious and production issues that can guide food manufacturers in understanding halal food production, but the country of Malaysia is progressing through preparation of a holistic approach with focusing on the Standard Operational Procedure and series of educational sessions with experts. Producing halal food is similar to producing regular foods, except for certain basic requirements. Halal foods can be processed by using the same equipment and utensils as regular food, with a few exceptions or changes.

2.3.1 The Kosher Diet

Food is kosher when it meets dietary requirements outlined by Jewish law or kashrut, making it acceptable for people observing those laws to eat. The role of a kosher supervisor is to ensure the food is kosher and remains kosher after preparation or processing. A kosher symbol on a food product means that the product has been certified kosher from an agency. Kosher food is divided into three groups: meat, dairy and pareve (neither meat nor dairy). Only animals that chew their cud, have cloven hooves and are free from disease are considered kosher. These restrictions also apply to animal flesh, organs, milk and any by-products. Domesticated fowl are considered kosher. Seafood with fins and scales are also allowed. According to Jewish law, meat and dairy products cannot be combined or eaten at the same meal. Many people wait between three to six

hours after eating a meal containing meat to have dairy products. Pareve foods do not contain any meat or dairy, therefore they can be eaten with either one.

Examples of foods allowed:

a. Meat: Kosher beef, game, lamb, chicken, turkey, duck, goose and fish
b. Dairy: Products Milk, cheese, yogurt (from a kosher certified animal)
c. Parve Fruits, vegetables, eggs, fish*, cereal products, nuts, grains
d. **Fish cannot be placed on the same plate as meat – however, it can be consumed during the same meal.*

Examples of forbidden foods:

a. Pork, reptiles, amphibians and insects
b. Shellfish (including lobster, oysters, mussels), shrimp and scallops
c. Animal products or by-products made from any non-certified animal

2.3.2 The Halal Diet

Islamic dietary laws define which foods are halal. Halal foods are lawful and permitted to be eaten by those observing Islamic teachings. Muslims are not allowed to consume foods or beverages that are Haram, or forbidden. Foods that carry a halal symbol on their packaging have been approved by an agency and are certified to be free of any forbidden components or ingredients. Halal claims on the nutrition label or the packaging must include the name of the certification body.

Examples of foods Halal (allowed) and Haram (forbidden):

Cereal products Halal:

a. Cereal products not containing haram ingredients
b. Rice
c. Pasta

Cereal products Haram:

a. Cereal products containing haram ingredients (alcohol animal fats, vanilla extract)

Fruits and vegetables Halal:

a. All (frozen, canned, raw, boiled, butter, vegetable, oil)
b. Juice

Fruits and vegetables Haram*:*

a. Fruits and vegetables containing Haram ingredients (alcohol, animal fats, gelatine, bacon)

Milk and dairy Halal:

a. Milk
b. Yogurt, cheese, and ice creame made with bacterial culture without animal rennet

Milk and dairy Haram:

a. Cheese, yogurt and ice cream made with animal rennet, vanilla extract, gelatine, pepsin, or lipase

Meats and alternatives Halal:

a. Certified meat and poultry
b. Seafood
c. Nuts
d. Eggs
e. Peanut butter
f. Tofu
g. Halal deli meats
h. Legumes

Meats and alternatives Haram:

a. Pork and port products (ham, sausage, bacon)
b. Non certified meat and poultry
c. Any product prepared with alcohol or animal fats

It is often difficult to classify processed food as strictly halal or haram because of the ingredients they contain. Therefore, it is important to check the product's label or packaging to see if it is halal certified. If no certification is specified, verify the list of ingredients and look for haram or forbidden ingredients. Some examples include: gelatin, lipase, pepsin, alcohol, vanilla extract (pure or artificial), animal fats, animal blood, animal rennet, mono and diglycerides from an animal source, whey powder, sodium stearoyl lactylate (SSL) or L-cysteine. By having food items and products available in your establishment that are allowed according to these religious principles, you will be able to satisfy the needs of a larger group of clients or customers.

It is hope that this research could shed some insights and hope in order to develop a more comprehensive system and to introduce marine derived products as an alternative ingredients in halal food.

Chapter 3: Oxidative Stability of Glyceroglycolipids; GL (MGDG & DGDG) from Spinach and Akamoku as Compared to Linseed Oil (TAG)

3.1 Introduction

Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG) are namely the constituents which made up the total acyl lipids of plant leaves (Figure 3.1), as previously reported by Harwood (1980). In addition, Harwood & Jones (1989) and Thompson Jr. (1996), mentioned that these polar lipids remain to be the major constituents in seaweeds as well. Both MGDG and DGDG account for more than half of the total membrane lipids in plant leaves, (Yamaguchi *et al.*; 2012). Dörmann & Benning (2002), also supported that these galactosyl diacylglycerol hold specifically important role in photosynthetic membranes of higher plants, including in algae and bacteria.

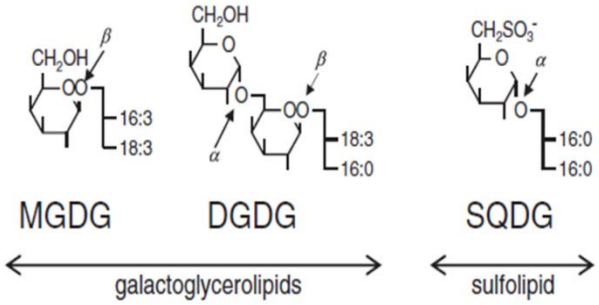


Figure 3.1: Main glyceroglycolipid (GL) classes conserved in photosynthetic membranes from cyanobacteria to primary chloroplasts of algae and plants. In this illustration of representative lipids from thylakoids of Arabidopsis chloroplasts, positions sn-1 and sn-2 of the glycerol backbone are esterified to fatty acids with 16 or 18 carbon atoms and position sn-3 harbors the polar head. Monogalactosyl diacylglycerol (MGDG); Digalactosyl diacylglycerol (DGDG); Sulfoquinovosyl diacylglycerol (SQDG); PG, phosphatidylglycerol. Image and content adapted from Boudière *et al.* (2013).

GL has also been characterized as having a uniquely high content of PUFAs especially α-linolenic acid (LN, 18:3n-3), (Yamaguchi *et al.* 2012). Harwood (1980) also included that this particular fatty acid is specially endowed with MGDG and DGDG. On the other hand, according to Harwood & Jones (1989) and Terasaki *et al.* (2009), majority seaweeds are composed of stearidonic acid (SA, 18:4n-3), arachidonic acid (AA, 20:4n-6) and eicosapentanoic acid (EPA, 20:5n-3). All these PUFAs are known to be easily oxidized as compared to the more stable unsaturated fatty acids namely; oleic acid (OA,

18:1n-9) and linoleic acid (LA, 18:2n-6) in studies mentioned by Miyashita & Takagi (1986), Cosgrove *et al.* (1987) and Cho *et al.* (1987) . Interestingly, GLs which are the main component in chloroplast, are continuously being disclosed to oxidative stress from the absorption of light energy during photosynthesis while harbouring these unusually high content of PUFA as highlighted by Yamaguchi *et al.* (2012).

With regard to the classical kinetic scheme by prominent researchers; Bateman & Gee (1951), Bateman *et al.* (1951 & 1953), Bateman (1954), Bolland (1946 & 1949), Bolland & Gee (1946a & 1946b), throughout the years with approval of contemporary knowledge as illustrated by Labuza (1971), it is generally accepted that autoxidation of PUFA proceeds in a chain reaction through three stages; (i) initiation, (ii) propagation and (iii) termination. However, complicated secondary phenomena including some details discovered through lipid oxidation studies, remains unsolved according to Chan (1987).

Free radical theory of autoxidation was defined by an attack of oxygen at the allylic position with the formation of unsaturated hydroperoxides. Benzie (1996), elaborated that these hydroperoxides would then have potential to decompose into peroxyl and alkoxyl radicals, which eventually advanced to secondary oxidation products which includes aldehydes, ketones, alcohols, acids and lactones (Figure 3.2). Impaired of taste, flavor and texture in foods are among the adverse effects of secondary oxidation products which could be potentially toxic compounds (Halliwell *et al.*, 1995; Frankel, 1998; Liu & Huang, 1995; Kubow, 1992 & 1993; Nawar, 1996). In other words, lipid oxidation remains to be one of the major concern that could shorten the shelf life of foods.

Plants through their natural control, possess an intrinsic antioxidant compounds as defense towards oxidation, as to mention a few; ascorbate, glutathione, phenolic compounds, tocopherols and carotenoids. Innate enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase are all capable of scavenging reactive oxygen species (ROS) and eventually protect them from oxidative stress, as reported by Meloni *et al.* (2003), Nunez *et al.* (2003) and Yasar *et al.* (2008).

Since there is only a handful literature reports regarding GL and its resistance towards oxidation, it is of extreme importance to acknowledge the role of PUFA in the form of GL to its characteristics response. Among the objectives of this study is also to establish more materials which exhibit higher oxidative stability and to understand ways

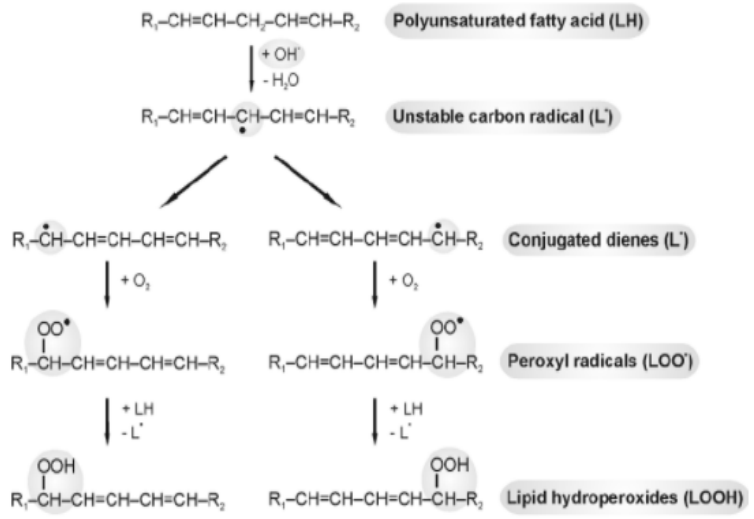


Figure 3.2: Basic reactions occurring during lipid peroxidation. Reactive species (e.g. hydroxyl radicals) abstract a hydrogen atom from a polyunsaturated fatty acid, yielding a lipid radical (L•) that may undergo some molecular rearrangements. Oxygen uptake by these radicals propagates the reaction via peroxyl radicals (LOO•), which leads to the formation of lipid hydroperoxides (LOOH). These can then combine and generate the final products of lipid peroxidation (e.g. MDA 4-HNE, acrolein, ethane/pentane, among others). Image and content adapted from Silva & Coutinho (2010).

on how to prevent lipid oxidation through inhibiting hydroperoxides from reacting with PUFA thus their cascade from transforming into other hazardous oxidation products. In the present study, we initially compared the oxidative stability of 5 types of lipids; Spinach MGDG, Spinach DGDG, Akamoku MGDG, Akamoku DGDG and Linseed TAG.

3.2 Materials and Methods

3.2.1 Sample

Spinach powder (GABAN Co. Ltd., Tokyo, Japan) was obtained from a local food market. Akamoku powder was obtained from Kohki Co., Tokyo, Japan. Linseed oil was obtain from Summit Oil Mill Co. Ltd., Chiba Japan.

3.2.2 Referenced Compound and Reagent

Silica gel (BW-60F) for the column chromatography was purchased from Fuji Sylysia Chem. Ltd. (Kasugai, Aichi, Japan). The activated Carbon and Celite (545 RVS) were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulphoquinovosyl diacylglycerol (SQDG) standards were purchased from Lipid Products (Redhill, United Kingdom) while Triolein was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

All other chemicals and solvents used in this study were of analytical grade and high-performance liquid chromatography (HPLC) grade solvents were used for HPLC analysis.

3.2.3 Sample Preparation

3.2.3.1 Extraction and Total Lipid Preparation of Spinach and Akamoku

The Spinach powder was extracted with six volumes (v/w) of methanol, followed by filtration afterwards in order to collect the filtrate. Initially, Spinach powder (*ca.* 2 kg) was soaked in methanol (12,000 mL) at room temperature, which was kept under the dark for overnight (about 16 hours). The green metanolic extract together with the residue was next filtered with a ceramic filter funnel, lined with filter paper (No. 2 Qualitative Filter Paper; 150 mm; Advantec®; Tokyo, Japan) and the green filtrate was removed from the residue under a vacuumed condition at 30 ^{o}C ± 1 ^{o}C.

The green filtrate was then pooled into a pilot scale rotary evaporator (Eyela Rotary Vacuum Evaporator N-11 and Eyela CA-2600; Tokyo Rikakikai Co. Ltd.; Tokyo, Japan) and traces of solvent remaining in the green filtrate was entirely removed in the dark, under vacuum, leaving the dark green viscous liquid obtained at the end. This dark green viscous liquid was collected with dissolving in equivalent volume of methanol which was finally designated as crude lipids of Spinach.

Crude lipids of Spinach was further dissolved into a separatory funnel using a cocktail mix solvents of chloroform-methanol-water (10:5:3, v/v/v) for a liquid-liquid distribution. After being shaken, the funnel was allowed to stand overnight. The lower layer; a mixed lipid layer with methanol and chloroform, was collected and again dissolved into a new separatory funnel. Same volume of water was again added and after being shaken, the funnel was further allowed to stand one more overnight separation (liquid-liquid distribution). The lower layer was then concentrated under vacuum, using a rotary evaporator. Remaining traces of organic solvents and water were removed in a desiccator (about 3 days) under a high vacuum with leaving the sample in an amber

coloured rotary flask. Total lipids of Spinach (*ca.* 20 g) was eventually collected with dissolving in equivalent volume of chloroform and subjected to consequent analysis or kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage).

The same overall procedure was done for Akamoku powder. The intensity of green colour including the total amount of the crude lipids and total lipids obtained, could also varies among each type of sample.

3.2.3.2 Lipid Class Analysis of Spinach and Akamoku

Total lipids of Spinach was subjected to lipid class profile by using preparative thin layer chromatography (TLC). The lipid fraction was dissolved in a cocktail mix solvents of chloroform-methanol-water (65:25:4, v/v/v) and spotted onto a 0.25 mm silica gel plate (Silica gel 60G; Merck, Darmstadt, Germany). The plate was developed with the same cocktail mix solvents of chloroform-methanol-water (65:25:4, v/v/v) and the spots were visualized by spraying the plate with orcinol-sulfuric acid or Dittmer reagent, followed by charring. The lipid sample was also analysed by preparative TLC with the same specification of silica gel plate mentioned previously but with using *n*-hexane-diethyl ether-acetic acid (80:20:1, v/v/v) as the developing solvent. The spots were detected using 60 % aqueous sulfuric acid charring.

The chromatogram was photographed with a digital camera and the image of the silica gel plate was acquired and transferred to the computer. The image was properly cropped and saved in bitmap format. The percentage ratio of each lipid fraction (as compared to standards) in the sample was expressed as the bitmap percentage of the total bitmap intensities.

The same overall procedure was done for total lipids of Akamoku.

3.2.3.3 Purification of Glyceroglycolipids from Spinach and Akamoku

Total lipids of Spinach which was collected with dissolving in equivalent volume of chloroform (*ca.* 20g) was first passed through a column (70 × 6 cm i.d.) packed with chloroform slurry mixture of silica gel. The whole column was wrapped with aluminium foil in order to protect the glyceroglycolipid (GL) from degradation with the exposure to light. The elution was first done with chloroform (about 3,000 mL) and then with acetone (about 13,000 L). Fractions eluted with acetone were used as GL (through a continuous

elution, with appropriately adjusting the flow rate manually, for about more than a week). The first whole dark green-black fraction was designated as MGDG of Spinach (1 fraction) and the consequent fractions of every 500 mL collected with acetone (clear light green solution) were designated as DGDG of Spinach (about 24 fractions). Fractions were concentrated under vacuum, using a rotary evaporator and was collected and kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage) or till further analysis.

Both MGDG and DGDG of Spinach were subjected to preparative TLC in order to choose the exact fraction with the desired GL. All GL fractions which was collected in ethanol were spotted onto a 0.25 mm silica gel plate. The plate was developed with a cocktail mix solvents of chloroform-methanol-water (65:25:4, v/v/v) and the spots were visualized by spraying the plate with orcinol-sulfuric acid or Dittmer reagent, followed by charring (as compared to standards; MGDG, DGDG & SQDG).

The absence of chlorophyll was also confirmed in all consequent fractions containing DGDG from prior TLC. Therefore, only the first whole fraction obtained; MGDG of Spinach (*ca.* 4 g) was again refined using a carbon column (70 × 6 cm i.d.) packed with 90 % of ethanol in chloroform slurry of activated carbon (*ca.* 250 g) in order to remove majority traces of chlorophyll. Carbon was first heated at 110 ^{o}C for 1 ½ hours prior to mixing with 90 % of ethanol in chloroform. Elutions were done with gradually decreasing concentration of ethanol; 90 % of ethanol in chloroform (about 1,000 mL), then 50 % ethanol in chloroform (about 1,500 mL) and finally 100 % of chloroform (about 10,000 mL). The fractions eluted with 50 % ethanol in chloroform and 100 % of chloroform were both used as MGDG of Spinach (clear light yellow solution) after consequent fractions of every 500 mL. Fractions were concentrated under vacuum, using a rotary evaporator and was collected and subjected to further analysis or kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage).

MGDG of Spinach were again subjected to preparative TLC. All MGDG fractions which was collected in ethanol were spotted, developed and visualized using the same procedure as described earlier, except that only MGDG was used as standard.

The same overall procedure was done for GL of Akamoku (MGDG & DGDG). The intensity of clear green (DGDG) and clear yellow (MGDG after the removal of

chlorophyll) colour of the solution, including the total amount of MGDG and DGDG collected could also vary among each type of sample.

3.2.3.4 Purification of TAG from Linseed Oil

Linseed oil undergone a direct double purification process with 2 types of system. In order to remove tocopherols and pigments, linseed oil (*ca.* 25 g) was first passed through a column (50 × 4 cm i.d.) packed with *n*-hexane slurry mixture of activated carbon (100 g) and Celite (100 g). The carbon was first heated at 110 ^{o}C for 1 ½ hours prior to mixing with Celite and *n*-hexane. The only elution was done with *n*-hexane (1,200 mL).

The obtained oil (*ca.* 10 g) was again refined using a silicic acid column (50 × 4 cm i.d.) packed with *n*-hexane slurry of silica gel (200 g). Elutions were done with *n*-hexane (200 mL), after that a mixture of *n*-hexane-diethyl ether (98:2, v/v) of 200 mL and again another mixture of *n*-hexane-diethyl ether (90:10, v/v) of 1,200 mL. The final fraction eluted with *n*-hexane-diethyl ether (90:10) was used as TAG.

In order to confirm the absence of impurities, the TAG fraction was subjected to preparative TLC. The lipid fraction was spotted onto a 0.25 mm silica gel plate. The plate was again developed with a cocktail mix solvents of *n*-hexane-diethyl ether (60:40, v/v) and the spot were detected with iodine vapour or 60% aqueous sulfuric acid charring (as compared to standard; Triolein).

3.2.3.5 Tocopherol Analysis of Spinach, Akamoku and Linseed TAG

Tocopherol analysis was performed for all 5 types of purified lipids; Spinach (MGDG & DGDG), Akamoku (MGDG & DGDG) including Linseed (TAG) with a Hitachi HPLC system equipped with a pump (Hitachi L-2130) and a fluorescence detector (Hitachi L-2485). The analysis was conducted on a silica column (Si 60, 250 × 4.6 mm i.d.; Kanto Chemical Co., Tokyo, Japan) protected with a guard column (15 × 3.2 mm) with the same stationary phase. The mobile phase was n-hexane-2-propanol (99.2:0.8, v/v) with a flow rate of 1.0 mL/min. The fluorescence detector was set at Ex. 298 nm and Em. 325 nm.

3.2.3.6 Fatty Acid Composition of Spinach, Akamoku and Linseed TAG

The fatty acids compositions of all 5 purified lipids; Spinach (MGDG & DGDG), Akamoku (MGDG & DGDG) including Linseed TAG was determined by gas chromatography (GC) after conversion of fatty acyl groups in the lipid to their methyl esters by the method Prevot and Modret (1976), with slight modification. Briefly, to an aliquot of total lipid (*ca.* 20 mg for GLs and 10 mg for TAG), 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol solution were added, vortexed (10 seconds) and incubated at 50 ^{o}C for 30 seconds. 0.2 mL of 2 N HCL in methanol solution was then added to the solution and again vortexed (1 minute). The mixture was separated by centrifugation at 1,000 x g for 5 minutes. The upper hexane layer containing fatty acid methyl esters was recovered and subjected to GC. The GC analysis was performed on a Shimadzu GC-2014 (Shimadzu Corporation, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (Supelco ™ Column; Omegawax-320; 30 m × 0.32 mm i.d.; Sigma-Aldrich, St. Louis, MO, USA). The detector, injector, and column temperatures were 260, 250, and 200 °C, respectively. The carrier gas was helium at a flow rate of 50 kPa. The fatty acid content was expressed as a weight percentage of total fatty acids.

Number of bisallylic position per molecule of lipids was calculated from the molar concentration of each PUFA and the mean molecular weight (MW) of each lipid. The molar concentration of PUFA was obtained on the basis of the weight percentage (%) of PUFA and the MW of each PUFA. The mean MW of Spinach (MGDG & DGDG), Akamoku (MGDG & DGDG) and Linseed TAG was calculated from the mean MW of all the fatty acyl moieties and the MW of galactosyl and glycerol moiety. The mean MW of all the fatty acyl moieties were calculated from the mol (%) of each fatty acid and the MW. In the case of GL, upon calculating the molar distribution of MGDG and DGDG from the weight percentage of each GL class, the mean MW of GL was calculated.

3.2.3.7 Oxidation Analysis of Purified Lipids

Each 300 mg of 5 types of all purified lipids were prepared as follows:

1) Sp.MGDG
2) Sp.DGDG
3) Aka.MGDG

4) Aka.DGDG
5) Lin.TAG

All samples were placed in a 20 mL aluminium sealed vial with a butyl gum septum (GL Science; Tokyo, Japan) and then incubated at 50 °C in the dark. Before the incubation, the level of oxygen in the headspace gas of the vial was estimated using a GC system (Shimadzu GC-14B) equipped with a thermal conductivity detector and a stainless steel column (3 m × 3.0 mm i.d.) packed with a molecular sieve 5A (GL Science) according to the method described by Cho *et al.* (1987). The temperatures at the injection port, detector port, and column oven were 120, 120, and 70 °C, respectively. The helium flow was 50 kPa. Three separate vials containing similar samples were prepared and incubated. A small portion (20 μL) of the headspace gas was taken from each vial using a microsyringe through the butyl gum septum at selected times during the oxidation. The decrease (%) in the oxygen was calculated from the changes in the oxygen to nitrogen ratio compared with the ratio before incubation. Three replicate measurements of each data and value at different oxidation times of the stored samples were expressed as the mean ± SD (n = 3).

After oxidation, polyunsaturated fatty acid contents were again analysed by GC. The preparation of fatty acid methyl esters and GC analysis was performed as described earlier. As for the statistical analysis, difference of before and after oxidation were statistically determined using one-way analysis of variance (ANOVA) by the SPSS IBM 20. Values of $p < 0.05$ were considered significant.

3.3 Results and Discussion

3.3.1 Lipid Class Analysis

Table 3.1: *Composition of GL from Spinach and Akamoku.*

GL Class	Spinach	Akamoku
MGDG	50	62
DGDG	43	8
SQDG	7	30

Values shown in Table 3.1 are represented as the mean of three independent experiments.
GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol); SQDG (Sulphoquinovosyl diacylglycerol).

When each lipid composition of both Spinach and Akamoku were roughly analysed based on the spot intensities of TLC, the overall distribution of their main lipid classes were documented as shown in Table 3.1. Only MGDG and DGDG from both samples; Spinach and Akamoku, were used in this study while SQDG was opted out due to the generally inferior amount in Spinach; which is not the intended focus of this research.

3.3.2 Purification of GL and Linseed TAG

Purified Spinach (MGDG & DGDG) and Akamoku (MGDG & DGDG) showed spots corresponding to MGDG and DGDG standards on the TLC (Figure 3.3, 3.4 and 3.5). In addition to this, visible spot in small streaks of chlorophyll was also observed during TLC analysis with Spinach MGDG and Akamoku MGDG while both Spinach DGDG and Akamoku DGDG were readily free from spot containing small streaks of chlorophyll after the first purification. However, after both MGDGs were again subjected to second purification with carbon column, only a single spot corresponded to MGDG standard was detected on the TLC for each type of sample.

Purified Linseed TAG on the other hand, showed only a single spot corresponding to lipid standard Triolein on analytical TLC as in Figure 3.6. No other impurities such as free fatty acids, monoacylglycerol or diacylglycerol were detected.

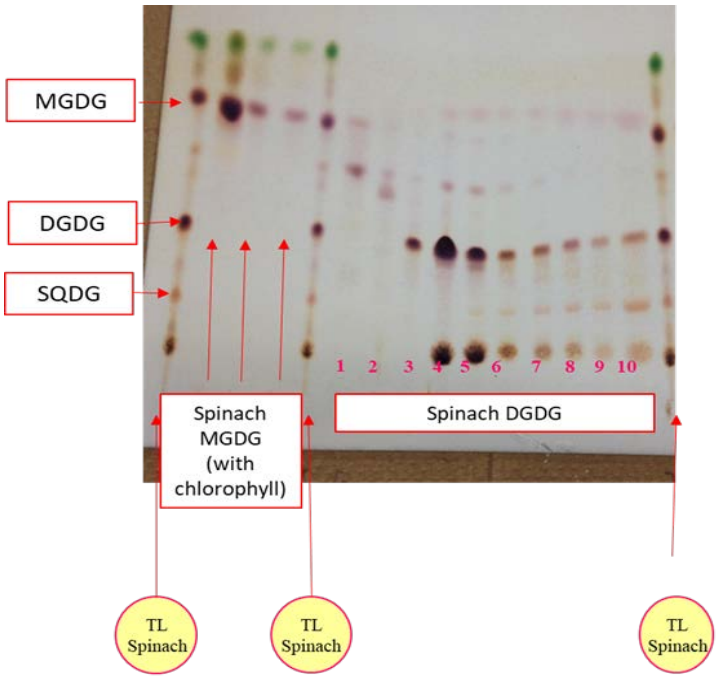


Figure 3.3: TLC plate after the purification of MGDG and DGDG from Spinach.
GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol).

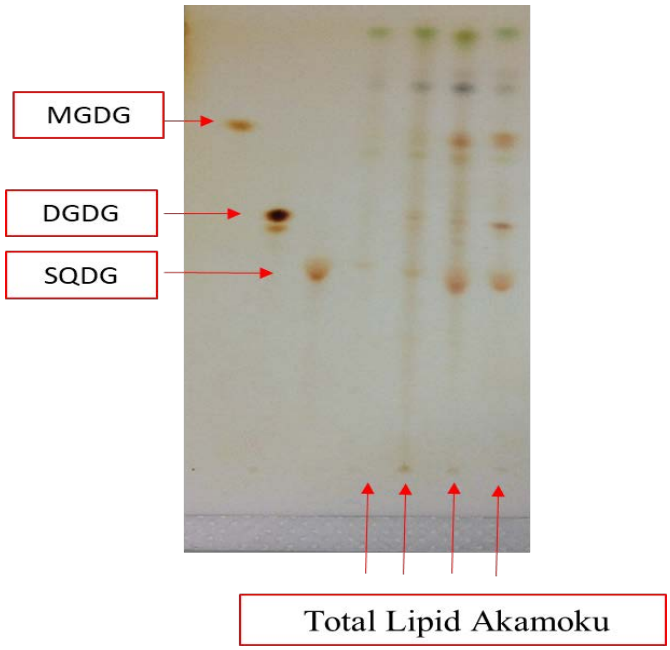


Figure 3.4: TLC plate after the first purification of MGDG and DGDG from Akamoku. GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol).

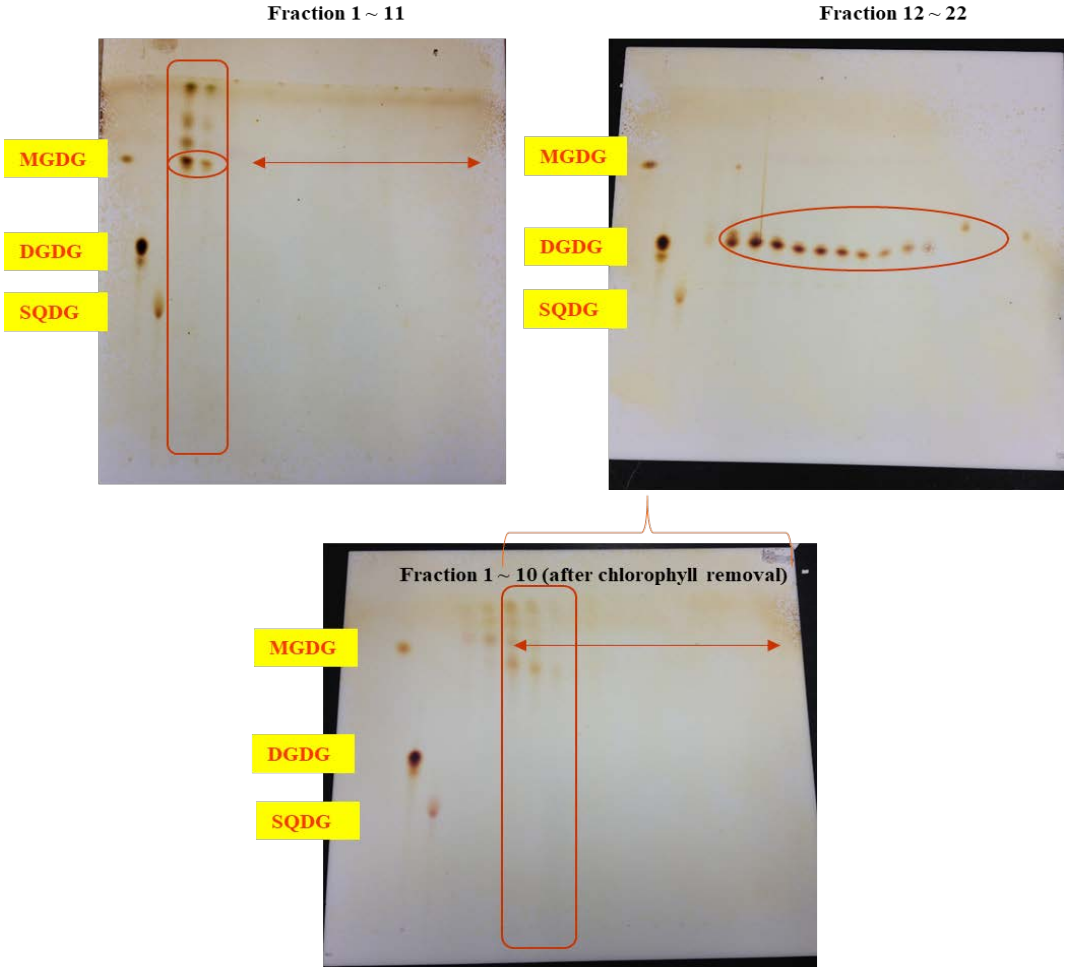


Figure 3.5: TLC plate after second purification of MGDG and DGDG from Akamoku. GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol).

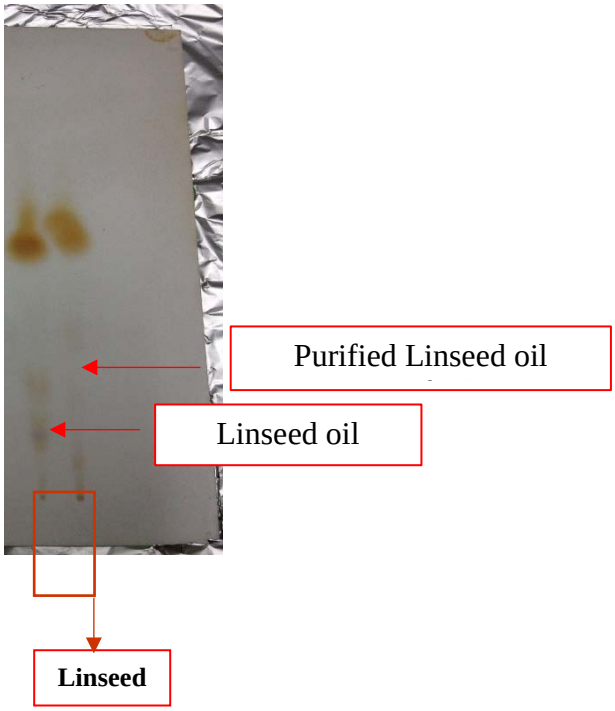


Figure 3.6: TLC plate after the first purification of Linseed oil TAG.
TAG (Triacylglycerol)

3.3.3 Tocopherol analysis

HPLC analysis either showed complete removal of tocopherols for almost all types of lipids or only minute amount of this antioxidant compound remaining for another few. Complete removal of this was especially confirmed after the double purification for all GL samples. Minute and negligible amount of tocopherol however, was detected for samples with Linseed TAG as reported by Shimajiri *et al.* (2013) which do not give rise for any contradiction in the comparisons of the oxidative stability result.

3.3.4 Fatty Acids Composition

The overall fatty acid compositions of purified GLs and Linseed TAG (prior to and after oxidation) used in this study are shown in Table 3.2 and 3.3. The main PUFA of Linseed TAG and both Spinach MGDG & DGDG prior to oxidation was α-linolenic acid (LN; 18:3n-3) which showed 43.47 %, 48.96 % and 77.53 % in weight percentage respectively. Those of Akamoku MGDG & DGDG, contained palmitic acid (PA; 16:0) as their main PUFA which made up to 35.92 % and 24.09 % in the overall weight percentage. A unique characteristic of Spinach GL was shown by the presence of

a n-3 PUFA; 16:3n-3 which accounted to 11.56 % and 2.91 % for Spinach MGDG and DGDG in weight percentage, as compared to the absence of this in all other purified lipid samples used in this study.

Upon prior to oxidation, Spinach; terrestrial lipid was in general, composed of four major fatty acids namely, LN (18:3n-3), 16:3n-3, PA (16:0) and LA (18:2n-6). Content of LN was recorded to be more in Spinach DGDG; 77.53 % as compared to its MGDG constituent; 48.96 %. On the other hand, Akamoku; marine seaweed lipid was especially abundant in five major fatty acids namely, PA (16:0), OA (18:1n-9), AA (20:4n-6), SDA (18:4n-3) and EPA. Content of PA was recorded to be more in Akamoku MGDG; 35.92 % as compared to its DGDG constituent; 24.09 %. Furthermore, Linseed TAG as terrestrial lipid was also composed mainly with LN (18:3n-3), OA (18:1n-9) and LA (18:2n-6). Content of LN was recorded to be at 43.47 %.

The content of LN for Spinach MGDG showed 5.49 % more while its DGDG showed 34.06 % in weight percentage more over the content of LN recorded by Linseed TAG, prior to oxidation. However, after the oxidation, LN amount of Linseed TAG was vastly reduced to only 1.75 % in weight percentage, making it almost 96 % loss in content ratio, from the mentioned content of fatty acid composition. Spinach MGDG showed a reduction of nearly half; about 47 % loss while its DGDG constituent showed only a slight reduction of about 3 % loss in content ratio from the amount of LN, as compared to prior and after oxidation process.

All samples recorded a significant reduction in comparison for the total content of PUFA; before and after oxidation. The differences were able to be calculated from Table 3.2 and 3.3. Spinach MGDG had a difference of 36.92 % while Spinach DGDG had a difference of only 4.96 % in weight percentage. Akamoku MGDG had a difference of 1.13 % and Akamoku DGDG had not much difference of 2.42 % in weight percentage. Linseed TAG showed the most significant difference of 55.50 % in weight percentage. In general, total loss in content ratio with respect to the difference calculated in weight percentage were about 51 % (Spinach MGDG), 6 % (Spinach DGDG), 10 % (Akamoku MGDG), 5 % (Akamoku DGDG) and 92 % (Linseed TAG). The maximal loss in content ratio was shown by Linseed TAG, followed by Spinach MGDG, Akamoku MGDG, then Spinach DGDG and finally Akamoku DGDG. Both DGDG from Spinach and Akamoku showed the least reduction in content ratio as compared to their MGDGs constituent.

As highlighted by Miyashita (2008), the reaction mechanisms involved in lipid oxidation; a 3 general step, which proceeds through initiation, propagation and termination, is usually very complex. Abstraction of hydrogen radical ($H^{\cdot}$) from substrate lipids (LH) to give rise to lipid-free radicals ($L^{\cdot}$) is regarded to be the crucial rate-limiting step. This hydrogen abstraction which if to occur at the bis-allylic positions present in PUFA ($CH=CH-CH_2-CH=CH$), would increase the susceptibility of this particular PUFA to oxidation. A higher susceptibility of PUFA to oxidation would mean a higher availability of bis-allylic hydrogens. Oxidative stability of PUFA as a whole is inversely related to the number of bis-allylic positions in the molecule. Several previous studies as highlighted by Cho *et al.*, (1987), Cosgrove *et al.*, (1987), Gunstone *et al.*, (1945), Holman *et al*, (1947), Miyashita & Takagi (1986) and Miyashita *et al.*, (1990), has confirmed that when the oxidative stability of PUFA decreased, the degree of unsaturation would increase.

As shown in Table 3.2 and 3.3, total content of PUFA in Linseed TAG was initially (prior to oxidation) very high as compared to both Akamoku MGDG and Akamoku DGDG. After the immediate total 7 days of oxidation, the total content of PUFA dropped significantly to 92 % since the initial content while in GL the reduction of amount was small; about 9 % (Akamoku MGDG) and about only 5 % (Akamoku DGDG).

Referring to Table 3.2 and 3.3, total content of PUFA in both terrestrial Spinach MGDG and DGDG were initially (prior to oxidation) in oppose to both GLs from Akamoku; are very high as compared to Linseed TAG. After the consecutive 7 days of oxidation altogether, the total content of PUFA in the former mentioned dropped to about 51 % while the latter mentioned reduced about only 6 % since the initial content. Even with this reduction, the loss in content was incomparable with the loss by Linseed TAG as the percentage remained to be higher; in fact, higher than that showed by Linseed TAG (prior to oxidation; Table 3.3).

Table 3.2 also showed GL of both Spinach MGDG and DGDG, contained considerable percentage of 16:3n-3 with especially Spinach MGDG showed a higher percentage of this fatty acid than the DGDG constituent. Both GLs from Spinach possess

Table 3.2: *Fatty acids composition (prior to and after oxidation) of GL from Spinach and Akamoku for both MGDG and DGDG.*

Fatty acid	Before oxidation			After Oxidation			Before oxidation			After oxidation			Before oxidation			After oxidation			Before oxidation			After oxidation		
	Spinach MGDG						Spinach DGDG						Akamoku MGDG						Akamoku DGDG					
	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD
6:0 / 8:0	0.00	±	0.01	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.03	±	0.06	ND	±	ND
10:0	0.01	±	0.02	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.02	±	0.02	ND	±	ND
11:0	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.00	±	0.01	ND	±	ND
12:0	0.08^{c}	±	0.02	0.30^{c}	±	0.04	0.00^{a}	±	0.01	0.01^{a}	±	0.01	0.06^{a}	±	0.00	0.04^{a}	±	0.01	0.03^{a}	±	0.00	0.02^{a}	±	0.02
13:0	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.01^{b}	±	0.01	ND	±	ND	ND	±	ND	ND	±	ND
14:0	0.28^{d}	±	0.00	1.00^{d}	±	0.09	0.06^{b}	±	0.00	0.08^{b}	±	0.00	10.23^{c}	±	0.21	11.07^{c}	±	0.27	5.56^{b}	±	0.10	3.91^{b}	±	3.38
14:1	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.28^{d}	±	0.03	0.29^{d}	±	0.07	0.12^{c}	±	0.04	0.10^{c}	±	0.11
15:0	0.08	±	0.00	ND	±	ND	0.05^{c}	±	0.01	0.07^{c}	±	0.00	0.50^{e}	±	0.02	0.59^{e}	±	0.02	0.29^{d}	±	0.01	0.21^{d}	±	0.18
15:1	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND
16:0	5.25^{e}	±	0.10	18.82^{e}	±	1.12	11.41^{d}	±	0.07	12.25^{d}	±	0.09	35.92^{f}	±	0.3538	35.88^{f}	±	0.73	24.09^{e}	±	0.30	15.60^{e}	±	13.51
16:1	0.26^{f}	±	0.03	0.84^{f}	±	0.05	0.16^{e}	±	0.03	0.17^{e}	±	0.02	8.90^{g}	±	0.0777	9.06^{g}	±	0.14	5.15^{f}	±	0.06	3.57^{f}	±	3.09
16:3n-3	11.56^{g}	±	0.22	6.12^{g}	±	0.63	2.91^{f}	±	0.07	3.35^{f}	±	0.04	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND
17:0	0.07^{h}	±	0.00	0.14^{h}	±	0.02	0.10^{g}	±	0.01	0.12^{g}	±	0.00	0.09^{h}	±	0.01	0.07^{h}	±	0.01	0.04^{g}	±	0.00	0.02^{g}	±	0.02
17:1	ND	±	ND	ND	±	ND	0.01^{h}	±	0.01	0.01^{h}	±	0.00	0.19^{i}	±	0.01	0.22^{i}	±	0.01	0.16^{h}	±	0.01	0.11^{h}	±	0.10
18:0	0.40^{i}	±	0.01	1.26^{i}	±	0.10	0.70^{i}	±	0.03	0.76^{i}	±	0.01	0.89^{j}	±	0.01	0.93^{j}	±	0.01	0.33^{i}	±	0.01	0.24^{i}	±	0.21
18:1n-9	1.47^{j}	±	0.07	3.87^{j}	±	0.23	0.86^{j}	±	0.04	0.97^{j}	±	0.01	14.8^{k}	±	0.13	14.58^{k}	±	0.13	7.26^{j}	±	0.04	4.83^{j}	±	4.19
18:1n-7	0.56^{k}	±	0.04	2.09^{k}	±	0.07	0.94^{k}	±	0.03	1.05^{k}	±	0.01	0.09^{l}	±	0.01	0.09^{l}	±	0.01	0.07^{k}	±	0.00	0.06^{k}	±	0.06
18:2n-6	3.60^{l}	±	0.09	4.07^{l}	±	0.10	3.60^{l}	±	0.07	3.83^{l}	±	0.02	5.88^{m}	±	0.03	5.36^{m}	±	0.06	6.43^{l}	±	0.02	4.22^{l}	±	3.66
18:3n-6	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.25^{n}	±	0.01	0.23^{n}	±	0.01	0.72^{m}	±	0.01	0.48^{m}	±	0.41

18:3n-3	48.96^{m}	±	1.37	26.08^{m}	±	2.31	77.53^{m}	±	0.14	75.28^{m}	±	0.12	1.13^{o}	±	0.02	1.02^{o}	±	0.02	5.47^{n}	±	0.02	3.57^{n}	±	3.09
18:4n-3	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.67^{p}	±	0.01	0.56^{p}	±	0.02	11.29^{o}	±	0.05	7.09^{o}	±	6.14
20:0	0.35^{n}	±	0.24	0.45^{n}	±	0.05	0.02^{n}	±	0.00	0.02^{n}	±	0.00	0.11^{q}	±	0.02	0.15^{q}	±	0.01	0.05^{p}	±	0.00	0.05^{p}	±	0.04
20:1n-9	ND	±	ND	ND	±	ND	0.01^{o}	±	0.01	0.01^{o}	±	0.01	1.83^{r}	±	0.03	1.86^{r}	±	0.04	1.77^{q}	±	0.03	1.17^{q}	±	1.02
20:2n-6	1.39^{o}	±	0.03	0.81^{o}	±	0.08	0.01^{p}	±	0.01	0.03^{p}	±	0.01	0.09^{s}	±	0.01	0.07^{s}	±	0.01	0.14^{r}	±	0.01	0.10^{r}	±	0.09
20:3n-6 / 21:0	0.06^{p}	±	0.01	0.15^{p}	±	0.03	ND	±	ND	ND	±	ND	0.24^{t}	±	0.01	0.18^{t}	±	0.02	0.52^{s}	±	0.01	0.34^{s}	±	0.30
20:4n-6	0.13^{q}	±	0.01	0.07^{q}	±	0.01	ND	±	ND	ND	±	ND	3.04^{u}	±	0.06	2.61^{u}	±	0.16	12.15^{t}	±	0.11	7.53^{t}	±	6.52
20:3n-3	0.11^{r}	±	0.01	0.23^{r}	±	0.05	0.20^{q}	±	0.01	0.21^{q}	±	0.01	0.03^{v}	±	0.03	0.05^{v}	±	0.01	0.11^{u}	±	0.01	0.07^{u}	±	0.06
20:5n-3 (EPA)	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.46^{w}	±	0.08	0.42^{w}	±	0.03	9.64^{v}	±	0.15	6.13^{v}	±	5.32
22:0	0.86^{s}	±	0.03	0.89^{s}	±	0.05	0.03^{r}	±	0.02	0.03^{r}	±	0.02	1.35^{x}	±	0.04	1.18^{x}	±	0.06	0.10^{w}	±	0.01	0.06^{w}	±	0.05
22:1n-9	4.24^{t}	±	0.09	0.88^{t}	±	1.52	ND	±	ND	ND	±	ND	0.16^{y}	±	0.14	0.33^{y}	±	0.07	ND	±	ND	ND	±	ND
22:2	5.91^{u}	±	0.13	3.48^{u}	±	0.35	0.05^{s}	±	0.08	0.01^{s}	±	0.01	0.13^{z}	±	0.15	0.28^{z}	±	0.01	ND	±	ND	ND	±	ND
23:0	1.82^{v}	±	3.16	0.31^{v}	±	0.06	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.09^{x}	±	0.16	0.01^{x}	±	0.02
22:5n-3 (DPA)	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND
24:0	0.40^{w}	±	0.02	0.91^{w}	±	0.09	0.14^{t}	±	0.01	0.08^{t}	±	0.07	0.06^{A}	±	0.0603	0.06^{A}	±	0.05	0.05	±	0.05	ND	±	ND
22:6n-3 (DHA)	0.12^{x}	±	0.10	0.01^{x}	±	0.02	0.17^{u}	±	0.23	0.04^{u}	±	0.06	ND	±	ND	ND	±	ND	0.29^{y}	±	0.00	0.00^{y}	±	0.01
24:1n-9	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	ND	±	ND	0.01	±	0.01
Identified	87.97	±	2.99	73.18	±	1.80	98.98	±	0.21	98.51	±	0.17	87.39	±	0.38	87.29	±	0.89	91.92	±	0.18	89.34	±	0.11
Unidentified	12.03	±	2.99	26.82	±	1.80	1.02	±	0.21	1.49	±	0.17	12.61	±	0.38	12.71	±	0.89	8.08	±	0.18	10.66	±	0.11
Total	100.00	±	0.00	100.00	±	0.00	100.00	±	0.00	100.00	±	0.00	100.00	±	0.00	100.00	±	0.00	100.00	±	0.00	100.00	±	0.00
∑ PUFA	71.83	±	1.90	34.91	±	2.74	84.47	±	0.19	79.51	±	0.18	11.92	±	0.10	10.79	±	0.20	46.75	±	0.21	44.33	±	0.46
∑ MUFA	6.54	±	0.23	7.79	±	1.62	1.99	±	0.04	2.22	±	0.03	26.26	±	0.21	26.52	±	0.15	14.52	±	0.09	14.86	±	0.19
∑ SFA	9.60	±	2.95	24.36	±	1.78	12.52	±	0.07	13.44	±	0.05	49.21	±	0.45	49.97	±	0.97	30.65	±	0.21	30.16	±	0.16

∑ w-3	49.19	±	1.46	26.33	±	2.35	77.90	±	0.18	75.64	±	0.16	2.30	±	0.13	2.05	±	0.04	26.80	±	0.11	25.31	±	0.49
∑ w-6	5.18	±	0.12	5.10	±	0.06	3.62	±	0.07	3.86	±	0.01	9.49	±	0.12	8.45	±	0.17	19.95	±	0.10	19.01	±	0.03
∑ w-9	5.71	±	0.15	4.78	±	1.62	0.87	±	0.04	0.99	±	0.01	16.79	±	0.28	16.82	±	0.05	9.03	±	0.03	9.06	±	0.15
Ratio w-3/w-6	9.49	±	0.09	5.16	±	0.43	21.54	±	0.42	19.60	±	0.02	0.24	±	0.01	0.24	±	0.00	1.34	±	0.00	1.33	±	0.03
Ratio SFA/PUFAs	0.13	±	0.04	0.70	±	0.10	0.15	±	0.00	0.17	±	0.00	4.13	±	0.07	4.63	±	0.17	0.66	±	0.01	0.68	±	0.01
Ratio PUFAs/SFA	7.91	±	2.13	1.44	±	0.23	6.75	±	0.05	5.92	±	0.03	0.24	±	0.00	0.22	±	0.01	1.53	±	0.02	1.47	±	0.02

Presented data are mean value of three replications ± standard deviation (n=3 ± s.d.)
Mean values in the same row with different superscript letters are differ significantly from each other ($p < 0.05$). ND= Not Detected

MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol)

Table 3.3: *Fatty acid composition of linseed TAG.*

Fatty acid	Before oxidation			After Oxidation		
	Linseed TAG					
	Mean		SD	Mean		SD
6:0 / 8:0	ND	±	ND	ND	±	ND
10:0	ND	±	ND	ND	±	ND
11:0	ND	±	ND	ND	±	ND
12:0	ND	±	ND	ND	±	ND
13:0	ND	±	ND	ND	±	ND
14:0	0.04[a]	±	0.01	3.08[a]	±	5.05
14:1	ND	±	ND	ND	±	ND
15:0	0.02	±	0.00	ND	±	ND
15:1	ND	±	ND	ND	±	ND
16:0	5.38[c]	±	0.06	18.46[c]	±	4.22
16:1	0.07[d]	±	0.00	0.16[d]	±	0.06
16:3n-3	ND	±	ND	ND	±	ND
17:0	0.05[e]	±	0.00	0.18[e]	±	0.04
17:1	0.04	±	0.01	ND	±	ND
18:0	3.62[f]	±	0.02	11.55[f]	±	2.46
18:1n-9	26.18[g]	±	0.06	40.61[g]	±	10.34
18:1n-7	0.85[h]	±	0.03	1.28[h]	±	0.33
18:2n-6	16.78[i]	±	0.04	3.03[i]	±	2.57
18:3n-6	ND	±	ND	ND	±	ND

18:3n-3	43.47[j]	±	0.03	1.75[j]	±	1.75
18:4n-3	ND	±	ND	ND	±	ND
20:0	0.16	±	0.01	ND	±	ND
20:1n-9	0.19[k]	±	0.01	0.43[k]	±	0.10
20:2n-6	0.02[l]	±	0.00	0.01[l]	±	0.00
20:3n-6 / 21:0	ND	±	ND	ND	±	ND
20:4n-6	ND	±	ND	ND	±	ND
20:3n-3	0.04[m]	±	0.01	0.00[m]	±	0.01
20:5n-3 (EPA)	ND	±	ND	ND	±	ND
22:0	0.14	±	0.01	ND	±	ND
22:1n-9	0.01[n]	±	0.01	0.16[n]	±	0.04
22:2	ND	±	ND	ND	±	ND
23:0	ND	±	ND	ND	±	ND
22:5n-3 (DPA)	ND	±	ND	ND	±	ND
24:0	0.09	±	0.01	ND	±	ND
22:6n-3 (DHA)	ND	±	ND	ND	±	ND
24:1n-9	ND	±	ND	ND	±	ND
Identified	97.16	±	0.06	82.43	±	9.34
Unidentified	2.84	±	0.06	17.57	±	9.34
Total	100.00	±	0.00	100.00	±	0.00
∑ PUFA	60.32	±	0.04	4.82	±	4.31
∑ MUFA	27.35	±	0.05	44.14	±	9.99
∑ SFA	9.49	±	0.04	33.48	±	5.73

∑ w-3	43.51	±	0.03	1.76	±	1.75
∑ w-6	16.80	±	0.04	3.05	±	2.56
∑ w-9	26.38	±	0.06	42.69	±	9.61
Ratio w-3/w-6	2.59	±	0.01	0.51	±	0.13
Ratio SFA/PUFAs	0.16	±	0.00	13.38	±	12.39
Ratio PUFAs/SFA	6.35	±	0.03	0.16	±	0.17

Presented data are mean value of three replications ± standard deviation (n=3 ± s.d.)
Mean values in the same row with different superscript letters are differ significantly from each other ($p < 0.05$). ND= Not Detected

(TAG) Triacylglycerol.

a higher mean number of bis-allylic position per molecule of lipid than did Linseed TAG (Table 3.4). Spinach MGDG had a difference of 0.45, higher as compared to Linseed TAG and also 1.35 higher as compared to Akamoku MGDG. On the other hand, Spinach DGDG, was recorded to be 0.62, higher than Linseed TAG and also 0.42 higher as compared to Akamoku DGDG.

3.3.5 Number of Bis-allylic Position

Table 3.4: *Average number of bis-allylic positions of lipids used in this study.*

Total number of bisalyllic positions	Spinach		Akamoku		TAG
	MGDG	DGDG	MGDG	DGDG	
Per molecule	1.54	1.71	0.19	1.29	1.09
Per g lipid (x6.02 × 10^{20})	1.98	1.91	0.25	1.41	1.31

Values shown in Table 3.4 are represented as the mean of three independent experiments.
MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol); TAG (Triacylglycerol).

Table 3.4 shows the number of bis-allylic positions per molecule. According to Miyashita (2008), oxidative stability of polyunsaturated lipids is known to be decreased with the increasing number of bis-allylic positions. With referring to the table mentioned, number of bis-allylic positions of a molecule of lipids could be computed from the molar concentration of each PUFA and the mean molecular weight (MW) of each lipid. Molar concentration of PUFA was calculated based on weight percentage of PUFA; before the oxidation (Table 3.2 and 3.3) including the MW of each PUFA.

The mean MW of all 5 samples; Spinach MGDG & DGDG, Akamoku MGDG and Linseed TAG was obtained from the mean MW of galactosyl or glycerol moiety. The mean MW of all fatty acyl moieties was directly calculated from the mol % of each fatty acid and the MW. The mean MW of each lipids were as follows: Spinach MGDG, 833.80; Spinach DGDG, 944.99; Akamoku MGDG, 784.8687; Akamoku DGDG, 956.3512 and Linseed TAG, 918.4956. Akamoku DGDG possessed the highest mean of MW followed by Spinach DGDG, Linseed TAG, Spinach MGDG and finally Akamoku MGDG.

GL of marine lipid source; both Akamoku MGDG and DGDG, contained considerable percentage of EPA with especially Akamoku DGDG showed a higher percentage of EPA and a higher mean number of bis-allylic position per molecule of lipid than did Linseed TAG (Table 3.4). Akamoku MGDG had a difference of 0.9, lower as

compared to Linseed TAG. On the other hand, Akamoku DGDG, was recorded to be 0.2, higher than Linseed TAG. Upon comparing the difference in the mean number of bis-allylic positions per molecule between Linseed TAG and with the notably Akamoku DGDG, the oxidative stability of the latter mentioned, was anticipated to be low. However in this case, stability of both Akamoku MGDG with the emphasize on its DGDG constituent, are far more higher than that of Linseed TAG as shown in the oxidation setting; Figure 3.7.

Upon comparing the difference in the mean number of bis-allylic positions per molecule between Linseed TAG, and Spinach MGDG, the stability is again anticipated to be low (Table 3.4). However in this case, stability of Spinach MGDG is much higher than that of Linseed TAG as shown in Figure 3.7. On the other hand, when comparing both Akamoku MGDG and Spinach MGDG, the result seemed to be in proportional as accordingly (in terms of stability and its relation to the mean number of bis-allylic positions).

In reference to the facts mentioned, when comparing the difference in the mean number of bis-allylic positions per molecule between Linseed TAG, and Spinach DGDG, the stability is again anticipated to be low. However in this case, stability of Spinach DGDG are much higher than that of Linseed TAG and in fact, higher than that of Akamoku DGDG, shown in Figure 3.7.

3.3.6 Stability of Purified Lipids

When the oxidative stability of all types of GLs including Linseed TAG at 50 ^{o}C were compared by measuring the decrease in oxygen concentration in the bulk phase (Figure 3.7), the oxidative stability was found to be the highest for Spinach DGDG, followed by Spinach MGDG, then by Akamoku DGDG and Akamoku MGDG. Linseed TAG was shown to have the lowest oxidative stability. This order however, was in total opposite with that of expected from the average number of bis-allylic positions calculated.

Both GLs with DGDG constituent were shown to be more stable as compared to their MGDG constituent when they only showed a mild decline over a brief time. The downward trend signifies the decrease of Oxygen level in the headspace with Akamoku DGDG decreased in stability at a sooner rate from the most stable; Spinach DGDG (98 %). Although Akamoku DGDG showed a sooner inclination downwards

(92 % of oxygen remained in headspace, throughout), it still retained the oxidative stability of about more than the strength of its MGDG constituent.

Spinach MGDG was much stable when compared to Linseed TAG. Akamoku MGDG marginally continued to level off at a much later rate to finally 88 % (of oxygen remained in headspace) before completing the oxidation process.

A study done by Lee *et al*., showed that Spinach GL in general, contain low ratio of saturated fatty acid (SFA) to unsaturated fatty acid (UFA) and yet this GL had a relatively high oxidative stability as compared to lipids of other classes such as neutral lipid (NL) and phospholipid (PL). This result might also suggest the special characteristics attributed to GL and eventually highlight that the mechanisms involve in oxidation of this particular class of lipids are unique in itself.

The intrinsic factor put forth by chlorophyll since this compound is reported to have both antioxidant as done by Endo *et al.*, (1985) and again Endo *et al*., (1985), and in some cases pro-oxidant as studied by Frankel (1998), could be ruled out as chlorophyll was in majority removed successfully by a total of double purification; by the first silicic acid with then carbon column as mentioned in methods of this study, for Akamoku MGDG and Spinach MGDG. In addition to that, experiment was done under the dark throughout, leaving less chances for traces of chlorophyll to react with light.

3.4 Conclusion

GL represents the most abundant lipid class of thylakoid membranes. Research by Jordan *et al.* (2001), have elucidated the x-ray crystallography, which signifies GL undertake specific role during photosynthetic electron transport, at a specific site. Stereochemistry of these sugar bound-lipid compound might be the underlining reasons for the high in stability thus protecting PUFA from further oxidized. A possible interaction between sugar moieties and the PUFA might exist, which in turn exerts in the protective mechanisms.

Protective interactions among same compounds; DGDG to DGDG, could be heavily dependent on the sugar moieties, the length and degree of unsaturation with also the regiospecificity of the two acyl chains in the sn-1 and sn-2 positions of the glycerol

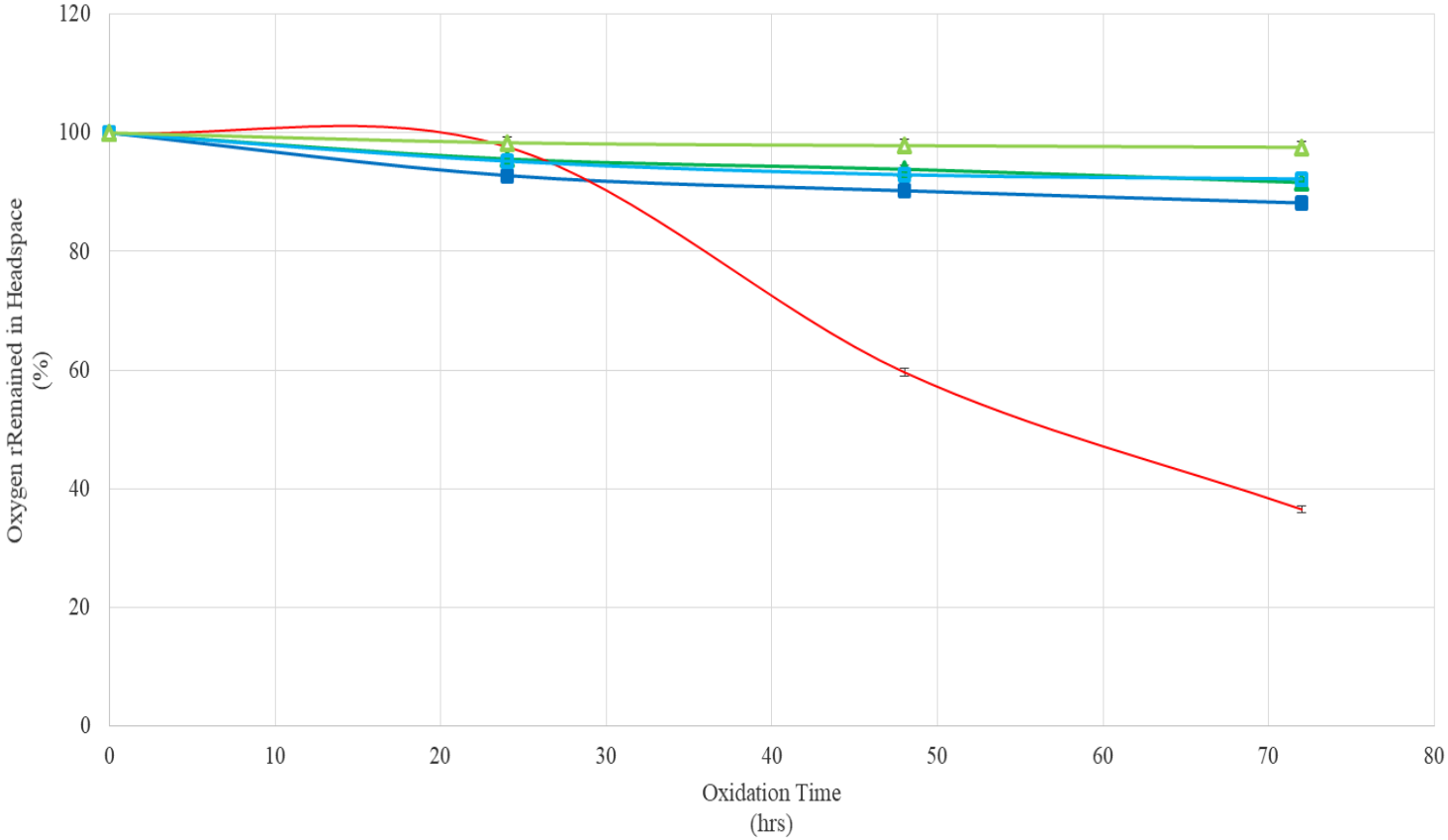


Figure 3.7: Oxidative stability of Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50ºC.

Value of graphs shown in Figure 3.7 are represented as mean of three independent experiments.
Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Sp.DGDG (bright green open triangle), Aka.MGDG (dark blue solid square) and Aka.DGDG (bright blue open square)

GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol); TAG (Triacylglycerol).

lipid bilayers, it probably suggest and favors the result of this research which shows that DGDG has a higher oxidative stability as compared to the MGDG constituents. Interactions between MGDG to MGDG, which tends to adapt a conical shape and could form inverted hexagonal structures in especially aqueous solutions, would probably limit the oxidative stability by exposing more sites to be exposed to oxidation although not much.

More research is needed concerning structural details of GL with especially the biosynthesis pathway which encodes specific roles of the membrane lipids. The polar lipids which is all inclusive with structural complexity would be one of the main reasons for this compound to be of a protective mechanisms over PUFA. In this study, we have managed to observe that the PUFA in the form of GLs were oxidatively more stable than those from TAGs. Even among different constituents of GLs; MGDG and DGDG with different terrestrial and marine source; Spinach and Akamoku, exhibit variation in properties and response towards oxidation. Further study is required to elucidate the fact of polar moieties; monogalactosyl and digalactosyl and their response towards delaying oxidation upon comparison to other TAG.

Chapter 4: Volatile Formation Involving Oxidative Stability of Glyceroglycolipids; GL (MGDG & DGDG) from Spinach and Akamoku as Compared to Linseed Oil (TAG)

4.1 Introduction

Oxidized lipids are responsible for the flavor deterioration of food and therefore, volatile oxidation products might be one of the best indicators of food spoilage. This is especially true for all n-3 PUFA containing lipids which forms the off-odors in the beginning oxidation stage. These volatile compounds are even disastrous as they could exert flavor deterioration at even a low concentration. Such example could be observed is fish oil and major contribution to all these deteriorative effects are especially short-chain saturated and unsaturated aldehydes and ketones which have undesirable earthy like odor, as elaborated by Hsieh *et al*., (1989). Shibata *et al*., (2015) reported that aldehydes and ketones were detected in oxidation involving four types of TAGs where pentane, was found to be formed the most other than hexanal, propanal and acrolein.

Static headspace methods is found to be effective in volatile analysis. They are primarily used to detect aldehydes and hydrocarbons. Jacobsen (1989), conclusively summarized and proved in her research that volatile compounds determined by the headspace methods with using GC could by the closest relate well with the sensory data. Few researchers; Hsieh *et al*., (1989); Karahadian & Lindsay (1989); Frankel (1993); Horiuchi (1998); Aidos *et al*., (2002); Venkateshwarlu *et al*., (2004a); Venkateshwarlu *et al*., (2004b), elaborated circumstances especially in fish oil oxidation, volatile compounds containing hydrocarbons, vinyl alcohols, alkenals, alkadienals, alkatrienals and vinyl ketones were managed to identified.

In this chapter, we initially compared the volatile compounds of 5 types of lipids; Spinach MGDG, Spinach DGDG, Akamoku MGDG, Akamoku DGDG and Linseed TAG. It is hope that the GC profiles of these samples might lead us to some highlights of volatiles involving GLs and educate us of how GLs are distinct from other types of lipids.

4.2 Materials and Methods

4.2.1 Sample

(Please refer to previous chapter; 3.2.1)

4.2.2 Referenced Compound and Reagent

(Please refer to previous chapter; 3.2.2)

4.2.3 Sample Preparation

4.2.3.1 Extraction and Total Lipid Preparation of Spinach and Akamoku

(Please refer to previous chapter; 3.2.3.1)

4.2.3.2 Purification of Glyceroglycolipids from Spinach and Akamoku

(Please refer to previous chapter; 3.2.3.3)

4.2.3.3 Purification of TAG from Linseed Oil

(Please refer to previous chapter; 3.2.3.4)

4.2.3.4 Tocopherol Analysis of Spinach, Akamoku and Linseed TAG

(Please refer to previous chapter; 3.2.3.5)

4.2.3.5 Oxidation Analysis of Purified Lipids

(Please refer to previous chapter; 3.2.3.7)

Each 300 mg of 5 types of all purified lipids were prepared as follows:

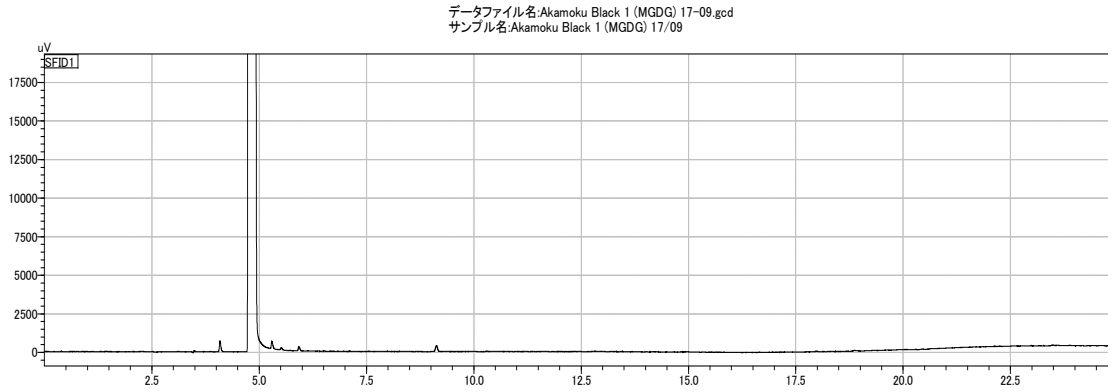
6) Sp.MGDG
7) Sp.DGDG
8) Aka.MGDG
9) Aka.DGDG
10) Lin.TAG

Apart from the method mentioned above, oxidation of samples were also monitored using the GC analysis of volatile compounds. Referring to the static headspace GC analysis, after a definite time of incubation, the sample vials were transferred into the HS-20 headspace autosampler (Shimadzu Corporation) of the GC apparatus. The headspace gas in the vials were automatically pressurized at 60 ^{o}C for 2 minutes and then immediately injected through a loop into a GC (Shimadzu GC-2014AFSC) equipped with a HP-1 capillary column (50-m length, 0.32 mm i.d and 1.05 μm film thickness; Agilent Technologies, CA, USA) and a flame ionization detector. An initial oven temperature of 40 ^{o}C for 5 minutes was used, followed by heating at 3 ^{o}C/min to 70 ^{o}C, then 200 ^{o}C/min to 200 ^{o}C and finally the temperature was held at 200 ^{o}C for 4 minutes. Both the injection port and the flame ionization detector were set at 250 ^{o}C. Three replicate measurements of each data and value at different oxidation times of the stored samples were expressed as the mean ± SD (n = 3).

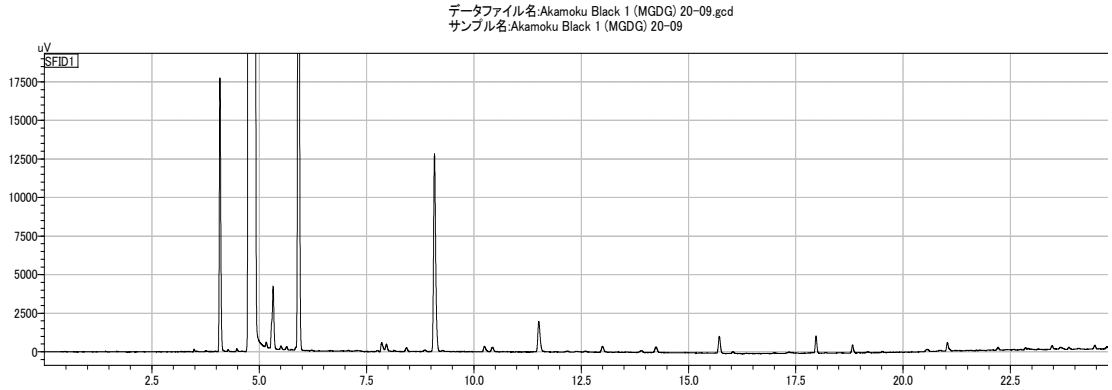
4.3 Results and Discussion

4.3.1 Akamoku MGDG

Akamoku MGDG 1; Day 0



Akamoku MGDG 1; Day 3



Akamoku MGDG 1; Day 7

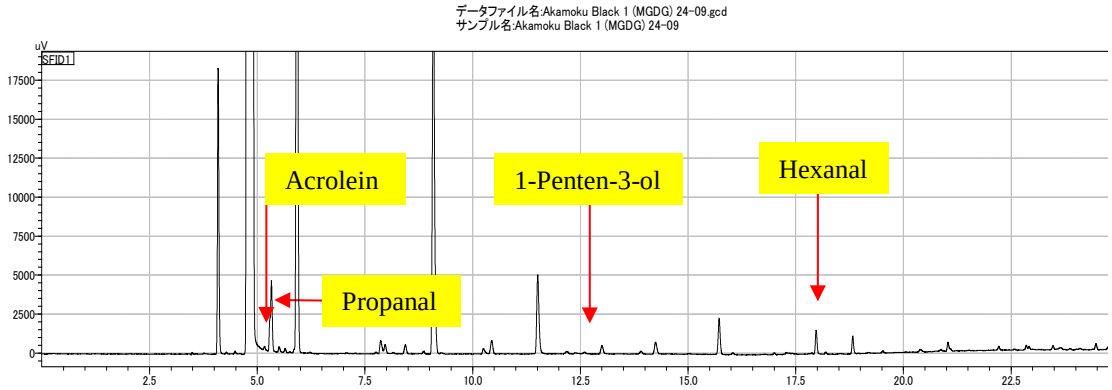
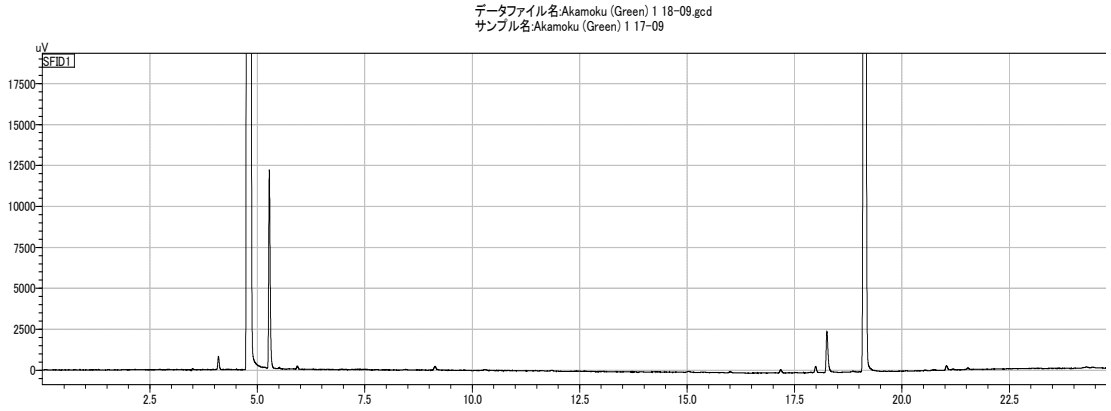


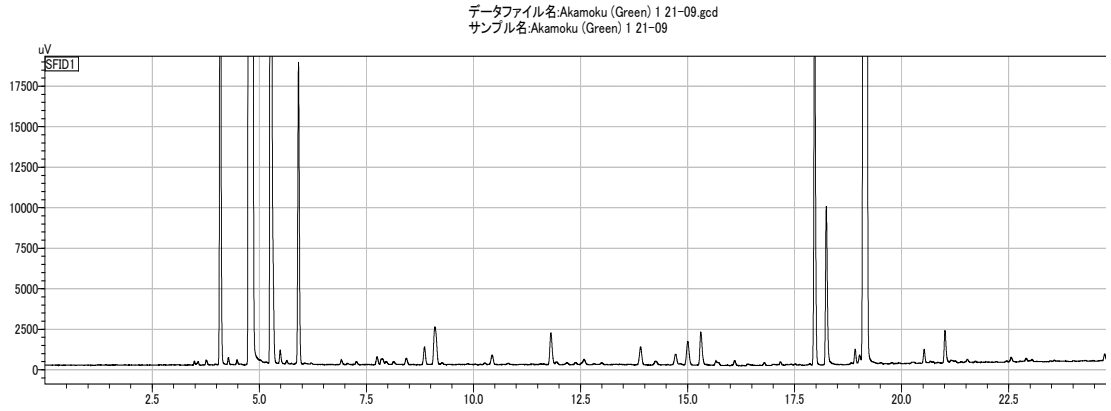
Figure 4.1: Chromatogram of GC volatile compounds from oxidized Akamoku MGDG at 0, 3 and 7 days of incubation at 50ºC. Volatile compounds from oxidized Akamoku MGDG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

4.3.2 Akamoku DGDG

Akamoku DGDG; Day 0



Akamoku DGDG; Day 3



Akamoku DGDG; Day 7

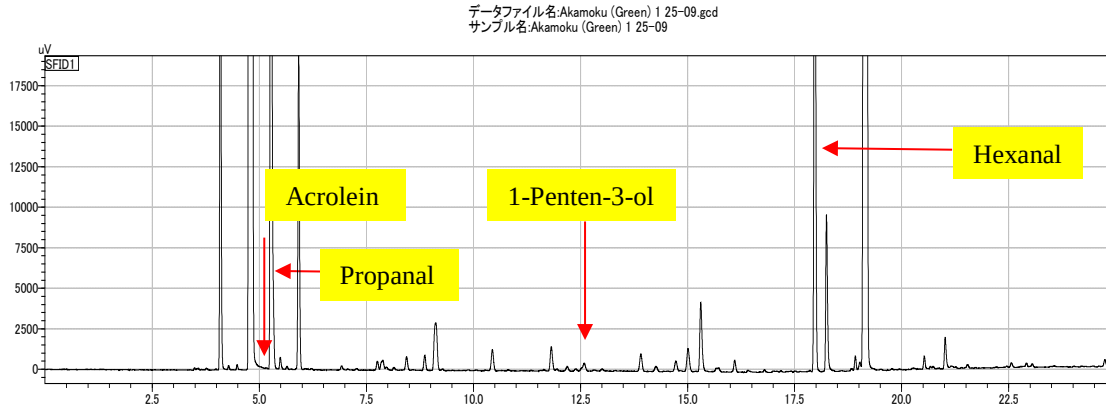
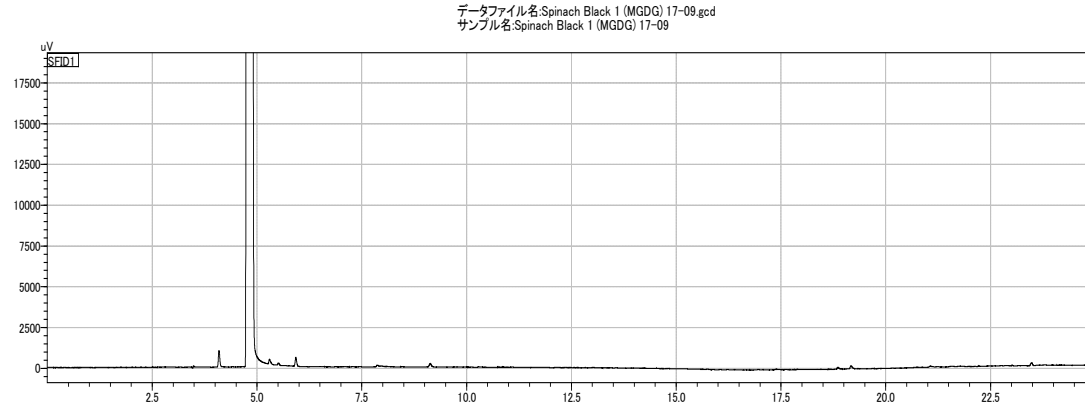


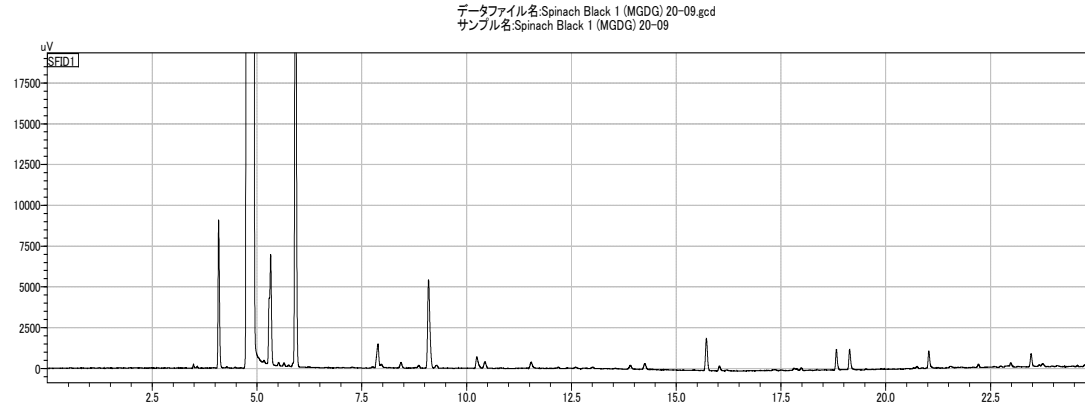
Figure 4.2: Chromatogram of GC volatile compounds from oxidized Akamoku DGDG at 0, 3 and 7 days of incubation at 50°C. Volatile compounds from oxidized Akamoku DGDG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

4.3.3 Spinach MGDG

Spinach MGDG; Day 0



Spinach MGDG; Day 3



Spinach MGDG; Day 7

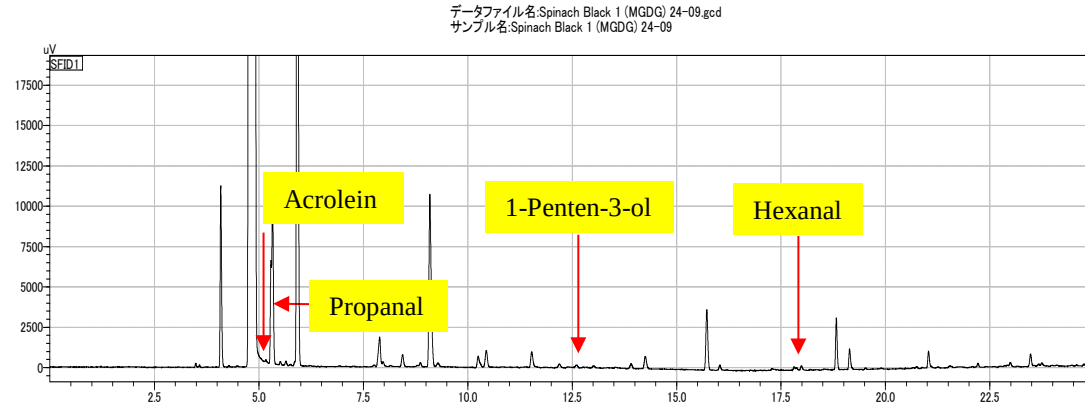
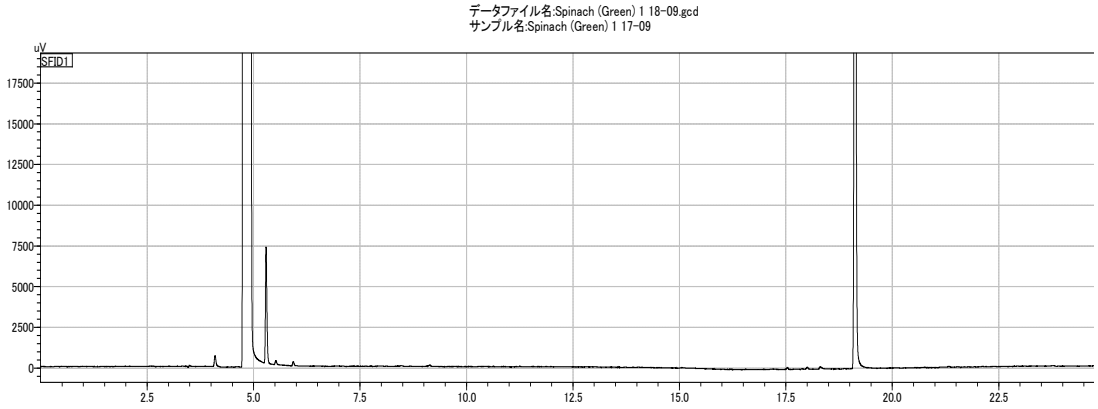


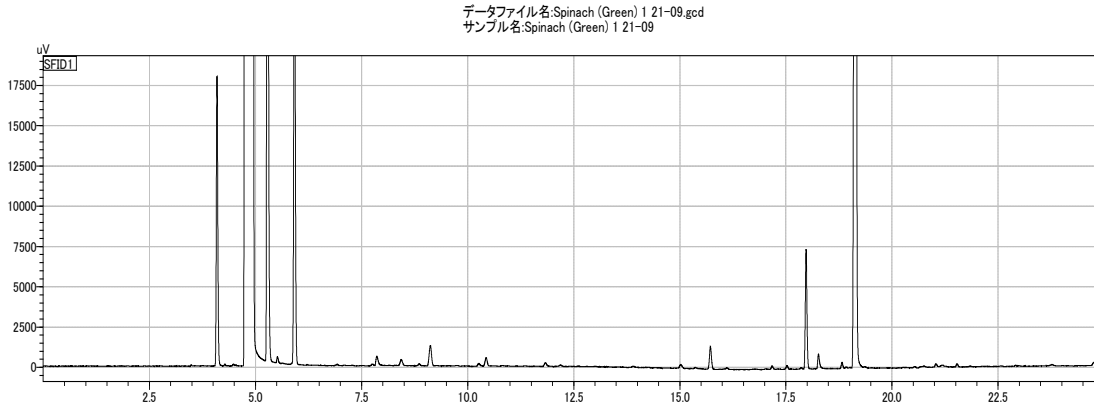
Figure 4.3: Chromatogram of GC volatile compounds from oxidized Spinach MGDG at 0, 3 and 7 days of incubation at 50ºC. Volatile compounds from oxidized Spinach MGDG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

4.3.4 Spinach DGDG

Spinach DGDG; Day 0



Spinach DGDG; Day 3



Spinach DGDG; Day 7

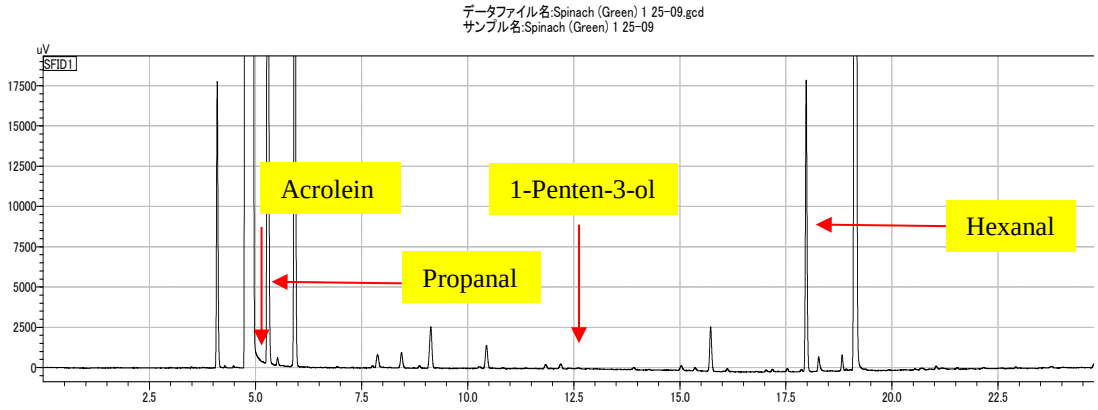
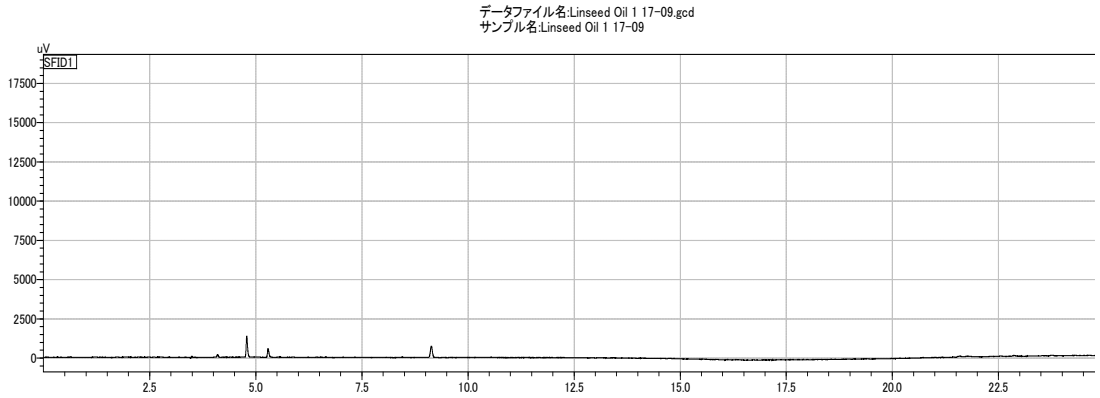


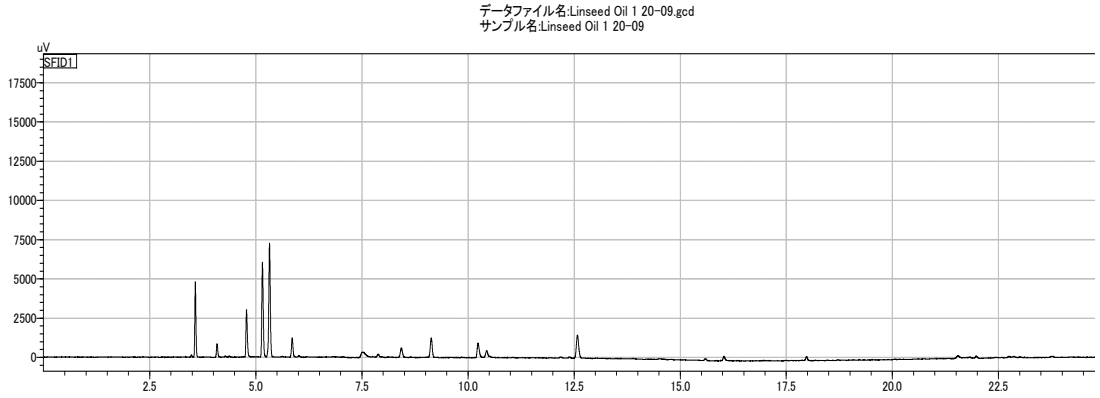
Figure 4.4: Chromatogram of GC volatile compounds from oxidized Spinach DGDG at 0, 3 and 7 days of incubation at 50ºC. Volatile compounds from oxidized Spinach DGDG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

4.3.5 Linseed TAG

TAG; Day 0



TAG; Day 3



TAG; Day 7

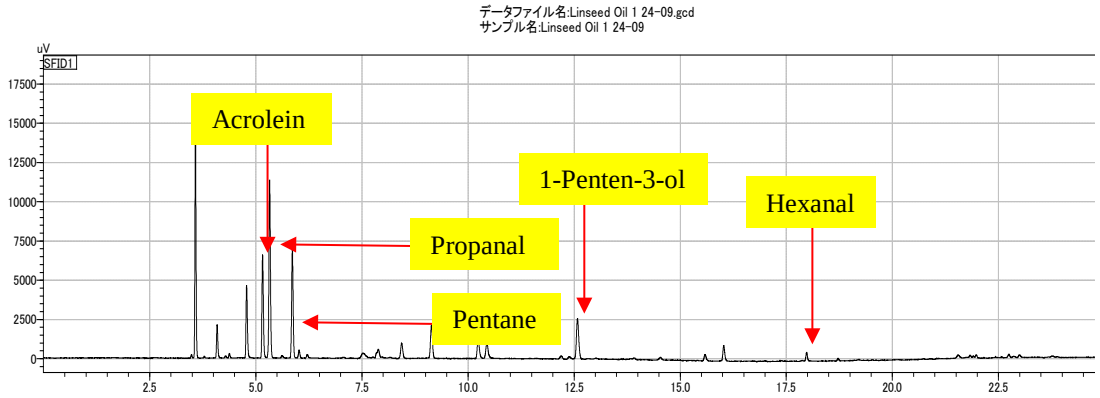


Figure 4.5: Chromatogram of GC volatile compounds from oxidized Linseed TAG at 0, 3 and 7 days of incubation at 50ºC. Volatile compounds from oxidized Linseed TAG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) Pentane, 4) 1-Penten-3-ol and 5) Hexanal.

Final total peak area of volatiles increased with the incubation of time for all samples (Figure 4.6). The next Figure 4.7; total peak area of 5 compounds; Acrolein, Propanal, Pentane, 1-Penten-3-ol and Hexanal of Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG), showed that Akamoku oxidised DGDG constituents having the most amount of those volatiles formed, followed by Lin.TAG as having the second most volatiles formed.

Akamoku oxidized MGDG constituents formed the least volatiles along the incubation then followed by the Spinach oxidized MGDG constituents and followed by Spinach oxidized DGDG constituents. Base on the volatiles result alone, this might not be in total agreement with the result showed by the oxidative stability previously, however, it clearly showed that more volatiles were released throughout the incubation

Results from the analysis of major volatile compounds using static headspace GC method are shown in Figure 4.1 to Figure 4.5. Peak intensities are used to express the amount of volatile compounds. All volatile compounds were found to be increased with a different trend on increment.

It is worth to highlight that the compound named acrolein of Lin.TAG relatively increase at initial time and however reached a plateau after a certain, given time (Figure 4.8 and 4.13). Shibata *et al.*, (2015) also mentioned that acrolein was the most abundant volatile in fish oil TAG. The pattern of the increased amount was reported to be the same with the ones analyzed of oxidised Lin.TAG.

On the contrary, acrolein was only detected in a small amount, in all types of oxidized GL (MGDG and DGDG) from both Spinach and Akamoku. This important point might be the distinct features which could be related to the protective mechanisms, which could also owed to the special stereochemistry configuration of GL towards PUFA.

Propanal was detected on the rise together with both Spinach and Akamoku oxidized MGDG constituents (Figure 4.9 and 4.13). An issue of equal importance to be highlighted with regard to both Spinach and Akamoku DGDG constituents is regarding to the retention time. Retention time for volatile acetone and volatile propanal were actually very near to each other. Acetone generally having the retention of about minute 5.27 to minute 5.30 while propanal having the retention of about minute 5.32 to minute 5.33.

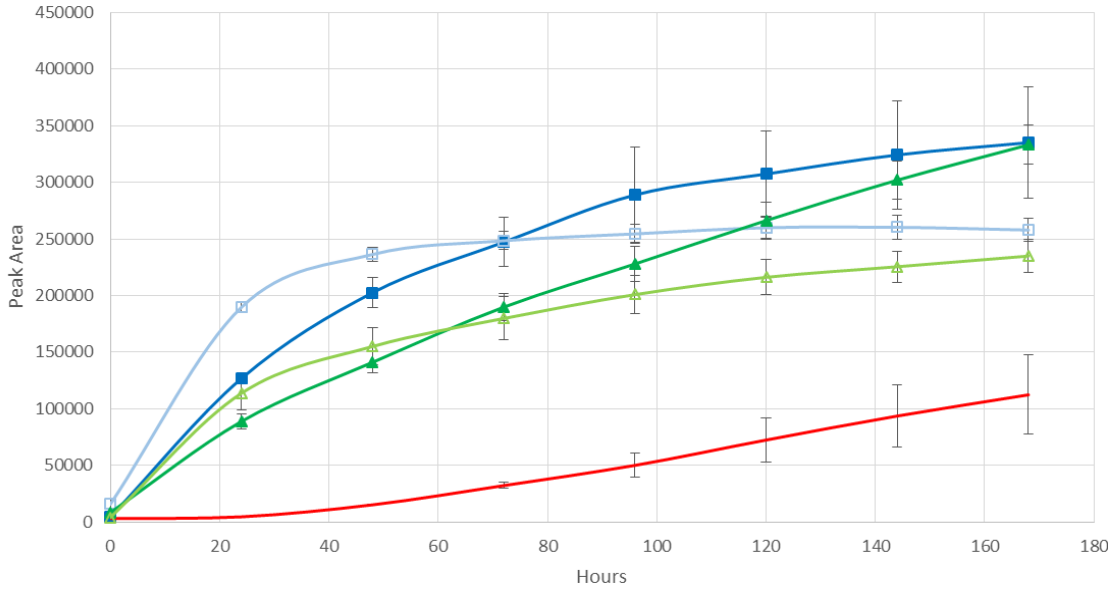


Figure 4.6: Final total peak area of Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50ºC.

Value of graphs shown in Figure 4.6 are represented as mean of three independent experiments. Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Sp.DGDG (light green open triangle), Aka.MGDG (dark blue solid square) and Aka.DGDG (light blue open square)

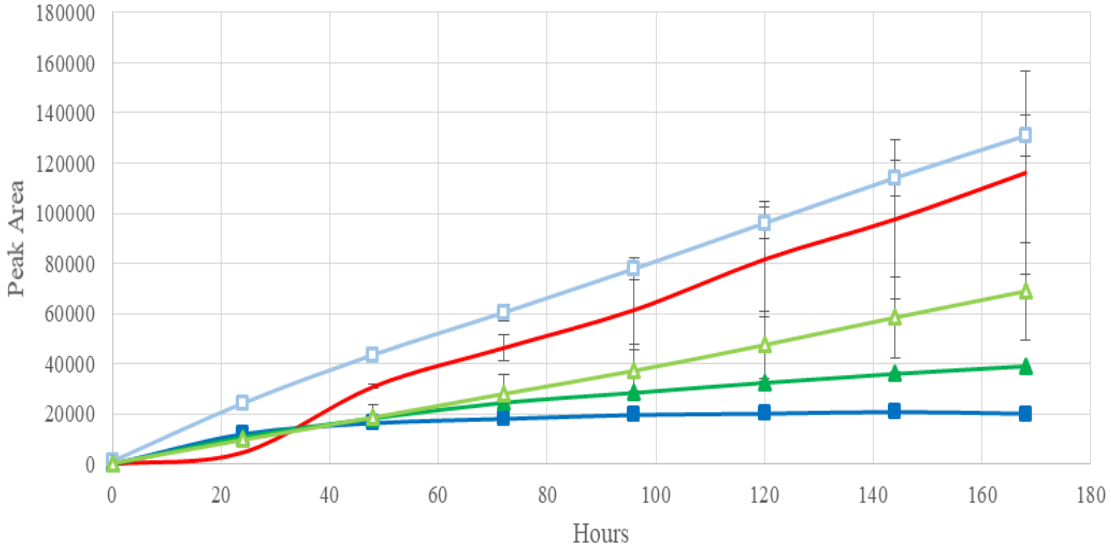


Figure 4.7: Total peak area of 5 compounds; Acrolein, Propanal, Pentane, 1-Penten-3-ol and Hexanal of Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50ºC.

Value of graphs shown in Figure 4.7 are represented as mean of three independent experiments. Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Sp.DGDG (light green open triangle), Aka.MGDG (dark blue solid square) and Aka.DGDG (light blue open square)

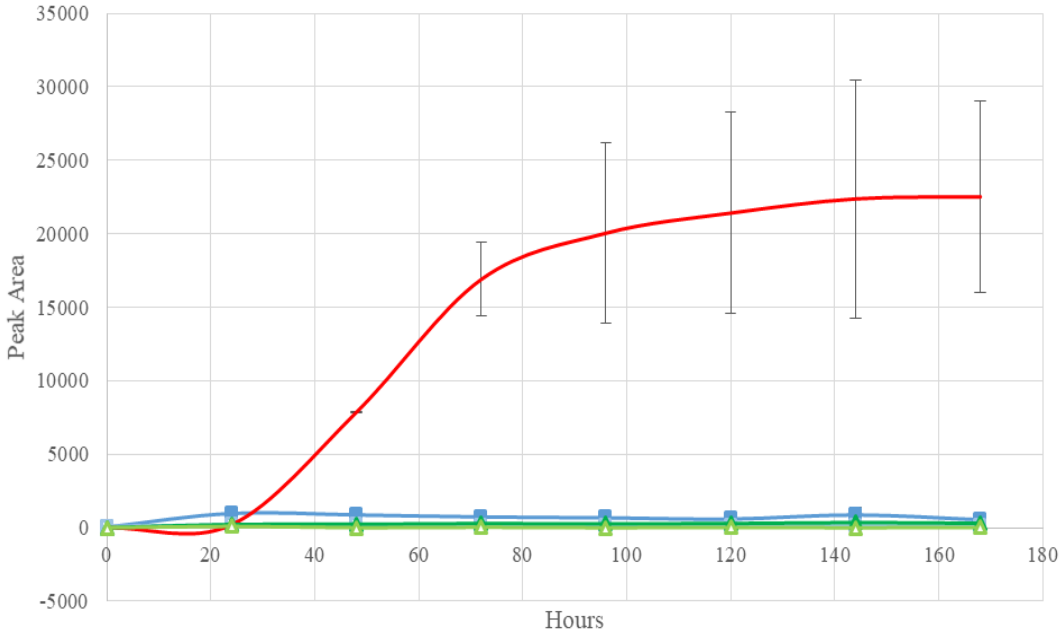


Figure 4.8: Peak area of Acrolein for Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50°C.

Value of graphs shown in Figure 4.8 are represented as mean of three independent experiments. Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Sp.DGDG (light green open triangle), Aka.MGDG (dark blue solid square) and Aka.DGDG (light blue open square)

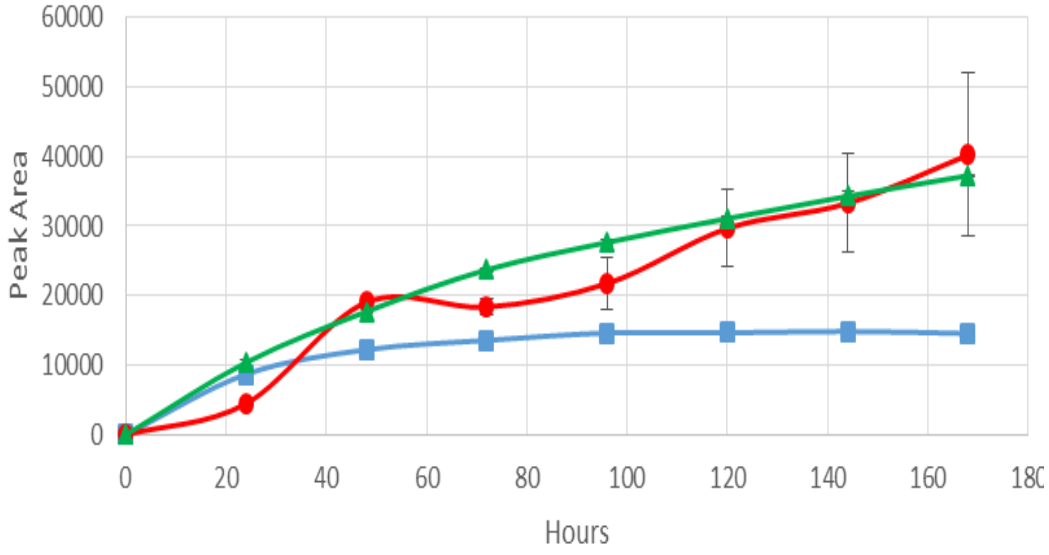


Figure 4.9: Peak area of Propanal for Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50°C.

Value of graphs shown in Figure 4.9 are represented as mean of three independent experiments. Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Aka.MGDG (dark blue solid square)

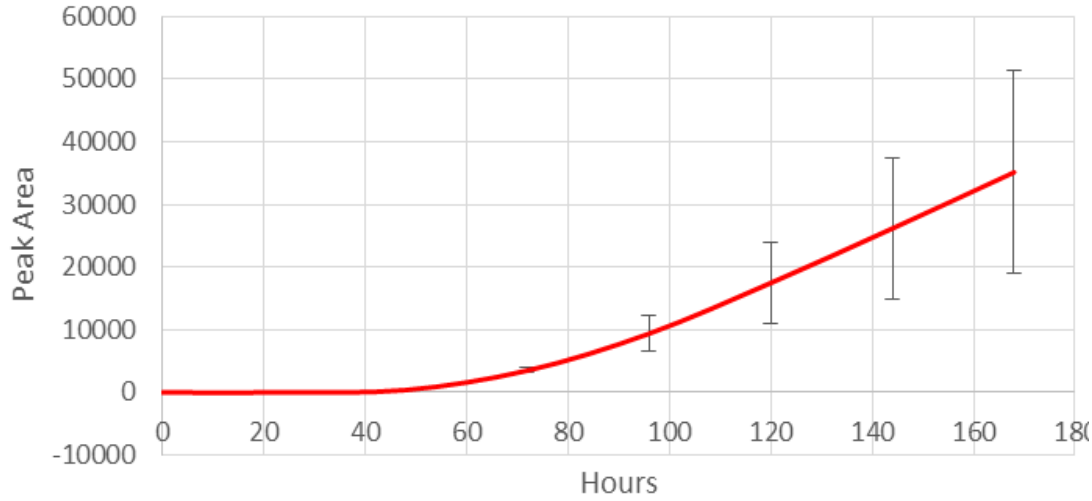


Figure 4.10: Peak area of Pentane for only Linseed Oil (TAG) under the dark at 50ºC.

Value of graphs shown in Figure 4.10 are represented as mean of three independent experiments. Lin.TAG (red line)

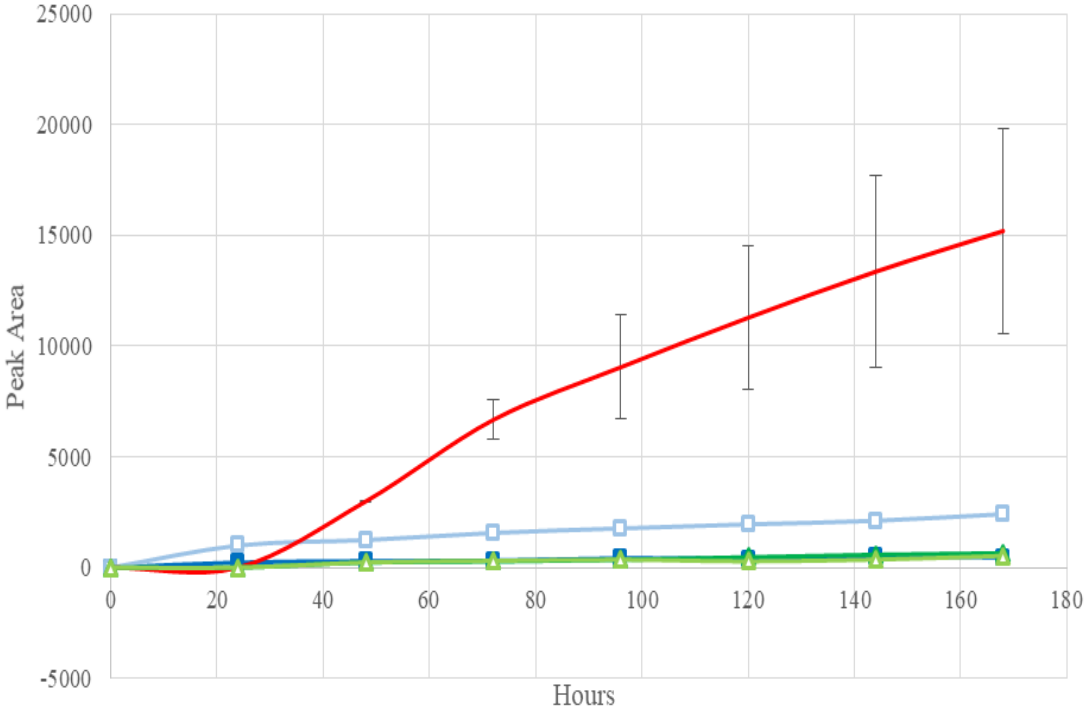


Figure 4.11: Peak area of 1-Penten-3-ol for Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50ºC.

Value of graphs shown in Figure 4.11 are represented as mean of three independent experiments. Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Sp.DGDG (light green open triangle), Aka.MGDG (dark blue solid square) and Aka.DGDG (light blue open square)

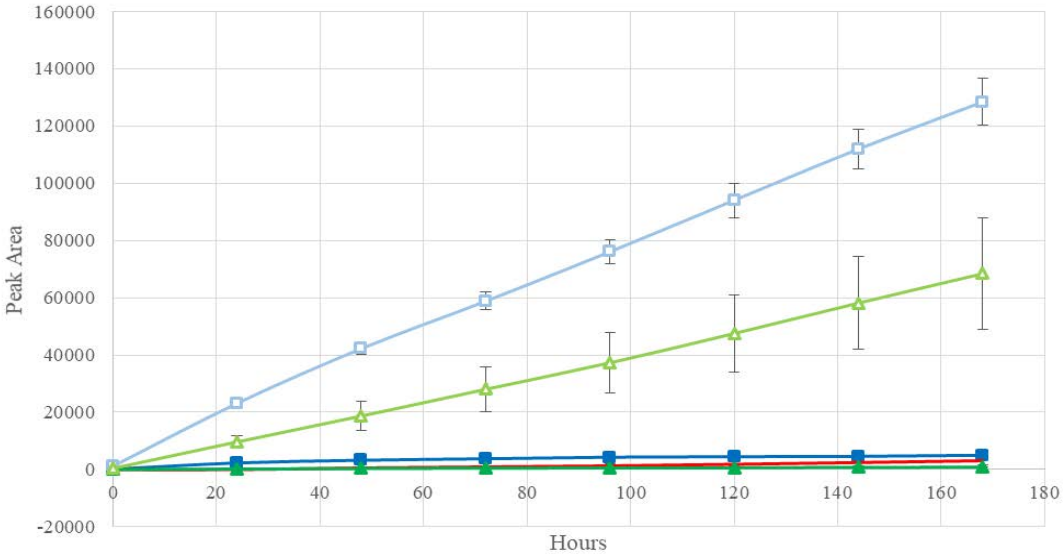


Figure 4.12: Peak Area of Hexanal for Glyceroglycolipids (MGDG & DGDG) from Spinach and Akamoku as compared to Linseed Oil (TAG) under the dark at 50°C.

Value of graphs shown in Figure 4.12 are represented as mean of three independent experiments. Lin.TAG (red line), Sp.MGDG (dark green solid triangle), Sp.DGDG (light green open triangle), Aka.MGDG (dark blue solid square) and Aka.DGDG (light blue open square)

Upon examining the chromatogram closely, peak for acetone (according to internal reference table of GC) was detected to be overlapping with propanal. Since a higher amount of acetone were detected in both Spinach and Akamoku oxidized DGDG constituents, the result of analysis was not recorded and was not clear in this sense. Not having any recorded amount of volatile propanal released, does not mean that both the Spinach and Akamoku oxidized DGDG constituents did not release them. In addition to this, overlapping peak between acetone and propanal were also found to be in both Spinach and Akamoku oxidized MGDG, however it was recorded in the analysis, as the amount of propanal was much higher.

By mentioning this, a complete separation of peaks for both mentioned compounds were recorded in Lin.TAG. Also, only Pentane was detected in Lin.TAG and not in any GL oxidized constituents (Figure 4.10 and 4.13).

Relative amount of 1-Penten-3-ol was also significant in the oxidized Lin.TAG. Major volatiles found in the oxidation of Lin.TAG were pentane and propanal (Figure 4.11). Snyder *et al*., (1988), mentioned that these volatiles could be form due to the decomposition of the monohydroperoxides from the n-3 PUFAs, namely; 18:3n-3

and 18:4n-3. According to the previously recorded fatty acids composition (Table 3.3. and 3.2) Spinach DGDG constituents contain unusually high level of the especially 18:3n-3, and the amount of propanal should be tremendous while the formation of pentane was probably suppressed and not detected in all the oxidized GLs constituents. Amount of propanal in both Spinach and Akamoku oxidized DGDG constituents could not be quantified due to the overlapping peaks mentioned in previous chapter. Shibata *et al*., (2015) added that pentane rapidly increased after 100 h of incubation in oxidized soybean oil eventhough, only small amount of volatiles in the earlier stage.

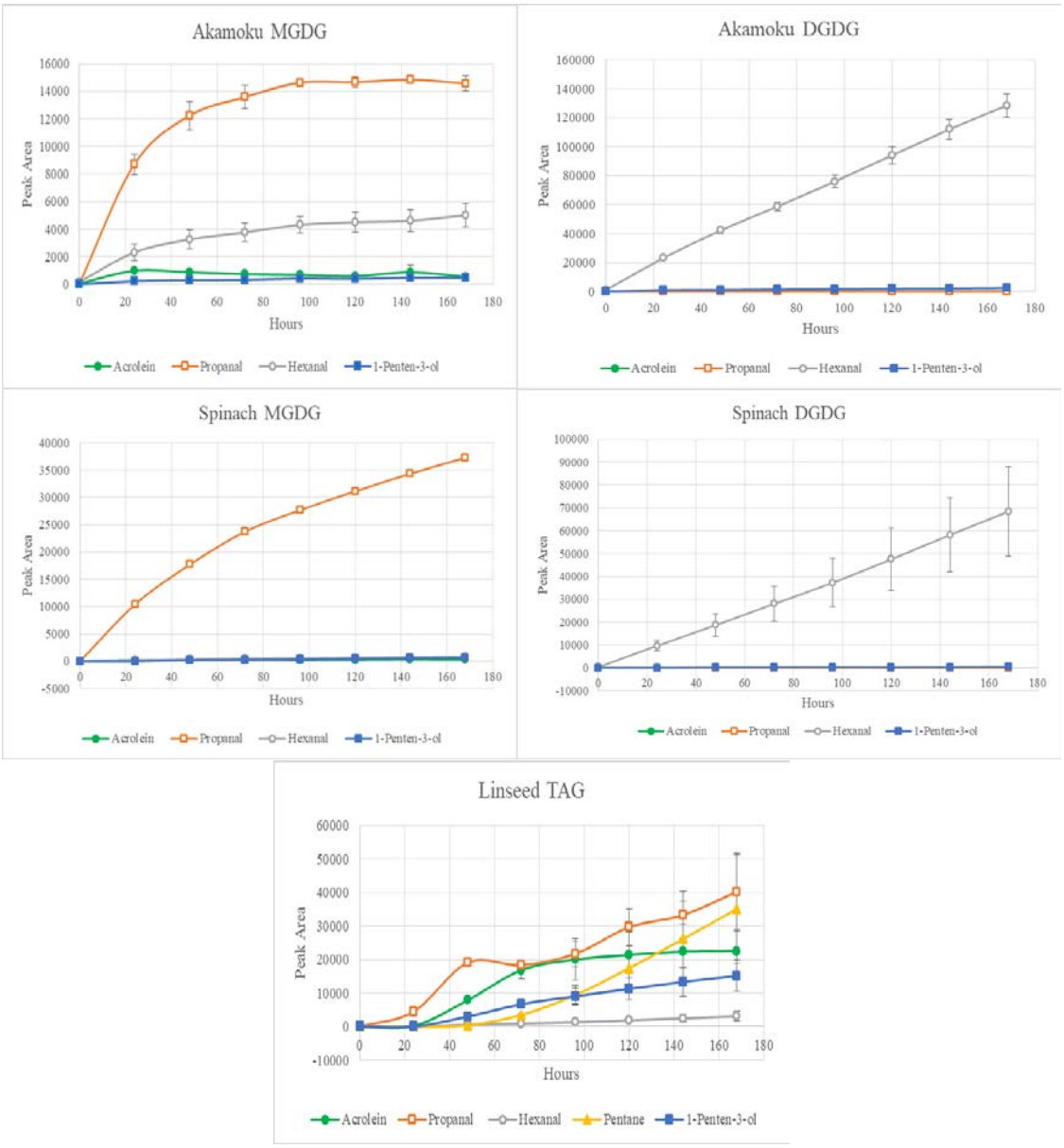


Figure 4.13: Peak area of 5 main volatile compounds according to the type of sample under the dark at 50°C.

Value of graphs shown in Figure 4.13 are represented as mean of three independent experiments.

Another distinctive features of GL with especially both Spinach and Akamoku DGDG constituents, might be due to the steadily increased in volatile compound (Figure 4.12 and 4.13) of hexanal. However, according to Frankel *et al*., (1981), hexanal is known to be the products of the decomposition of n-6 PUFA oxidation which unfortunately not high in Spinach oxidized DGDG constituents. Only Akamoku oxidized DGDG constituents recorded a considerable amount which ironically, not much.

4.4 Conclusion

The total increase in volatiles (which would probably come from the sugar moieties of GL) further elucidate the complexities involved in the protective mechanisms of GL towards PUFA since it might bring a slightly different set of understanding regarding the stereochemistry configuration as compared to the result of oxidative stability which could be explain by common oxidation process. With involving few overlaps peaks, progress in further interpretation and the relation between this uniquely stable compounds makes the quantification of volatiles difficult to understand at the moment.

Since GL samples were not subjected to GC-MS, we could not deduced or either confirmed each peaks responsible for the especially 5 mentioned volatiles usually produce in autoxidation; acrolein, propanal, pentane, 1-penten-3-ol and hexanal. Similarly, peaks especially coming from both Spinach and Akamoku oxidized DGDG constituents showed an un-identified profile where they were not refered to any of the internal standards. These peaks might perhaps a show of some impurities or could also come from the sugar moieties of this yet–to-be-understood lipid with sugar bound compound.

Decomposition of products in biological system remains to be major problem faced by consumers in all areas. This area of biological lipid too, is continuing to gain focus of many researchers. A cascade of multitude-complex product which remains to be unexplored, would in the future waiting to be challenged.

Chapter 5: Oxidative Stability of Glyceroglycolipid from Spinach (MGDG) and Linseed Oil (TAG) with the Addition of Different Concentrations of (Medium Chain Triacylglycerol (MCT)

5.1 Introduction

Mechanisms of lipid oxidation have been broadly studied in bulk oils and oil-in-water emulsions by Abdalla and Roozen (1999), Frankel *et al*., (1994), Fritsch (1994), Halliwell *et al*., (1995), Naz *et al*., (2005), McClements and Decker (2000) and Hu *et al.*, (2004), just to mention a few. A better understanding of oxidation reactions in bulk oils was able to achieve with more current studies on mechanisms of oxidation in oil-in-water emulsions. The very first study however, which leads us to understand of the mechanisms of lipid oxidation were observed in bulk oils.

Food lipids do not exist in a homogenized form, but also, most of the times exist as association colloids; milk, mayonnaise, varieties of dipping and sauce, ice cream, margarine and butter including beverages. They consist of dispersion, between two immiscible liquids, commonly oil and water. One form would be dispersed in another, in a form of small spherical droplets. Positive free energy is needed in order to increase the surface area between oil and water phase, due to this, Dickinson (1992), stressed that they would be thermodynamically unstable and eventually would separate.

The oxidative stability of these GLs in bulk oil were also compared with that of corresponding to addition of varying concentration of Medium Chain Triacylglycerol (MCT). Through this, reactions between GL components with other component set in the same environment could be further understood and predicted.

5.2 Materials and Methods

5.2.1 Sample

Spinach powder (GABAN Co. Ltd., Tokyo, Japan) was obtained from a local food market. Linseed oil was obtain from Summit Oil Mill Co. Ltd., Chiba Japan.

5.2.2 Referenced Compound and Reagent

Silica gel (BW-60F) for the column chromatography was purchased from Fuji Sylysia Chem. Ltd. (Kasugai, Aichi, Japan). The activated Carbon and Celite (545 RVS) were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulphoquinovosyl diacylglycerol (SQDG) standards were purchased from Lipid Products (Redhill, United Kingdom) while Triolein and Tricaprilin (as MCT) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

All other chemicals and solvents used in this study were of analytical grade and high-performance liquid chromatography (HPLC) grade solvents were used for HPLC analysis.

5.2.3 Sample Preparation

5.2.3.1 Extraction and Total Lipid Preparation of Spinach

The Spinach powder was extracted with six volumes (v/w) of methanol, followed by filtration afterwards in order to collect the filtrate. Initially, Spinach powder (*ca.* 2 kg) was soaked in methanol (12,000 mL) at room temperature, which was kept under the dark for overnight (about 16 hours). The green metanolic extract together with the residue was next filtered with a ceramic filter funnel, lined with filter paper (No. 2 Qualitative Filter Paper; 150 mm; Advantec®; Tokyo, Japan) and the green filtrate was removed from the residue under a vacuumed condition at 30 ^{o}C ± 1 ^{o}C.

The green filtrate was then pooled into a pilot scale rotary evaporator (Eyela Rotary Vacuum Evaporator N-11 and Eyela CA-2600; Tokyo Rikakikai Co. Ltd.; Tokyo, Japan) and traces of solvent remaining in the green filtrate was entirely removed in the dark, under vacuum, leaving the dark green viscous liquid obtained at the end. This dark green viscous liquid was collected with dissolving in equivalent volume of methanol which was finally designated as crude lipids of Spinach.

Crude lipids of Spinach was further dissolved into a separatory funnel using a cocktail mix solvents of chloroform-methanol-water (10:5:3, v/v/v) for a liquid-liquid distribution. After being shaken, the funnel was allowed to stand overnight. The lower layer; a mixed lipid layer with methanol and chloroform, was collected and again dissolved into a new separatory funnel. Same volume of water was again added and after

being shaken, the funnel was further allowed to stand one more overnight separation (liquid-liquid distribution). The lower layer was then concentrated under vacuum, using a rotary evaporator. Remaining traces of organic solvents and water were removed in a desiccator (about 3 days) under a high vacuum with leaving the sample in an amber coloured rotary flask. Total lipids of Spinach (*ca.* 20 g) was eventually collected with dissolving in equivalent volume of chloroform and subjected to consequent analysis or kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage).

5.2.3.2 Purification of Glyceroglycolipids from Spinach

Total lipids of Spinach which was collected with dissolving in equivalent volume of chloroform (*ca.* 20g) was first passed through a column (70 × 6 cm i.d.) packed with chloroform slurry mixture of silica gel. The whole column was wrapped with aluminium foil in order to protect the glyceroglycolipid (GL) from degradation with the exposure to light. The elution was first done with chloroform (about 3,000 mL) and then with acetone (about 13,000 L). Fractions eluted with acetone were used as GL (through a continuous elution, with appropriately adjusting the flow rate manually, for about more than a week). The first whole dark green-black fraction was designated as MGDG of Spinach (1 fraction) and the consequent fractions of every 500 mL collected with acetone (clear light green solution) were designated as DGDG of Spinach (about 24 fractions). Fractions were concentrated under vacuum, using a rotary evaporator and was collected and kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage) or till further analysis.

Both MGDG and DGDG of Spinach were subjected to preparative TLC in order to choose the exact fraction with the desired GL. All GL fractions which was collected in ethanol were spotted onto a 0.25 mm silica gel plate. The plate was developed with a cocktail mix solvents of chloroform-methanol-water (65:25:4, v/v/v) and the spots were visualized by spraying the plate with orcinol-sulfuric acid or Dittmer reagent, followed by charring (as compared to standards; MGDG, DGDG & SQDG).

The absence of chlorophyll was also confirmed in all consequent fractions containing DGDG from prior TLC. Therefore, only the first whole fraction obtained; MGDG of Spinach (*ca.* 4 g) was again refined using a carbon column (70 × 6 cm i.d.) packed with 90 % of ethanol in chloroform slurry of activated carbon (*ca.* 250 g) in order

to remove majority traces of chlorophyll. Carbon was first heated at 110 ^{o}C for 1 ½ hours prior to mixing with 90 % of ethanol in chloroform. Elutions were done with gradually decreasing concentration of ethanol; 90 % of ethanol in chloroform (about 1,000 mL), then 50 % ethanol in chloroform (about 1,500 mL) and finally 100 % of chloroform (about 10,000 mL). The fractions eluted with 50 % ethanol in chloroform and 100 % of chloroform were both used as MGDG of Spinach (clear light yellow solution) after consequent fractions of every 500 mL. Fractions were concentrated under vacuum, using a rotary evaporator and was collected and subjected to further analysis or kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage).

MGDG of Spinach were again subjected to preparative TLC. All MGDG fractions which was collected in ethanol were spotted, developed and visualized using the same procedure as described earlier, except that only MGDG was used as standard.

5.2.3.3 Purification of TAG from Linseed Oil

Linseed oil undergone a direct double purification process with 2 types of system. In order to remove tocopherols and pigments, linseed oil (*ca.* 25 g) was first passed through a column (50 × 4 cm i.d.) packed with *n*-hexane slurry mixture of activated carbon (100 g) and Celite (100 g). The carbon was first heated at 110 ^{o}C for 1 ½ hours prior to mixing with Celite and *n*-hexane. The only elution was done with *n*-hexane (1,200 mL).

The obtained oil (*ca.* 10 g) was again refined using a silicic acid column (50 × 4 cm i.d.) packed with *n*-hexane slurry of silica gel (200 g). Elutions were done with *n*-hexane (200 mL), after that a mixture of *n*-hexane-diethyl ether (98:2, v/v) of 200 mL and again another mixture of *n*-hexane-diethyl ether (90:10, v/v) of 1,200 mL. The final fraction eluted with *n*-hexane-diethyl ether (90:10) was used as TAG.

In order to confirm the absence of impurities, the TAG fraction was subjected to preparative TLC. The lipid fraction was spotted onto a 0.25 mm silica gel plate. The plate was again developed with a cocktail mix solvents of *n*-hexane-diethyl ether (60:40, v/v) and the spot were detected with iodine vapour or 60% aqueous sulfuric acid charring (as compared to standard; Triolein).

5.2.3.4 Tocopherol Analysis of Spinach and Linseed TAG

Tocopherol analysis was performed for all types of purified lipids; Spinach (MGDG & DGDG), including Linseed (TAG) with a Hitachi HPLC system equipped with a pump (Hitachi L-2130) and a fluorescence detector (Hitachi L-2485). The analysis was conducted on a silica column (Si 60, 250 × 4.6 mm i.d.; Kanto Chemical Co., Tokyo, Japan) protected with a guard column (15 × 3.2 mm) with the same stationary phase. The mobile phase was n-hexane-2-propanol (99.2:0.8, v/v) with a flow rate of 1.0 mL/min. The fluorescence detector was set at Ex. 298 nm and Em. 325 nm.

5.2.3.5 Oxidation Analysis of Purified Lipids

Each 300 mg of 6 types of all purified lipids were prepared as follows:

1) Sp.MGDG

2) Sp.MGDG (100) + MCT (200)
@
Sp.MGDG + MCT (67 wt %)

3) Sp.MGDG (25) + MCT (275)
@
Sp.MGDG + MCT (92 wt %)

4) Lin.TAG
5) Lin.TAG (100) + MCT (200)
@
Lin.TAG + MCT (67 wt %)

6) Lin.TAG (25) + MCT (275)
@
Lin.TAG + MCT (92 wt %)

All samples were placed in a 20 mL aluminium sealed vial with a butyl gum septum (GL Science; Tokyo, Japan) and then incubated at 50 °C in the dark. Before the incubation, the level of oxygen in the headspace gas of the vial was estimated using a GC system (Shimadzu GC-14B) equipped with a thermal conductivity detector and a stainless steel column (3 m × 3.0 mm i.d.) packed with a molecular sieve 5A (GL Science) according to the method described by Cho *et al.* (1987). The temperatures at the injection port, detector port, and column oven were 120, 120, and 70 °C, respectively. The helium flow was 50 kPa. Three separate vials containing similar samples were

prepared and incubated. A small portion (20 μL) of the headspace gas was taken from each vial using a microsyringe through the butyl gum septum at selected times during the oxidation. The decrease (%) in the oxygen was calculated from the changes in the oxygen to nitrogen ratio compared with the ratio before incubation. Three replicate measurements of each data and value at different oxidation times of the stored samples were expressed as the mean ± SD (n = 3).

5.3 Results and Discussion

Oxidative stability of Spinach MGDG, including Linseed TAG at 50 ^{o}C was determined by measuring the decrease in oxygen concentration, this time, with the addition of Medium Chain Triacylglycerol (MCT) at varying concentration (Figure 5.1). Two out of the total 6 types of samples; Lin.TAG + MCT (92 wt %) and Sp.MGDG + MCT (92 wt %) showed similar high oxidative stability without being able to clearly distinguish the rate of decrease over time between the 2 mentioned samples. However, upon comparison of the specific data value, Sp.MGDG + MCT (92 wt %) showed a tendency of declining yet with a constant and steady bottom value of 91 % of Oxygen remained in the headspace, throughout the period.

The sample Sp.MGDG + MCT (67 wt %) showed a gradual decline over time to a 78 % of Oxygen remained in the headspace upon analyses of data throughout the oxidation processed. Not forgetting, Sp.MGDG also showed a similar decline and drop to a value of 80 % during the oxidation period. Lin.TAG showed the least oxidative stability among all samples which corresponds to the initial oxidation process in the first oxidation experiment setting (Chapter 3).

Results of this second oxidation setting where 6 types of lipids with some samples added with MCT in varying concentration of GL, showed gradual increase in oxidative stability (Figure 5.1). Oxidative stability of mixed samples were proven to have improved and to a certain extent delayed oxidation as compared to sample in bulk oil; Sp.MGDG only and Lin.TAG only. Sp.MGDG + MCT (67 wt %) showed an improved in oxidative stability also the stability of Sp.MGDG + MCT (92 wt %) improved more thus a sign of delaying oxidation further. Same trends was observed in 2 samples with MCT; Lin.TAG + MCT (67 wt %) and Lin.TAG + MCT (92 wt %) where the stability increased and surpass their bulk oil sample.

If by any means, the abstraction of hydrogen from the bis-allylic position by free radical could be prevented, therefore, lipid oxidation would most probably be curbed. By mixing of samples, now the molecules of MCT would perhaps be in vicinity and spread apart along with molecules of GLs or TAGs. With other molecules in together with the sample, those double bonds could be restricted by them which would eventually be a defense against the attack by free radicals.

Kobayashi *et al*., (2004) and Azuma *et al*., (2009), reported their observation through a study of aqueous oxidation of DHA. Miyashita *et al*., (1993) in his report observed that DHA; although with higher number of bis-allylic position where it is usually oxidized at a more speedy rate than LA, gave a completely opposite results. Thus, making DHA more oxidatively stable than LA due to the specific conformation of this molecule in aqueous system. DHA molecules in micelles was depicted by NMR to be loosely packed as compared to LA molecules by which water would be feasibly permeate into the DHA micelles. Through this, hydrogen abstraction from the bis-allylic positions of DHA could be obstructed to later hindered oxidation from occurring.

Much controversy has surround the literature on the oxidative stability of long-chain n-3 PUFA as different polyunsaturated fats shows a diversity of results which are challenging to be interpret. Frankel *et al.*, (2002), clearly pointed out that variation in oxidation conditions, the extent of the application of experimental methods that reaffirms different endpoints of the specific oxidation effects, to the uncertain methods used to rule stability may give rise in inconsistency of results.

Mechanisms of lipid oxidation have been broadly studied in bulk oils and oil-in-water emulsions by Abdalla and Roozen (1999), Frankel *et al*., (1994), Fritsch (1994), Halliwell *et al*., (1995), Naz *et al*., (2005), McClements and Decker (2000) and Hu *et al*., (2004), just to mention a few. A better understanding of oxidation reactions in bulk oils was able to achieve with more current studies on mechanisms of oxidation in oil-in-water emulsions. The very first study however, which leads us to understand of the mechanisms of lipid oxidation were observed in bulk oils.

Majority of the studies as highlighted by Chaiyasit *et al.*, (2007), came to assume that lipid oxidation in bulk oil occurs in a homogenous medium while in actual, these polar lipids and polar amphiphilic products that form during lipid oxidation reactions. By having both polar and non-polar region on the same molecule with most of these,

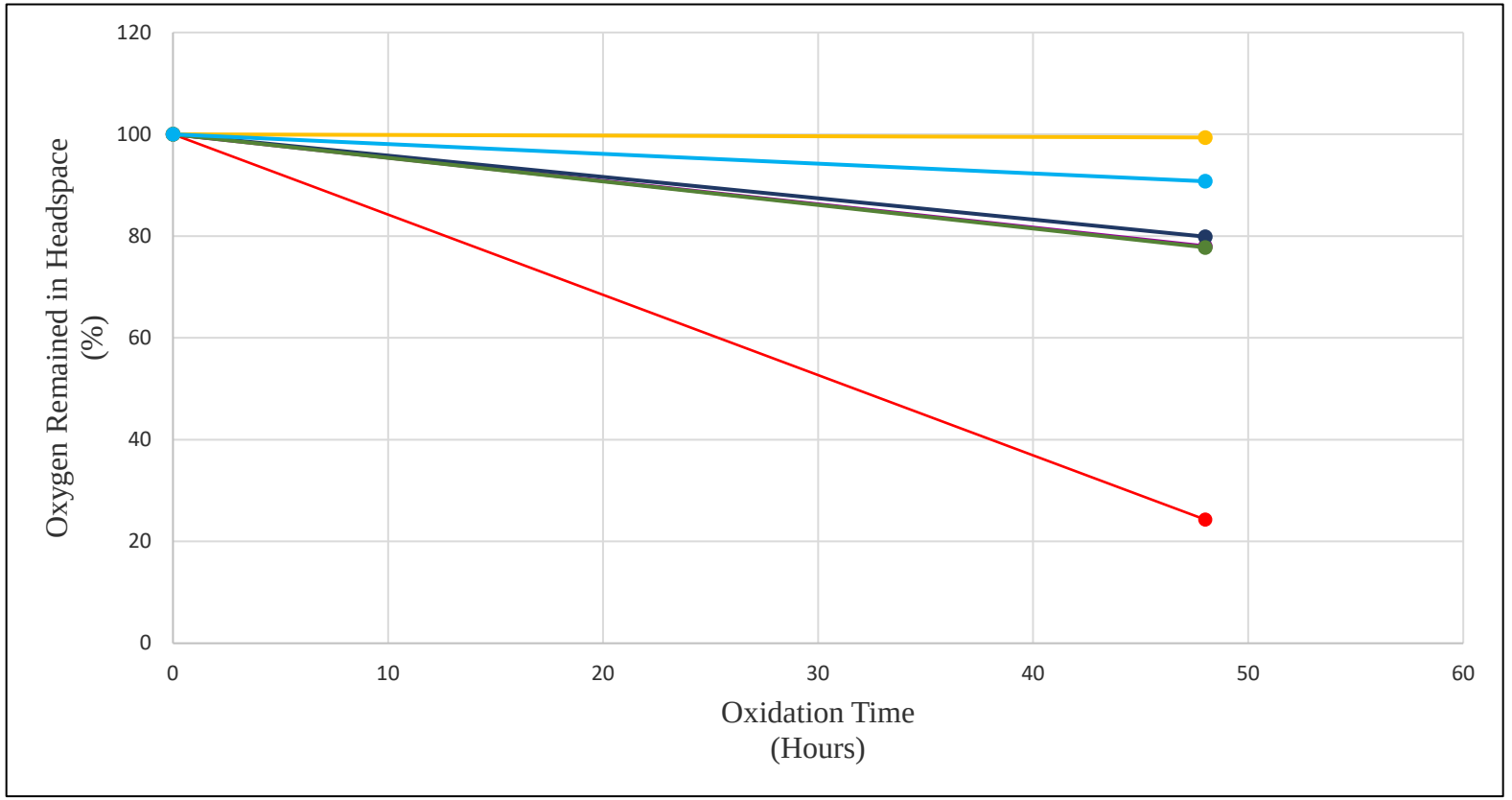


Figure 5.1: Oxidative Stability of Glyceroglycolipids (MGDG) of Spinach and Triacylglycerol (TAG) of Linseed Oil with various concentration of MCT.

Value of graphs shown in Figure 5.1 are represented as mean of three independent experiments.
Lin.TAG + MCT (92 wt %) (dark yellow line), Lin.TAG + MCT (67 wt %) (orange line), Lin.TAG (red line),
Sp.MGDG + MCT (92 wt %) (bright blue line), Sp.MGDG + MCT (67 wt %) (dark purple line) and Sp.MGDG (dark blue line).
GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); TAG (Triacylglycerol).

amphiphilic compounds, self-assemble is possible (Chaiyasit *et al.*; 2007). Considering hydrophobic interaction, these molecules could form an assortment of different types of association colloids; namely, lamellar structures and reverse micelles. According to report by by Gulik-Krzywicki and Larsson (1984), monoacylglycerols and water are able to form reverse micelles in bulk oil. Researchers are anticipating that since the nano or microenvironments is able to modify the physical location of the hydroperoxides, perhaps structures in bulk oil play a crucial role in the chemistry of lipid oxidation. In addition to this, unravelling the physical nature of how bulk oil influence lipid oxidation reactions, could further lead to more effective ways in applying existing antioxidant ingredients and to develop new antioxidant technologies.

5.4 Conclusion

In this study, we have managed to observe that the PUFA in the form of GLs were oxidatively more stable than those from TAGs. Even among different constituents of GLs; MGDG and DGDG with different terrestrial and marine source; Spinach and Akamoku, exhibit variation in properties and response towards oxidation. In addition to this, oxidation in bulk oils and in mix samples in the presence of other molecules, used in this study; MCT, was investigated. Oxidative stability was clearly improved with all samples according to the different combination amount added as compared with the ones in bulk oils. Further study is required to elucidate the fact of polar moieties; monogalactosyl and digalactosyl and their response towards delaying oxidation upon comparison to other TAG.

Chapter 6: Volatile Formation Involving Oxidative Stability of Glyceroglycolipids; GL (MGDG) from Spinach and Linseed Oil (TAG) with the Addition of Different Concentrations of (Medium Chain Triacylglycerol (MCT)

6.1 Introduction

Oxidative stability of PUFA; especially fish and algae oils varies widely according to their fatty acids composition the physical and colloidal state of the lipids, the content of intrinsic antioxidant and the presence and activity of transition metals, Caroll, (1986); Kinsella, (1986); Kinsella, (1987); Harris, (1989); Simoupaulos, (1991); Moffat, (1995) and Horrocks, (1999). Lipid oxidation, however limits the utilization of these oils in processed foods and as nutritional supplements in fortified food. Therefore, in many cases it leaves a debatable question which may be attributed to the wide variation in fatty acid and triglyceride composition of fish or algal oils including the wide range of methods and lipid systems used in oxidative stability test.

In this chapter, we compared the volatile compounds of 6 types of lipids; 3 variations having the base of Sp.MGDG including the mixed samples and the other remaining there samples were of Lin.TAG including the mixed samples. It is hope that the GC profiling of these samples would again render us to understand the major types of volatiles formation in autoxidation process.

6.2 Materials and Methods

6.2.1 Sample

(Please refer to previous chapter; 3.2.1)

6.2.2 Referenced Compound and Reagent

(Please refer to previous chapter; 3.2.2)

6.2.3 Sample Preparation

6.2.3.1 Extraction and Total Lipid Preparation of Spinach and Akamoku

(Please refer to previous chapter; 3.2.3.1)

6.2.3.2 Purification of Glyceroglycolipids from Spinach and Akamoku

(Please refer to previous chapter; 3.2.3.3)

6.2.3.3 Purification of TAG from Linseed Oil

(Please refer to previous chapter; 3.2.3.4)

6.2.3.4 Tocopherol Analysis of Spinach, Akamoku and Linseed TAG

(Please refer to previous chapter; 3.2.3.5)

6.2.3.5 Oxidation Analysis of Purified Lipids

(Please refer to previous chapter; 3.2.3.7)

Each 300 mg of 5 types of all purified lipids were prepared as follows:

2) Sp.MGDG

2) Sp.MGDG (100) + MCT (200)
@
Sp.MGDG + MCT (67 wt %)

3) Sp.MGDG (25) + MCT (275)
@
Sp.MGDG + MCT (92 wt %)

4) Lin.TAG

5) Lin.TAG (100) + MCT (200)
@
Lin.TAG + MCT (67 wt %)

6) Lin.TAG (25) + MCT (275)
@
Lin.TAG + MCT (92 wt %)

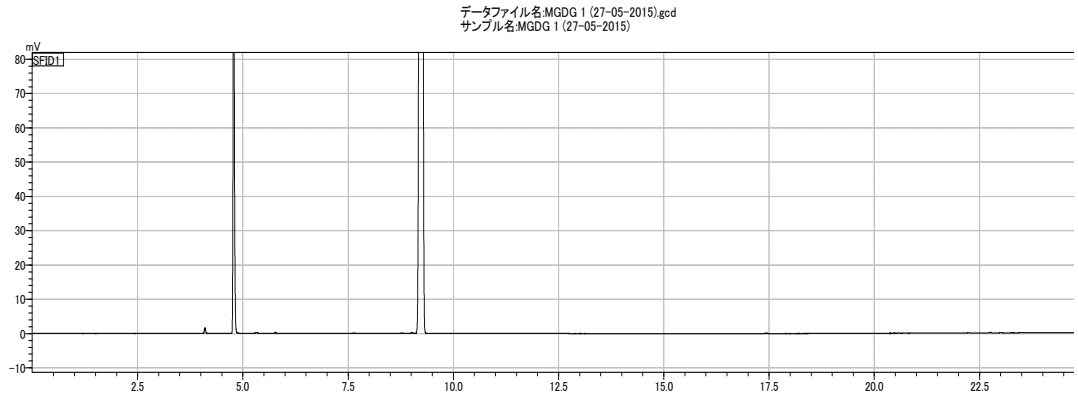
Apart from the method mentioned above, oxidation of samples were also monitored using the GC analysis of volatile compounds. Referring to the static headspace GC analysis, after a definite time of incubation, the sample vials were transferred into the HS-20 headspace autosampler (Shimadzu Corporation) of the GC apparatus. The

headspace gas in the vials were automatically pressurized at 60 ^{o}C for 2 minutes and then immediately injected through a loop into a GC (Shimadzu GC-2014AFSC) equipped with a HP-1 capillary column (50-m length, 0.32 mm i.d and 1.05 µm film thickness; Agilent Technologies, CA, USA) and a flame ionization detector. An initial oven temperature of 40 ^{o}C for 5 minutes was used, followed by heating at 3 ^{o}C/min to 70 ^{o}C, then 200 ^{o}C/min to 200 ^{o}C and finally the temperature was held at 200 ^{o}C for 4 minutes. Both the injection port and the flame ionization detector were set at 250 ^{o}C. Three replicate measurements of each data and value at different oxidation times of the stored samples were expressed as the mean ± SD (n = 3).

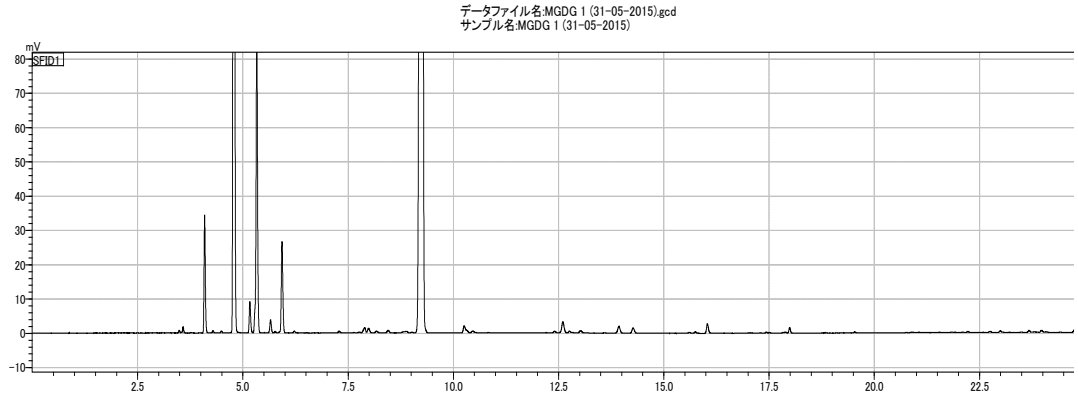
6.3 Results and Discussion

6.3.1 Spinach MGDG

MGDG; Day 0



MGDG; Day 4



MGDG; Day 8

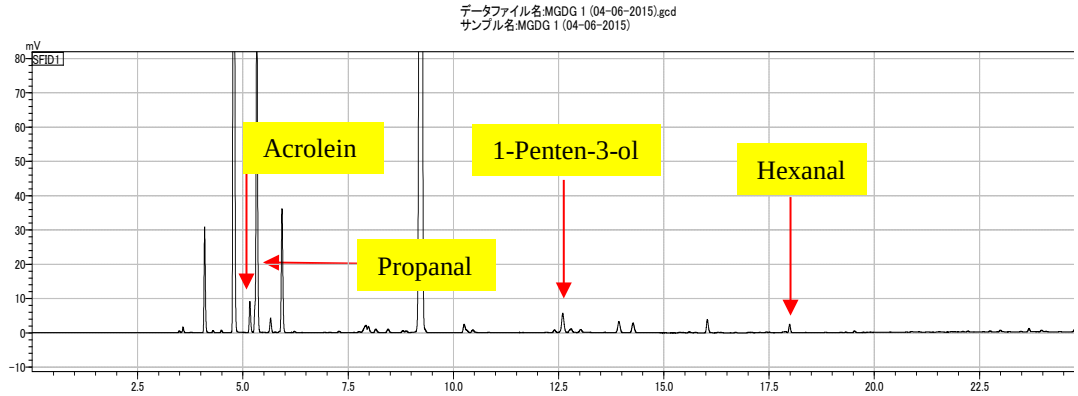
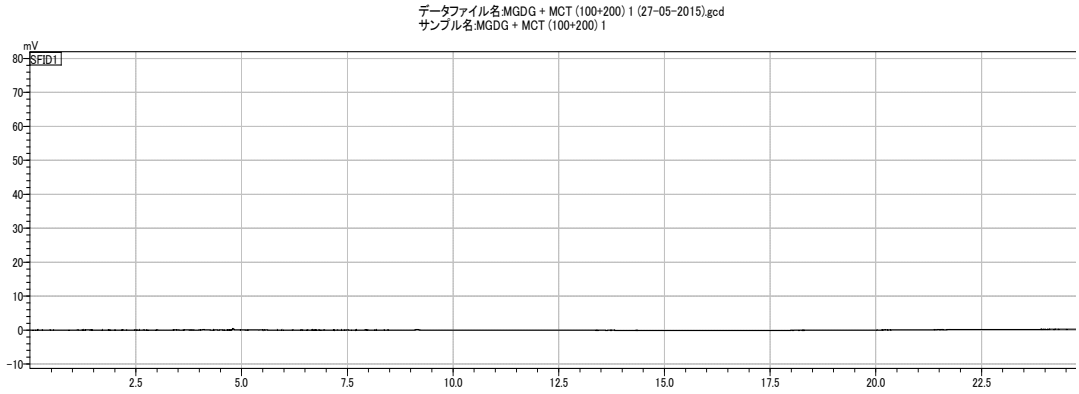


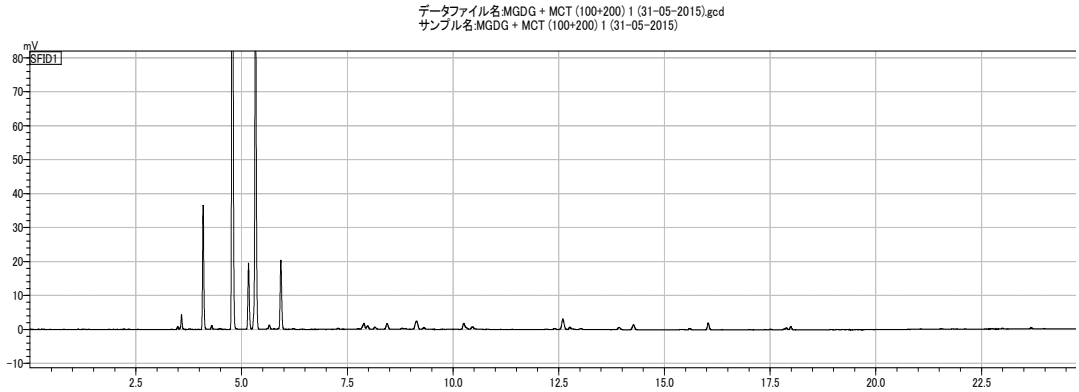
Figure 6.1: Chromatogram of GC volatile compounds from oxidized Spinach MGDG at 0, 4 and 8 days of incubation at 50ºC. Volatile compounds from oxidized Spinach MGDG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

6.3.2 Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %)

MGDG + MCT (100 + 200); Day 0



MGDG + MCT (100 + 200); Day 4



MGDG + MCT (100 + 200); Day 8

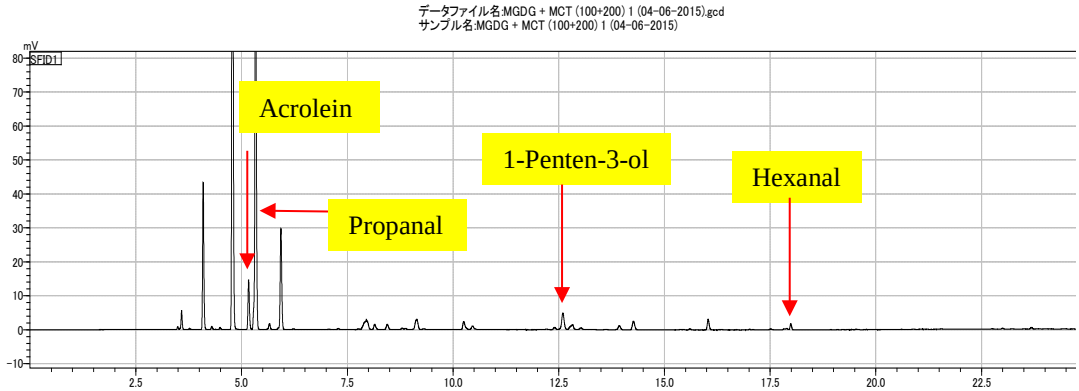
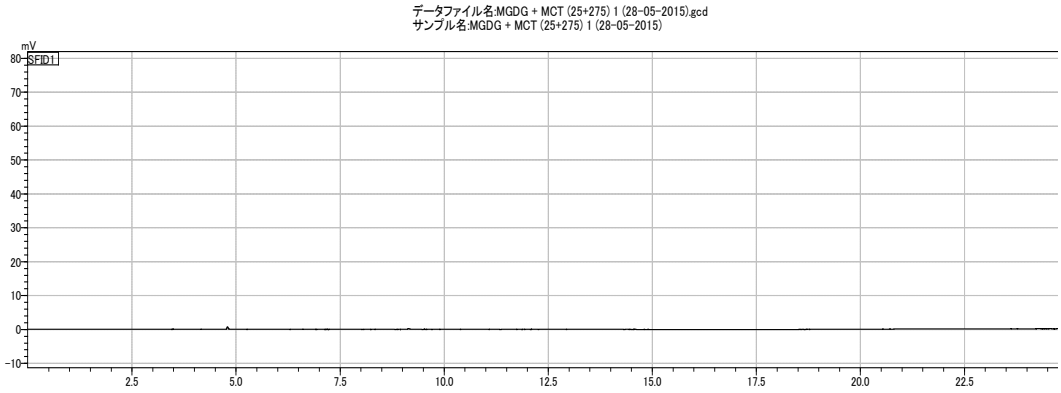


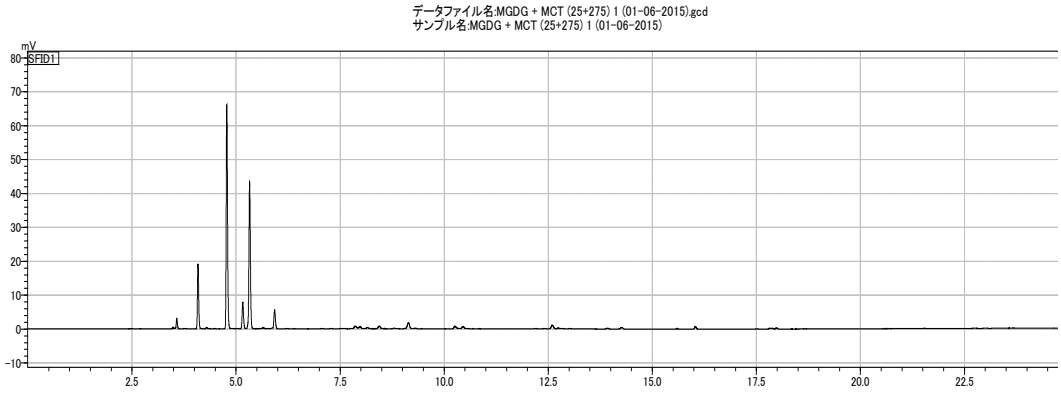
Figure 6.2: Chromatogram of GC volatile compounds from oxidized Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) at 0, 4 and 8 days of incubation at 50ºC. Volatile compounds from oxidized Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

6.3.3 Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %)

MGDG + MCT (25 + 275); Day 0



MGDG + MCT (25 + 275); Day 4



MGDG + MCT (25 + 275); Day 8

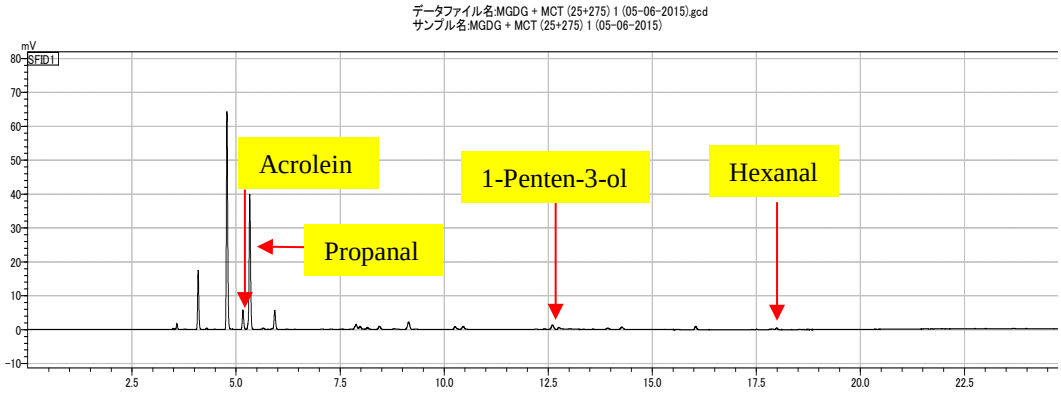
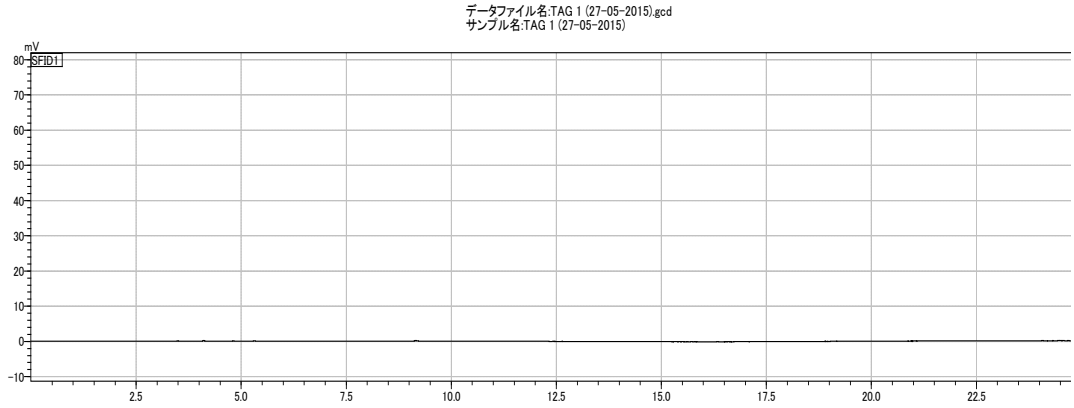


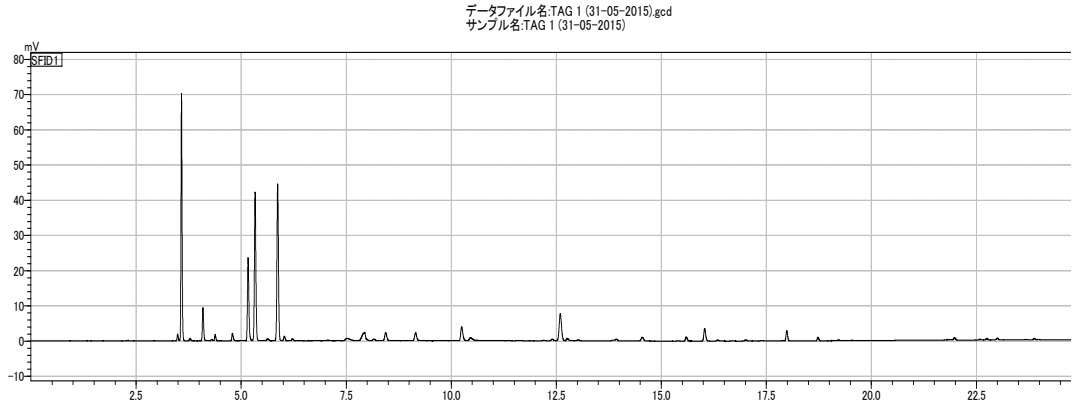
Figure 6.3: Chromatogram of GC volatile compounds from oxidized Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) at 0, 4 and 8 days of incubation at 50ºC. Volatile compounds from oxidized Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) 1-Penten-3-ol and 4) Hexanal.

6.3.4 Linseed TAG

TAG; Day 0



TAG; Day 4



TAG; Day 8

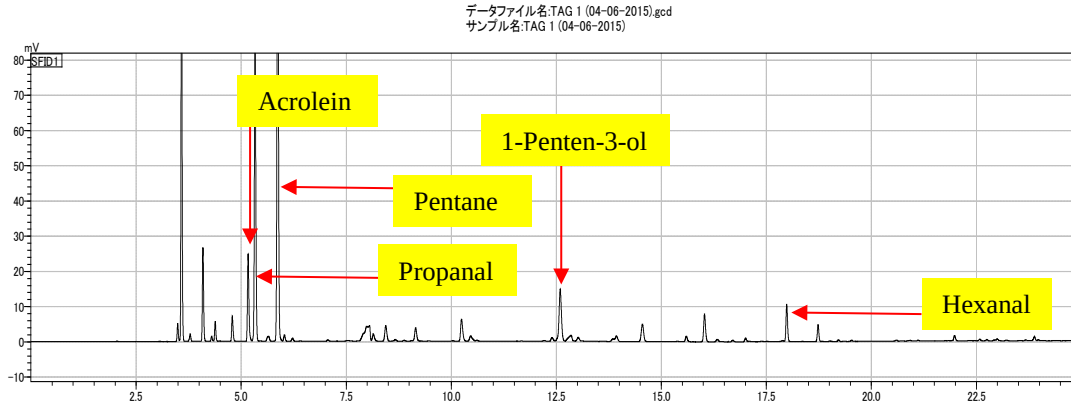
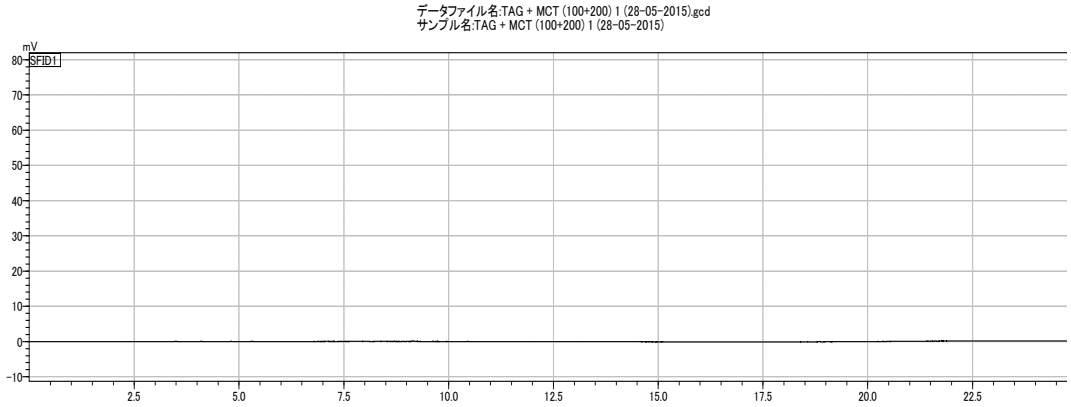


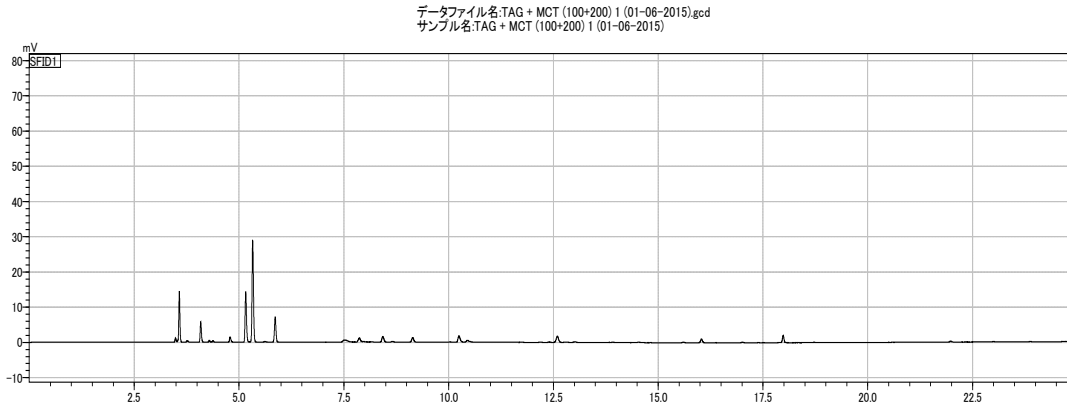
Figure 6.4: Chromatogram of GC volatile compounds from oxidized Linseed TAG at 0, 4 and 8 days of incubation at 50ºC. Volatile compounds from oxidized Linseed TAG were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) Pentane, 4) 1-Penten-3-ol and 5) Hexanal.

6.3.5 Linseed TAG (100) + MCT (200) @ Lin.TAG + MCT (67 wt %)

TAG + MCT (100 +200); Day 0



TAG + MCT (100 +200); Day 4



TAG + MCT (100 +200); Day 8

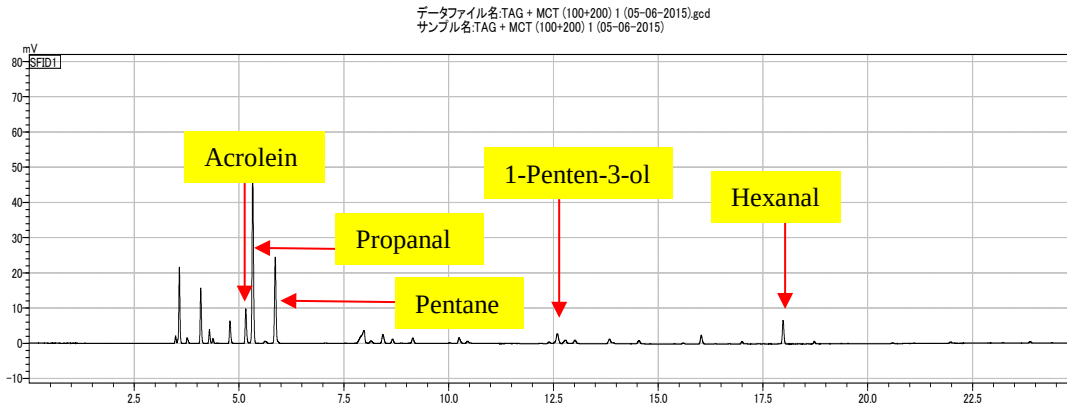
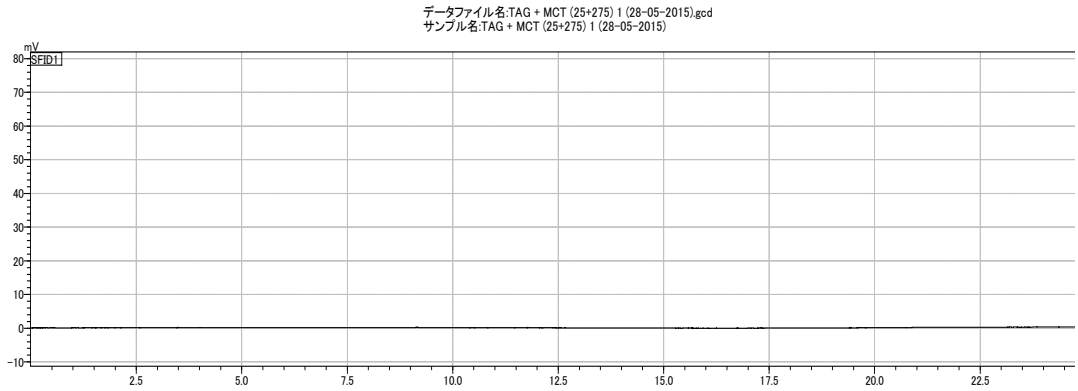


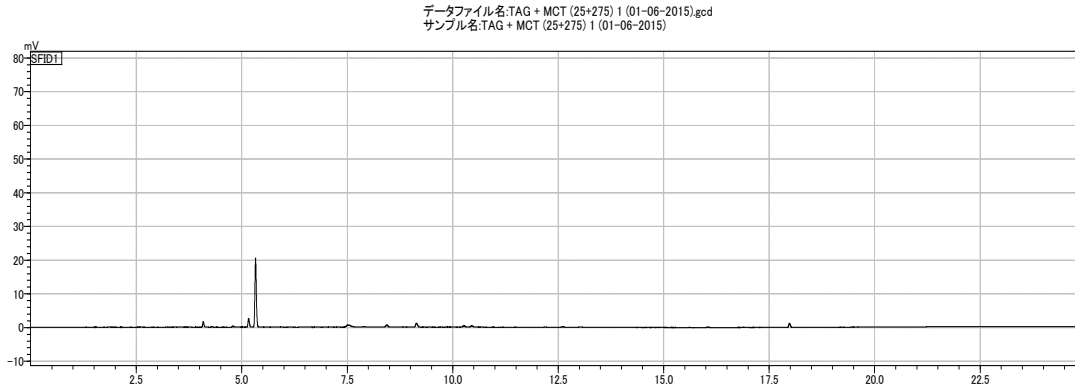
Figure 6.5: Chromatogram of GC volatile compounds from oxidized Linseed TAG (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) at 0, 4 and 8 days of incubation at 50ºC. Volatile compounds from oxidized Linseed TAG (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) Pentane, 4) 1-Penten-3-ol and 5) Hexanal.

6.3.6 Linseed TAG (25) + MCT (275) @ Lin.TAG + MCT (92 wt %)

TAG + MCT (25 + 275); Day 0



TAG + MCT (25 + 275); Day 4



TAG + MCT (25 + 275); Day 8

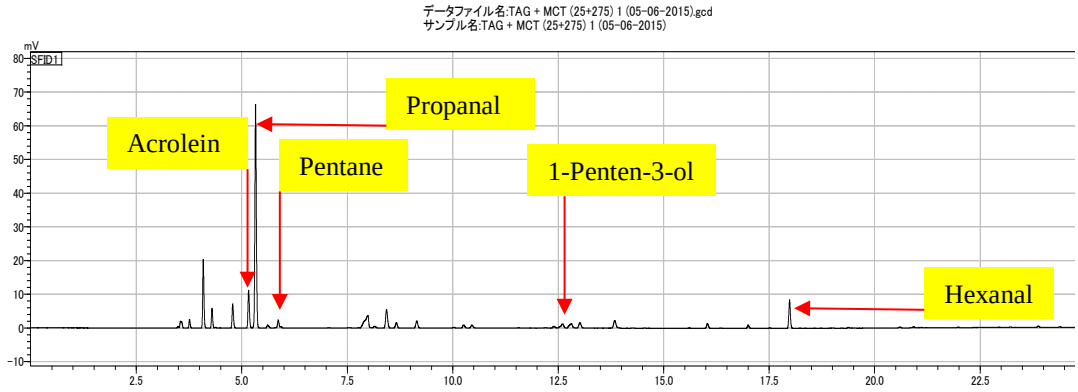


Figure 6.6: Chromatogram of GC volatile compounds from oxidized Linseed TAG (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) at 0, 4 and 8 days of incubation at 50ºC. Volatile compounds from oxidized Linseed TAG (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) were analyzed by static headspace GC. Major volatiles; 1) Acrolein, 2) Propanal, 3) Pentane, 4) 1-Penten-3-ol and 5) Hexanal.

Final total peak area of volatiles increased with the incubation of time for all samples (Figure 6.7). The next Figure 6.8; total peak area of 6 compounds; Acrolein, Propanal, Pentane, 1-Penten-3-ol and Hexanal of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG), showed that oxidised Lin.TAG having the most amount of those volatiles formed, followed by Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) as having the second most volatiles formed.

The total oxidisable compound formed from Lin.TAG, relatively increase throughout but the other five samples were shown to have increase in the initial incubation before they settled into somewhat a plateau phase and shows inclination of less volatiles forming (Figure 6.7 and Figure 6.8). Sample containing TAG mixed with the highest amount of MCT showed a much reduced in formation of volatiles, so as for samples containing Sp.MGDG mixed with the highest amount of MCT. However, samples containing Sp. MGDG mixed with considerable amount of MCT showed formation of more volatiles compared to the prime sp.MGDG only.

Oxidized Linseed TAG (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) formed the least volatiles along the incubation then followed by Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) and then followed by Spinach oxidized MGDG constituents and Linseed TAG (100) + MCT (200) @ Lin.TAG + MCT (67 wt %).

Results from the analysis of major volatile compounds using static headspace GC method are shown in Figure 6.1 to Figure 6.6. Peak intensities are used to express the amount of volatile compounds. All volatile compounds were found to be increased with a different trend on increment.

It is worth to highlight that the compound named acrolein of Lin.TAG relatively increase at initial time and however reached a plateau after a certain, given time (Figure 6.9 and 6.14). Acrolein was found to be the most abundant volatile in fish oil TAG, Shibata *et al*., (2015). The pattern of the increased amount was reported to be the same with the ones analyzed of oxidised Lin.TAG.

On the contrary, acrolein was only detected in a small amount, in all oxidized GL (MGDG) from both Spinach only and Spinach with mixed MCT samples. This important was also shown to be the distinct features which could be related to the protective mechanisms, which could also owed to the special stereochemistry configuration of GL towards PUFA (Chapter 4).

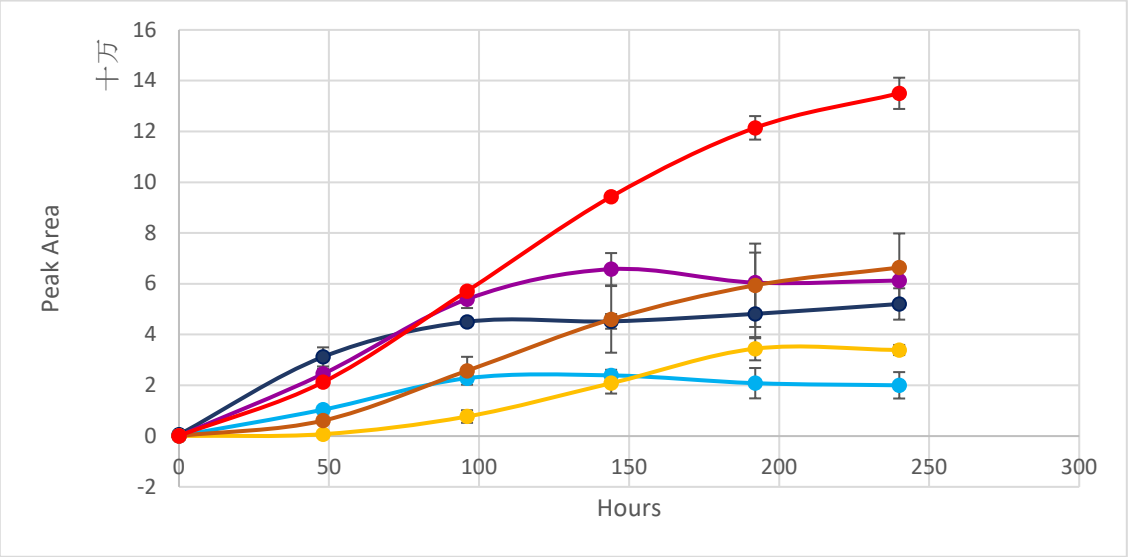


Figure 6.7: Final total peak area of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50ºC.

Value of graphs shown in Figure 6.7 are represented as mean of three independent experiments. Sp.MGDG (dark blue line), Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) (dark purple line), Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) (bright blue line), Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

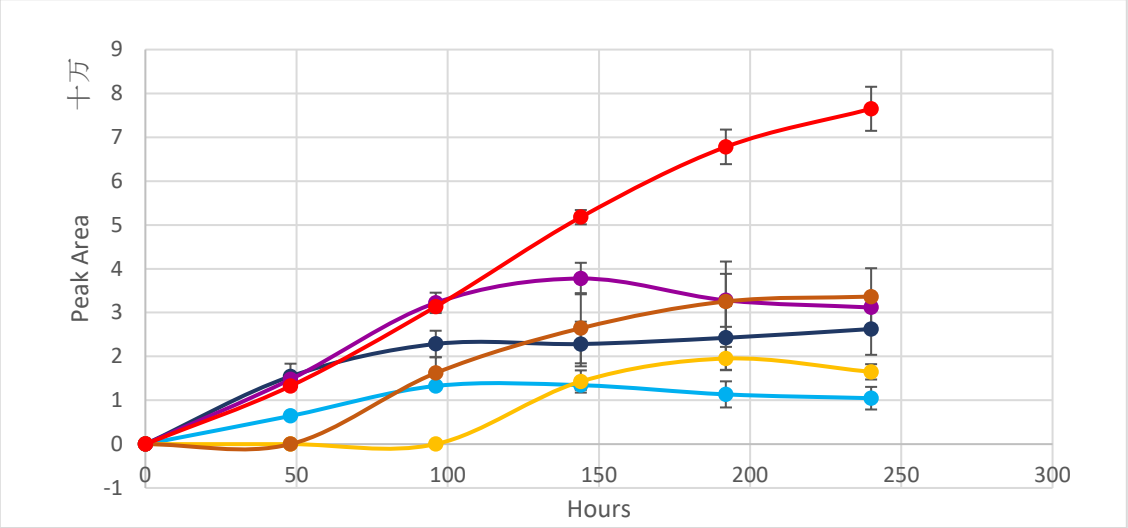


Figure 6.8: Total peak area of 5 compounds; Acrolein, Propanal, Pentane, 1-Penten-3-ol and Hexanal of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50ºC.

Value of graphs shown in Figure 6.8 are represented as mean of three independent experiments. Sp.MGDG (dark blue line), Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) (dark purple line), Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) (bright blue line), Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

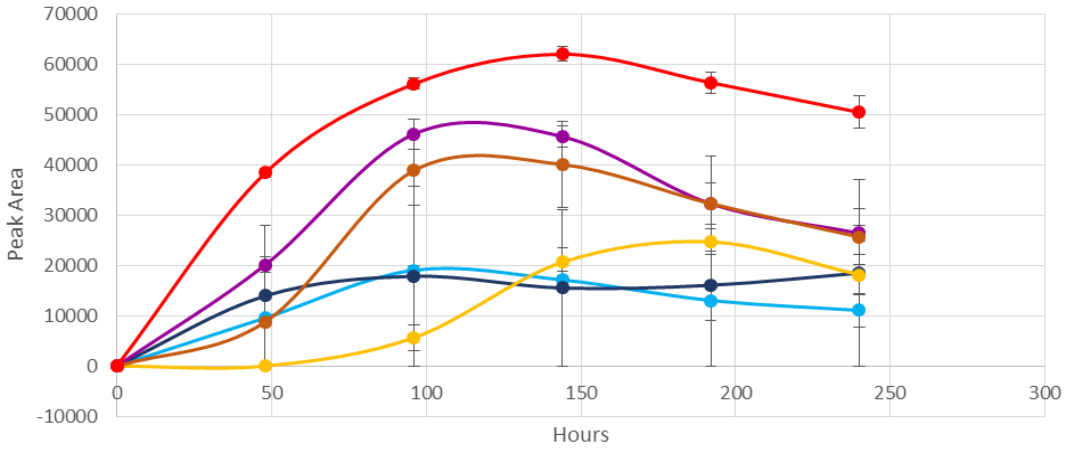


Figure 6.9: Peak area of Acrolein of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50ºC.

Value of graphs shown in Figure 6.9 are represented as mean of three independent experiments. Sp.MGDG (dark blue line), Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) (dark purple line), Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) (bright blue line), Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

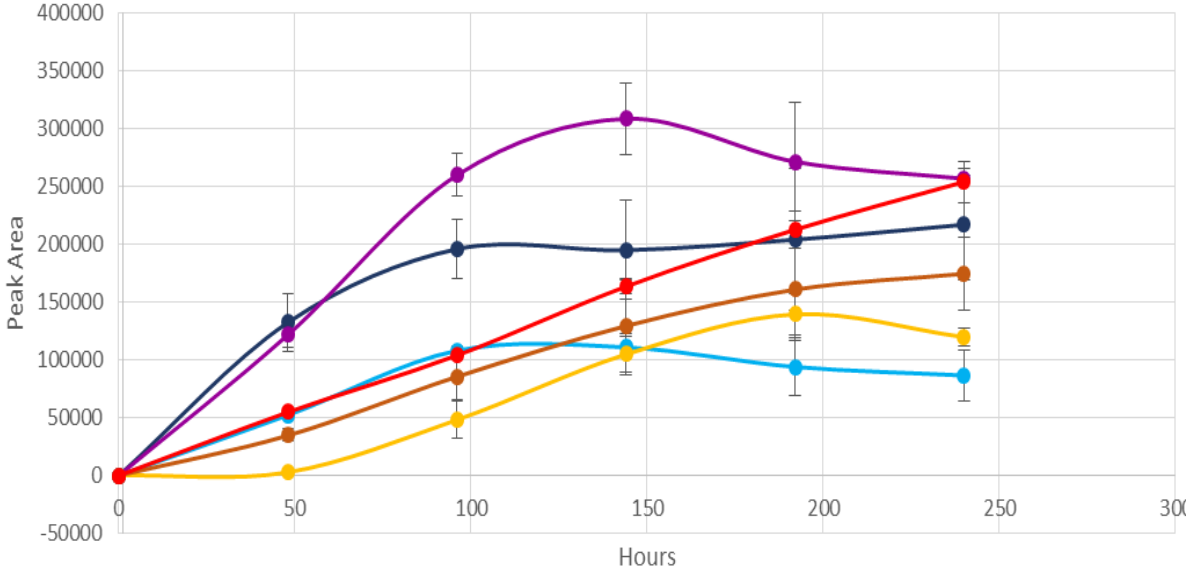


Figure 6.10: Peak area of Propanal of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50ºC.

Value of graphs shown in Figure 6.10 are represented as mean of three independent experiments. Sp.MGDG (dark blue line), Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) (dark purple line), Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) (bright blue line), Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

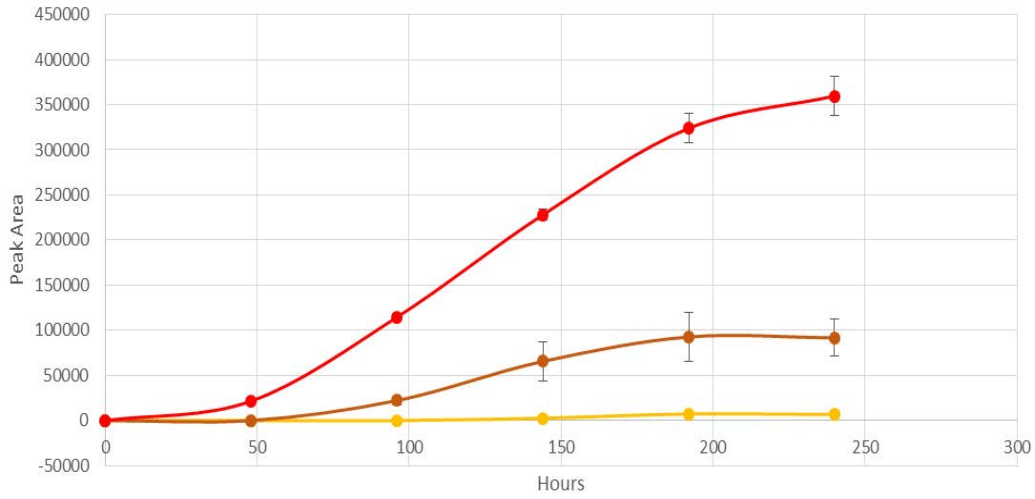


Figure 6.11: Peak area of Pentane of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50ºC.

Value of graphs shown in Figure 6.11 are represented as mean of three independent experiments. Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

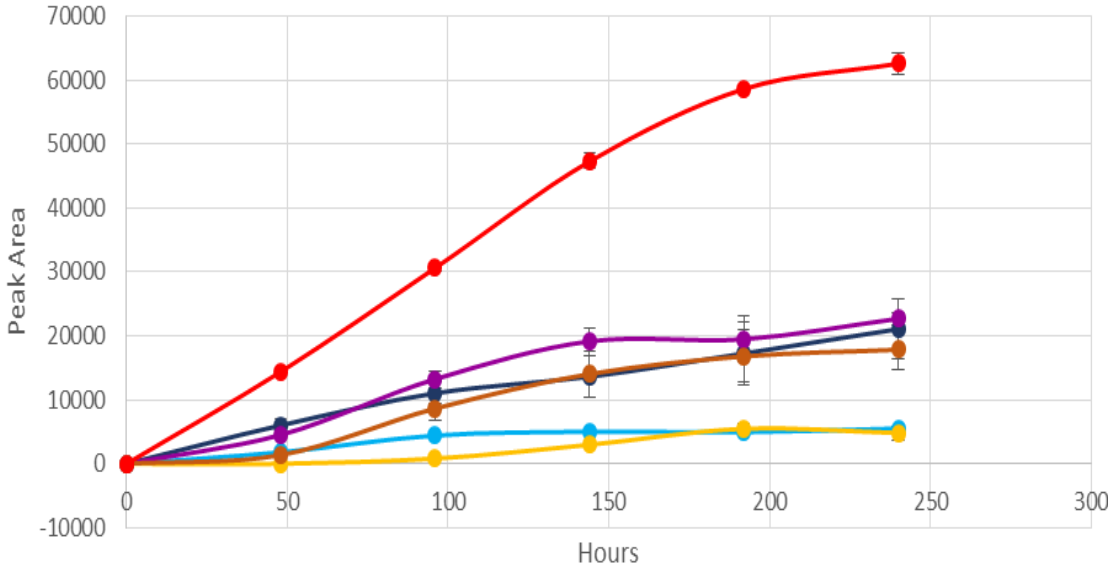


Figure 6.12: Peak area of 1-Penten-3-ol of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50ºC.

Value of graphs shown in Figure 6.12 are represented as mean of three independent experiments. Sp.MGDG (dark blue line), Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) (dark purple line), Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) (bright blue line), Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

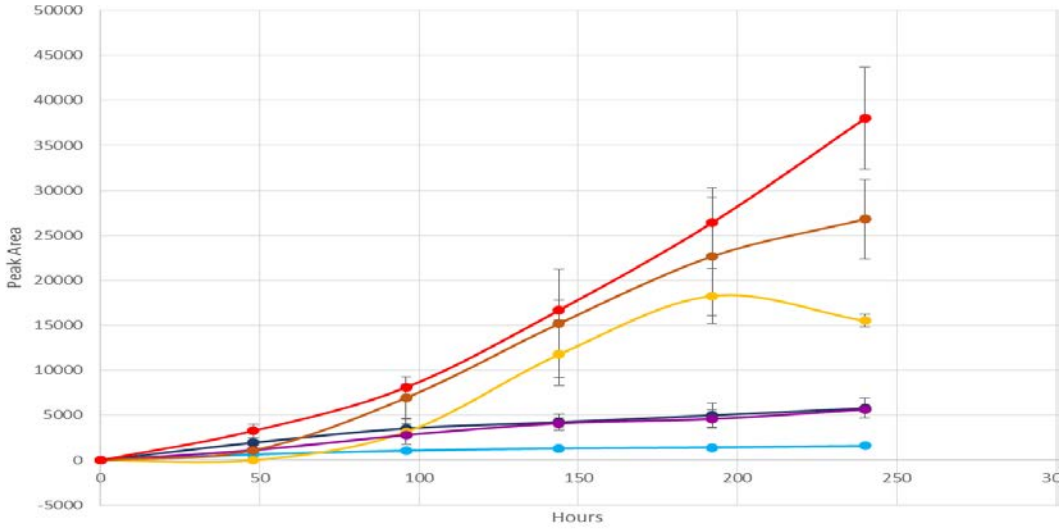


Figure 6.13: Peak Area of Hexanal of Glyceroglycolipids (MGDG) from Spinach and Linseed Oil (TAG) with the addition of different concentrations of (Medium Chain Triacylglycerol (MCT) under the dark at 50^{o}C.

Value of graphs shown in Figure 6.13 are represented as mean of three independent experiments. Sp.MGDG (dark blue line), Spinach MGDG (100) + MCT (200) @ Sp.MGDG + MCT (67 wt %) (dark purple line), Spinach MGDG (25) + MCT (275) @ Sp.MGDG + MCT (92 wt %) (bright blue line), Linseed TAG (red line), Linseed TAG + (100) + MCT (200) @ Lin.TAG + MCT (67 wt %) (brown line) and Linseed TAG + (25) + MCT (275) @ Lin.TAG + MCT (92 wt %) (dark yellow line).

Propanal was detected on the rise together with 2 samples containing GL; Sp.MGDG and Sp.MGDG (100) + (200) (Figure 6.10 and 6.14). Yet again, the peaks of acetone overlaps with the peaks of propanal for all samples containing Sp.MGDG (as mentioned previously in Chapter 4).

Pentane was detected in all samples containing Lin.TAG and not in any GL oxidized constituents (Figure 6.11 and 6.14).

Relative amount of 1-Penten-3-ol was also significant in the oxidized Lin.TAG as Snyder *et al*., (1988) reported that major volatiles found in the oxidation of Lin.TAG were pentane and propanal (Figure 6.14). He also added that these volatiles could be form due to the decomposition of the monohydroperoxides from the n-3 PUFAs, namely; 18:3n-3 and 18:4n-3, which coincides with fatty acids composition (Table 3.3. and 3.2).

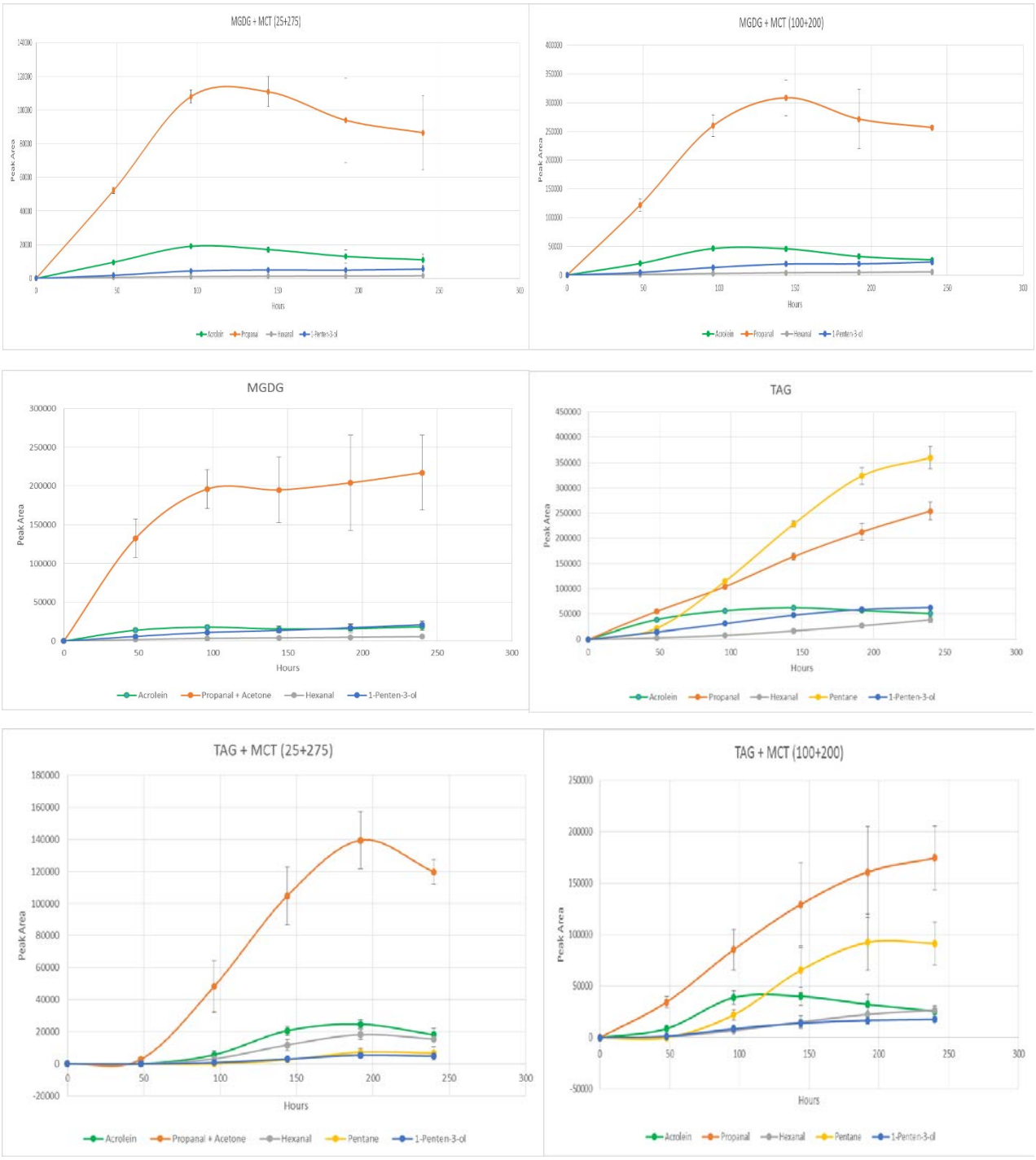


Figure 6.14: Peak area of 5 main volatile compounds according to the type of sample under the dark at 50ºC.

Value of graphs shown in Figure 6.14 are represented as mean of three independent experiments.

6.4 Conclusion

Quantification of total increase of volatiles in oxidized Spinach involving MGDG constituents, is much less complex as compared to the DGDG constituents reported in the previous chapter (Chapter 4). Sp.MGDG kept on with the distinct features of showing a protective mechanisms towards PUFA with much less volatiles formed rather than in Lin.TAG. Perhaps again, stereochemistry configuration including dilution factors of GL with other combined samples, play an important role in keeping this mechanism intactly function.

In evaluating the volatile composition of all the 3 variations of Sp.MGDG, sample mixed with considerable amount of MCT showed a peculiar contradiction when the CG profiles showed a much more volatiles produced as oppose to the prime Sp.MGDG. This could be due to some irregularities of the mix in the incubation vials or either the formation of aggregates among themselves.

Method of GC used in determining volatiles play a crucial role as relative volatile composition would change accordingly. Static headspace method is more common to determine a low molecular weight volatiles including acrolein, propanal, pentane, 1-penten-3-ol.

Chapter 7: Effect of α-Tocopherol Addition on the Oxidative Stability of Glyceroglycolipids (MGDG & DGDG) from Spinach as Compared to Linseed Oil (TAG)

7.1 Introduction

Oxidation causes the loss of quality in lipid foods. The two main compositional factors of oils which determine their susceptibility to oxidation are fatty acid composition and presence of antioxidant compounds. Lipid oxidation remains to be a highly deteriorative process in foods. It could lead to unacceptable properties for customers, a loss in nutritional value and could eventually cause serious health disorders; atherosclerosis and carcinogenesis.

Ironically, GLs of higher plants, algae and bacteria have been proven to incorporate unusually high content of PUFA, namely; α-Linolenic acid (18:3n-3), Stearidonic acid (SA,18:4n-3), Arachidonic acid (AA, 20:4n-6) and Eicosapentanoic acid (EPA, 20:5n-3) but yet exhibiting opposite response to oxidation. In the previous chapters of this research, it showed that the major constituents of these lipids, especially DGDG and MGDG from Spinach, showed a high oxidative stability as compared to Linseed Oil (TAG).

With regard to this, the addition of antioxidant in foods is crucial to retain its quality and safety (Koleva *et al*.. 2003). Adverse effects of synthetic antioxidant with the raging consumer preferences for natural products have demanded in increased interest in the application of natural antioxidants (Arabshahi *et al.*, 2007). According to Frankel, (1998), the search for using natural antioxidants have been rampant to prevent oxidative deterioration of lipids. Among natural antioxidants widely used are vitamin E, carotenoids, flavonoids, anthocyanins and phenolic compounds (Ahn *et al.*, 2008).

Antioxidants exert their effects through a few means. The mechanisms involved are through radical scavenging, metal chelation and oxygen scavenging. There are in some cases whereby some antioxidants are able to offer more than one possible mode of action. As highlighted by Frankel, (2005) antioxidative efficacy would be highly influenced by the actual location of the antioxidant in a given food matrix, which in turn, is again highly dependent on polarity and solubility of the particular antioxidant. In

reality, it takes many complicated factors to predict antioxidant efficacy in real food systems (Let *et al.*, 2007).

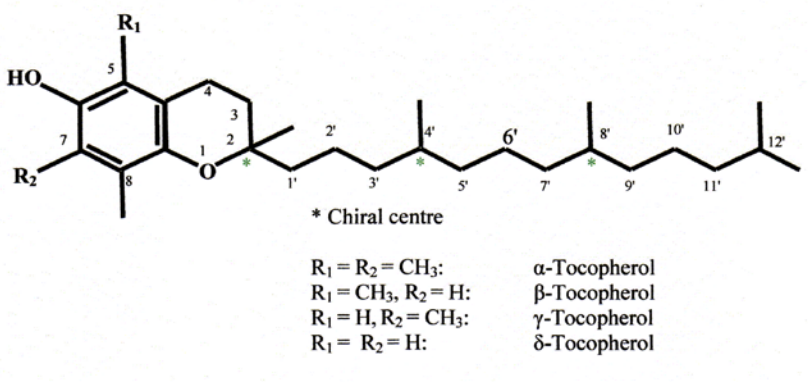


Figure 7.1: Structure of Tocopherol. Image and content adapted from Palm Neutraceuticals Sdn.Bhd. (http://www.palmnutraceuticals.com/vite.htm)

Tocopherols are among the most favored antioxidants used for its wide application in foods and cosmetics. α-tocopherols in particular has the highest biological activity. They are lipid soluble antioxidants which function as the most effective chain breaker. The ability of α-tocopherols as an antioxidant, be it neutral or pro-oxidant effect in foods depends on temperature, lipid composition, physical state (bulk phase or emulsion) and its concentration.

This chapter is intended to access the efficacy of α-tocopherol in oxidative stability and to establish its relation with both constituents of especially DGDG and MGDG from Spinach.

7.2 Materials and Methods

7.2.1 Sample

Spinach powder (GABAN Co. Ltd., Tokyo, Japan) was obtained from a local food market. Linseed oil was obtain from Summit Oil Mill Co. Ltd., Chiba Japan.

7.2.2 Referenced Compound and Reagent

Silica gel (BW-60F) for the column chromatography was purchased from Fuji Sylysia Chem. Ltd. (Kasugai, Aichi, Japan). The activated Carbon and Celite (545 RVS) were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulphoquinovosyl diacylglycerol (SQDG) standards were purchased from Lipid Products (Redhill, United Kingdom) while Triolein was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). α-tocopherol was also the product of Wako Pure Chemical Ind. Ltd., Osaka, Japan.

All other chemicals and solvents used in this study were of analytical grade and high-performance liquid chromatography (HPLC) grade solvents were used for HPLC analysis.

7.2.3 Sample Preparation

7.2.3.1 Extraction and Total Lipid Preparation of Spinach

The Spinach powder was extracted with six volumes (v/w) of methanol, followed by filtration afterwards in order to collect the filtrate. Initially, Spinach powder (*ca.* 2 kg) was soaked in methanol (12,000 mL) at room temperature, which was kept under the dark for overnight (about 16 hours). The green metanolic extract together with the residue was next filtered with a ceramic filter funnel, lined with filter paper (No. 2 Qualitative Filter Paper; 150 mm; Advantec®; Tokyo, Japan) and the green filtrate was removed from the residue under a vacuumed condition at 30 ^{o}C ± 1 ^{o}C.

The green filtrate was then pooled into a pilot scale rotary evaporator (Eyela Rotary Vacuum Evaporator N-11 and Eyela CA-2600; Tokyo Rikakikai Co. Ltd.; Tokyo, Japan) and traces of solvent remaining in the green filtrate was entirely removed in the dark, under vacuum, leaving the dark green viscous liquid obtained at the end. This dark green viscous liquid was collected with dissolving in equivalent volume of methanol which was finally designated as crude lipids of Spinach.

Crude lipids of Spinach was further dissolved into a separatory funnel using a cocktail mix solvents of chloroform-methanol-water (10:5:3, v/v/v) for a liquid-liquid distribution. After being shaken, the funnel was allowed to stand overnight. The lower layer; a mixed lipid layer with methanol and chloroform, was collected and again dissolved into a new separatory funnel. Same volume of water was again added and after being shaken, the funnel was further allowed to stand one more overnight separation (liquid-liquid distribution). The lower layer was then concentrated under vacuum, using a rotary evaporator. Remaining traces of organic solvents and water were removed in a

desiccator (about 3 days) under a high vacuum with leaving the sample in an amber coloured rotary flask. Total lipids of Spinach (*ca.* 20 g) was eventually collected with dissolving in equivalent volume of chloroform and subjected to consequent analysis or kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage).

7.2.3.2 Purification of Glyceroglycolipids from Spinach

Total lipids of Spinach which was collected with dissolving in equivalent volume of chloroform (*ca.* 20g) was first passed through a column (70 × 6 cm i.d.) packed with chloroform slurry mixture of silica gel. The whole column was wrapped with aluminium foil in order to protect the glyceroglycolipid (GL) from degradation with the exposure to light. The elution was first done with chloroform (about 3,000 mL) and then with acetone (about 13,000 L). Fractions eluted with acetone were used as GL (through a continuous elution, with appropriately adjusting the flow rate manually, for about more than a week). The first whole dark green-black fraction was designated as MGDG of Spinach (1 fraction) and the consequent fractions of every 500 mL collected with acetone (clear light green solution) were designated as DGDG of Spinach (about 24 fractions). Fractions were concentrated under vacuum, using a rotary evaporator and was collected and kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage) or till further analysis.

Both MGDG and DGDG of Spinach were subjected to preparative TLC in order to choose the exact fraction with the desired GL. All GL fractions which was collected in ethanol were spotted onto a 0.25 mm silica gel plate. The plate was developed with a cocktail mix solvents of chloroform-methanol-water (65:25:4, v/v/v) and the spots were visualized by spraying the plate with orcinol-sulfuric acid or Dittmer reagent, followed by charring (as compared to standards; MGDG, DGDG & SQDG).

The absence of chlorophyll was also confirmed in all consequent fractions containing DGDG from prior TLC. Therefore, only the first whole fraction obtained; MGDG of Spinach (*ca.* 4 g) was again refined using a carbon column (70 × 6 cm i.d.) packed with 90 % of ethanol in chloroform slurry of activated carbon (*ca.* 250 g) in order to remove majority traces of chlorophyll. Carbon was first heated at 110 ^{o}C for 1 ½ hours prior to mixing with 90 % of ethanol in chloroform. Elutions were done with gradually decreasing concentration of ethanol; 90 % of ethanol in chloroform (about 1,000 mL),

then 50 % ethanol in chloroform (about 1,500 mL) and finally 100 % of chloroform (about 10,000 mL). The fractions eluted with 50 % ethanol in chloroform and 100 % of chloroform were both used as MGDG of Spinach (clear light yellow solution) after consequent fractions of every 500 mL. Fractions were concentrated under vacuum, using a rotary evaporator and was collected and subjected to further analysis or kept in -30 ^{o}C freezer with equivalent volume of ethanol (for long term storage).

MGDG of Spinach were again subjected to preparative TLC. All MGDG fractions which was collected in ethanol were spotted, developed and visualized using the same procedure as described earlier, except that only MGDG was used as standard.

7.2.3.3 Purification of TAG from Linseed Oil

Linseed oil undergone a direct double purification process with 2 types of system. In order to remove tocopherols and pigments, linseed oil (*ca.* 25 g) was first passed through a column (50 × 4 cm i.d.) packed with *n*-hexane slurry mixture of activated carbon (100 g) and Celite (100 g). The carbon was first heated at 110 ^{o}C for 1 ½ hours prior to mixing with Celite and *n*-hexane. The only elution was done with *n*-hexane (1,200 mL).

The obtained oil (*ca.* 10 g) was again refined using a silicic acid column (50 × 4 cm i.d.) packed with *n*-hexane slurry of silica gel (200 g). Elutions were done with *n*-hexane (200 mL), after that a mixture of *n*-hexane-diethyl ether (98:2, v/v) of 200 mL and again another mixture of *n*-hexane-diethyl ether (90:10, v/v) of 1,200 mL. The final fraction eluted with *n*-hexane-diethyl ether (90:10) was used as TAG.

In order to confirm the absence of impurities, the TAG fraction was subjected to preparative TLC. The lipid fraction was spotted onto a 0.25 mm silica gel plate. The plate was again developed with a cocktail mix solvents of *n*-hexane-diethyl ether (60:40, v/v) and the spot were detected with iodine vapour or 60% aqueous sulfuric acid charring (as compared to standard; Triolein).

7.2.3.4 Tocopherol Analysis of Spinach and Linseed TAG

Tocopherol analysis was performed for all types of purified lipids; Spinach (MGDG & DGDG), including Linseed (TAG) with a Hitachi HPLC system equipped with a pump (Hitachi L-2130) and a fluorescence detector (Hitachi L-2485). The analysis

was conducted on a silica column (Si 60, 250 × 4.6 mm i.d.; Kanto Chemical Co., Tokyo, Japan) protected with a guard column (15 × 3.2 mm) with the same stationary phase. The mobile phase was n-hexane-2-propanol (99.2:0.8, v/v) with a flow rate of 1.0 mL/min. The fluorescence detector was set at Ex. 298 nm and Em. 325 nm.

7.2.3.5 Oxidation Analysis of Purified Lipids

α-tocopherol was dissolved into n-hexane. A certain volume of tocopherol solutions was added to all purified lipids with each 100 mg of Spinach (MGDG & DGDG), including Linseed TAG making it a total of 9 types of lipid samples as follows:

1) Lin.TAG
2) Lin.TAG + α-Toc (0.05%)
3) Lin.TAG + α-Toc (1%)
4) Sp.MGDG
5) Sp.MGDG + α-Toc (0.05%)
6) Sp.MGDG + α-Toc (1%)
7) Sp.DGDG
8) Sp.DGDG + α-Toc (0.05%)
9) Sp.DGDG + α-Toc (1%)

All samples were placed in a 2 mL aluminium sealed vial with a butyl gum septum (GL Science; Tokyo, Japan) and then incubated at 50 °C in the dark. Before the incubation, the level of oxygen in the headspace gas of the vial was estimated using a GC system (Shimadzu GC-14B) equipped with a thermal conductivity detector and a stainless steel column (3 m × 3.0 mm i.d.) packed with a molecular sieve 5A (GL Science) according to the method described by Cho *et al.* (1987). The temperatures at the injection port, detector port, and column oven were 120, 120, and 70 °C, respectively. The helium flow was 50 kPa. Three separate vials containing similar samples were prepared and incubated. A small portion (20 μL) of the headspace gas was taken from each vial using a microsyringe through the butyl gum septum at selected times during the oxidation. The decrease (%) in the oxygen was calculated from the changes in the oxygen to nitrogen ratio compared with the ratio before incubation. Three replicate

measurements of each data and value at different oxidation times of the stored samples were expressed as the mean ± SD (n = 3).

7.3 Results and Discussion

When oxidative stability of all the 9 types of lipids samples; 3 types of TAG mix; (1) Lin.TAG, (2) Lin.TAG + α-Toc (0.05%) and (3) Lin.TAG + α-Toc (1%), 3 types of MGDG mix; (4) Sp.MGDG, (5) Sp.MGDG + α-Toc (0.05%) and (6) Sp.MGDG + α-Toc (1%) also 3 types of DGDG mix; (7) Sp.DGDG, (8) Sp.DGDG + α-Toc (0.05%) and (9) Sp.DGDG + α-Toc (1%) were compared by measuring the decreased in oxygen concentration, the stability was found to be the highest for DGDG sample when mixed with tocopherol; (8) Sp.DGDG + α-Toc (0.05%) with the indication of blue solid square while the lowest stability was markedly showed by prime Lin.TAG (without any addition of α-tocopherol, with dramatic decreased in graph, indicated by the dark blue line in Figure 7.2.

Referring to the analysis of the oxygen consumption rate, the addition of α-tocopherol increased the oxidative stability of Sp.DGDG. A more significant antioxidative activity was obtain by only adding 0.05% of α-tocopherol. Instead, with the addition of more amount of the mentioned antioxidant substance (1%), proved to lead to otherwise; resulted in a more reduced in stability. This could be clearly deduced from Figure 7.2.

Based on the declining trend of Sp.DGDG, the sample Sp.DGDG + α-Toc (0.05%) showed a final stability of 91 %, even after about 600 hours of oxidation; a total of 12 % increase in oxidative stability with the addition of the mentioned antioxidant. While the sample Sp.DGDG itself showed gradual decline, following closely after the most stable sample as aforementioned. On the other hand, Sp.DGDG + α-Toc (1%) showed an inferior stability with a decline difference of 27 % as compared to the prime Sp.DGDG. The sequence of oxidative stability of GL with DGDG constituents in this research could be summarized as follows:

Sp.DGDG + α-Toc (0.05%) > Sp.DGDG > Sp.DGDG + α-Toc (1%)

Consequently, attributing to the decreased oxygen level in the headspace with Sp.MGDG, the sample Sp.MGDG + α-Toc (0.05%) showed a stability of 68 %, after about 96 hours of oxidation; a total of 40 % increase in oxidative stability as compared to the prime Sp.MGDG with the addition of the mentioned antioxidant before continued to level of at 37 % for after about 384 hours. Sp.MGDG + α-Toc (1%) also reached a total of 37 % earlier, at about after only 192 hours. Both samples of Sp.MGDG with the addition of α-tocopherol, showed an increased in oxidative stability as compared to the prime Sp.MGDG. Samples of MGDG without any addition of the mentioned antioxidant showed somewhat sharp decline with a slight difference in the declining trend with Lin.TAG. The sequence of oxidative stability of GL with MGDG constituents in this research could be summarized as follows:

Sp.MGDG + α-Toc (0.05%) > Sp.MGDG + α-Toc (1%) > Sp.MGDG

GLs with DGDG constituents were again generally shown to be more stable as compared to their MGDG constituent when they steeply differ between each other in their declining trend; Sp.MGDG marked with the bright blue line while Sp.DGDG marked with the yellow line, according to Figure 7.2.

With regard to Lin.TAG, both samples with the addition of α-tocopherol showed the same sequence in declining trend; the more stable being Lin.TAG + α-Toc (1%) then followed by Lin.TAG + α-Toc (0.05%). Effect of the addition of the aforementioned antioxidant, significantly increase oxidative stability of the latter to 70 % compared to the prime Lin.TAG at about after 96 hours before further experienced a dramatic decrease at after 168 hours as observed. The former also increased in stability and remained to be more stable throughout till about after 432 hours. The sequence of oxidative stability of TAG in this research could be summarized as follows:

Lin.TAG + α-Toc (1%) > Lin.TAG + α-Toc (0.05%) > Lin.TAG

Under common circumstances, unsaturated fatty acids would be competing with tocopherol for lipid peroxy radicals. Peroxy radical would be receiving the hydrogen atom from tocopherol by the 6-hydroxy group on its chroman ring. Jung & Min, (1990)

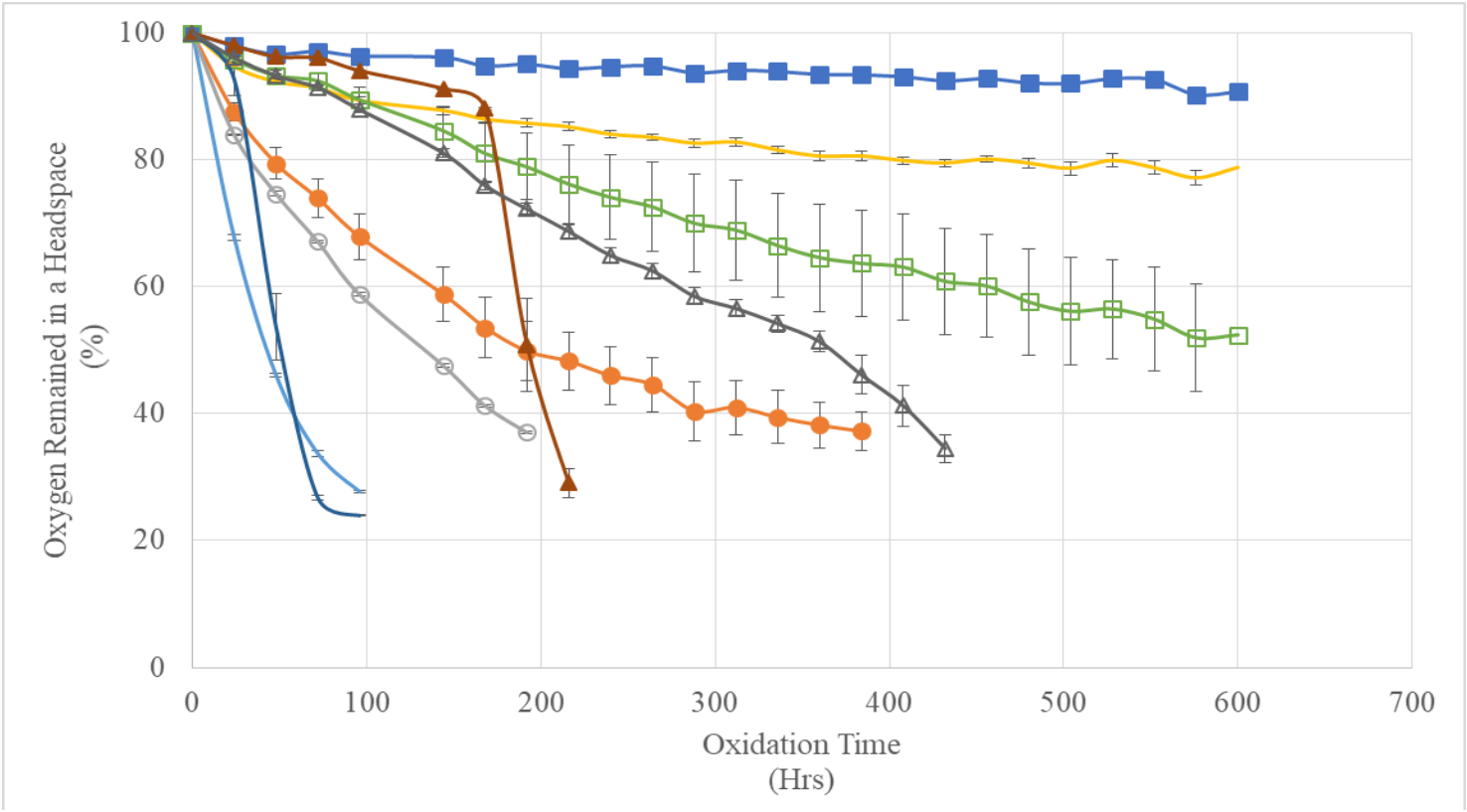


Figure 7.2 : Oxidative stability of Glyceroglycolipids (MGDG & DGDG) from Spinach as compared to Linseed Oil (TAG) with the addition of α-toc under the dark at 50°C.

Value of graphs shown in Figure 7.1 are represented as mean of three independent experiments.
Lin.TAG (dark blue line), Lin.TAG + α-Toc (0.05%) (dark brown solid triangle), Lin.TAG + α-Toc (1%) (dark grey open triangle).
Sp.MGDG (bright blue line), Sp.MGDG + α-Toc (0.05%) (orange solid circle) and Sp.MGDG + α-Toc (1%) (light grey open circle).
Sp.DGDG (yellow line), Sp.DGDG + α-Toc (0.05%) (blue solid square) and Sp.DGDG + α-Toc (1%) (green open square).

GL (Glyceroglycolipids); MGDG (Monogalactosyl diacylglycerol); DGDG (Digalactosyl diacylglycerol); TAG (Triacylglycerol).

mentioned that, since tocopherol poses a reduction potential of 300-400 mV, it readily donates hydrogen to lipid peroxy radical (ROO$^{\bullet}$) to further produces lipid hydroperoxide (ROOH) and tocopheroxy radical (T$^{\bullet}$).

$$TH + ROO^{\bullet} \rightarrow T^{\bullet} + ROOH^{\bullet}$$

Tocopheroxy radicals (T$^{\bullet}$) are more stable than lipid peroxy radicals (ROO$^{\bullet}$), because of their resonance structures. As researched by Niki *et al.*, (1984), Choe *et al.*, (2005) and Naumov *et al.* (2003), reaction rate of α-tocopherol with lipid peroxy radical is shown to be 10^5-10^6 times faster than that of unsaturated lipid with peroxy radical. Radicals from the oxidizing fatty acids would be taken away by tocopherols and this would prevent radical chain reaction from progressing further. Kamal-Eldin & Appelqvist, (1996), highlighted that, a tocopherol molecule could protect over 10^3-10^8 polyunsaturated fatty acid molecules at low peroxide value. Tocopheroxy radicals (T$^{\bullet}$) as stated by Kamal-Eldin & Appelqvist, (1996), could also interact with each other among themselves or exert reaction with other compounds. All these reaction would depend on their lipid oxidation rates. Tocopheroxy radicals (T$^{\bullet}$) may eventually have reaction with lipid peroxy radicals and form nonradical products, as elaborated by Kamal-Eldin & Appelqvist, (1996).

$$T^{\bullet} + ROO^{\bullet} \rightarrow T - OOR^{\bullet}$$

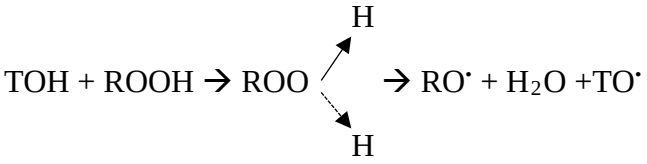
On the overall, the effectiveness of tocopherol as antioxidants depends on the chemical and physical characteristics such as the structural characteristics of tocopherol, bond dissociation energy and then finally, the reduction potential.

On the contrary, tocopherols could also act as prooxidant, as reported by many; Reische *et al.*, (2002), Gregory (1996), Verleyen *et al.*, (2001) and Kamal-Eldin & Appelqvist (1996), depending on temperature, pH, concentration, presence of other compounds near tocopherols and their chemical characteristics. This finding has been proposed by Terao & Matsushita, (1986), to be induced by hydrogen abstraction between the tocopheroxy radical (T$^{\bullet}$) and lipid molecules or lipid hydroperoxides.

$$RH + T^{\bullet} \rightarrow TH + RO^{\bullet}$$

$$ROOH + T^{\bullet} \rightarrow TH + ROO^{\bullet}$$

Kamal-Eldin & Appelqvist, (1996), also reported that reaction rate constants of α-tocopheroxyl radicals with polyunsaturated fatty acids or with hydroperoxides of polyunsaturated fatty acids were reported to be very slow as compared to the antioxidative reaction of α-tocopherol and the termination reaction of lipid autooxidation. This however, is not sufficient to fully explain the prooxidant effect of α-tocopherol. Inspite of this, an alternative prooxidant mechanism of tocopherol has been suggested where it is more significant in the presence of high levels of hydroperoxides, as earlier reported by Hicks & Gebicki, (1981). Hydrogen bonding between tocopherol (TO-H) and lipid hydroperoxide (RO-O-H) are involved in this reaction mechanisms. Hydrogen is eventually abstracted by the peroxide from the tocopherol, and the O-O bond in the peroxide is cleaved. Eventually, alkoxyl radical (RO$^{\bullet}$) is formed and thus propagates lipid oxidation.



In the presence of molecular oxygen, tocopherols would be degraded as a result from the loss of antioxidant activity which leads to the prooxidant effect of the oxidized tocopherol product. Jung and Min (1992), reported that the addition of oxidized of α-, γ- and δ-tocopherols to soybean oil, lowered the oxidative stability. Even strong oxidizing agents; chromic acid, nitric acid and ferric chloride which oxidized tocopherols, would produce lactones, quinines, and many other degradation products as elaborated by Kamal-Eldin & Appelqvist, (1996). Among the oxidation products of α-tocopherols as reported by Fautsman *et al.,* (1999) and Liebler *et al.*, (1996) are; α-tocopherolquinone, α-tocopherolhydroquinone, 4a,5-epoxy-αtocopherolquinone and 7,8-epoxy-α-tocopherolquinone. Also, it was suggested that increased levels of oxidized α-tocopherol would also probably resulted in increased levels of intermediate radicals. This increased levels would give rise to lipid oxidation, (Rietjens *et al*.; 2002).

The degree of tocopherol being an antioxidant or prooxidant substance, significantly dependent on the concentration of it. Generally, the action of tocopherols triumphs at lower concentration where it would increase oxidative stability and would likewise acts as the opposite at a higher concentration. Jung & Min, (1990), Bowry &

Stocker (1993), Evans *et al.*, (2002) and Yoshida *et al.*, (2003) showed that an increase of hydroperoxide levels and conjugated dienes due to a high level of α-tocopherol would result in prooxidant activity.

The higher the concentration of α-tocopherol in lipids, the higher the amount of intermediate radicals namely; alkyl, alkoxyl and peroxy radicals formed from tocopherol oxidation during storage of lipids. These intermediate radicals are able to initiate lipid oxidation. By detailing all these, it is vital to prevent the oxidation of tocopherol and to remove the oxidized tocopherols.

In determining the efficacy measurement test of antioxidant substance could at some points, lead to ambiguities in measurements and interpretation of results. The nonlinear synergistic and antagonistic effects may arise when the substance are mixed together. Laguerre *et al.* (2007) stressed that these substances, may unknowingly exert some tendency which may be induced due to their efficacy when analyzed. Referring to Uri, (1961), synergism in general is the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than that which would be derived from a simple additivity concept. While antagonism may be defined by having adverse effect even with more addition of antioxidant substance. Laguerre *et al.* (2007) deduced four main types of observed in many occasions, and they are as follows:

1) Synergy by regeneration of highly active antioxidants by less active forms (synergy between α-tocopherol with some phenolic compounds)
2) Synergy created through the interaction of antioxidants with different mechanisms of action (singlet oxygen quenchers and chain breaking antioxidants)
3) Synergy through interaction of antioxidants with different polarities in multiphase media (ascorbate-induced regeneration of α-tocopherol)
4) Synergy between antioxidants and substances without any antioxidant activity (bovine serum albumin)

The nonlinear synergistic and antagonistic effects clearly highlights the need to assess the situation on global (mixed samples) and individual (isolated substances) levels.

7.4 Conclusion

It is interesting to highlight here that, compounds such as glycerophospholipids (PL) and sphingolipids (SL); with respect to the amine containing polar lipids, have been reported by literature to have positive influence in inhibiting oxidation of PUFAs with the presence or perhaps addition of α-tocopherol. On the other hand, Lu *et al.,* (2011) and Shimajiri *et al*., (2013) reported that only trivial antioxidant activity is found in these polar lipids without α-tocopherol. Although the detail mechanism involved for the combination effect of these compounds are readily unavailable at this moment, this amine containing polar lipids, taking the example of dihydrosphingosine (d18:0) has been assumed to have important role as a hydrogen or electron donor which is responsible to regenerate and recycle tocopheroxyl radical intermediate to the initial phenol; tocopherol as observed by Takenaka *et al.* (2007).

The scarce literature on GL makes it more challenging to determine precise antioxidative function and mechanism of these lipids. Perhaps another possible assumption of these polar compounds as a source of antioxidants would formed between the interaction sugar constituents of the GL with the oxidised lipids. This might be the reason for the increased in stability of especially DGDG also MGDG. Therefore, the formation of antioxidant compound from both the interaction of oxidised lipids and sugar constituents might be enhanced with the addition of α-tocopherol. Oxidation condition controlled by mild addition α-tocopherol may be important for the formation of antioxidant compounds in DGDG. While in MGDG, similar protective mechanisms is possible however, with a little more than mild addition of α-tocopherol required.

Although the structure of the antioxidant compounds have not been elucidated, they could effectively inhibit DGDG and MGDG oxidation by probably regeneration of α-tocopherol in order to inhibit the lipid oxidation or through a direct inhibition of lipid oxidation.

When α-tocopherol was added to TAG; Lin.TAG + α-Toc (0.05%), a steady decrease then a steep decline of especially after about 168 hours were observed. This again, suggest the formation of antioxidant compounds during the first stage of TAG incubation, before it drastically slump in decline.

Chapter 8: Conclusion

Different chemical mechanisms are responsible for the oxidation of fats and oils during processing, storage, and cooking. Two types of oxygen, atmospheric triplet oxygen and singlet oxygen, can react with fats and oils. Triplet oxygen, having a radical character, reacts with radicals and causes autoxidation. The non-radical electrophilic singlet oxygen does not require radicals to react with; it directly reacts with the double bonds of unsaturated fats and oils with high electron densities, which is called type II photosensitized oxidation.

Plant leaves contain up to 7 % of lipid per dry weight. Major constituents which made up these lipids are monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG) and sulfoquinovosyl diacylglycerol (SQDG). These constituents, collectively called Glyceroglycolipids (GL), made up the photosynthetic membrane (chloroplast; in the layers of the thylakoids) of higher plants, algae and bacteria. The fatty acid composition of each of these GLs have been proven to have unusually high in polyunsaturated fatty acid (PUFA) namely; α-Linolenic acid (18:3n-3) as the main fatty acid. In seaweeds, the main fatty acid composition comprise of Stearidonic acid (SA,18:4n-3), Arachidonic acid (AA, 20:4n-6) and Eicosapentanoic acid (EPA, 20:5n-3). All these PUFA are easily oxidized as they are structured in the chloroplast. Continual exposure to oxidative stress with involving in the absorbtion of light energy would surely exhaust the fatty acid through oxidation. However, instead of it being oxidized, the opposite response was found to happen. Protective mechanisms underlining this contradictory response are still being research upon.

On the same note, oxidation decreases consumer acceptability of foods by producing low-molecular-weight off-flavor compounds, as well as by destroying essential nutrients, and it produces toxic compounds and dimers or polymers of lipids and proteins. Oxidation of foods can be minimized by removing prooxidants such as free fatty acids, metals, and oxidized compounds, and by protecting foods from light.

The major objectives, as listed in **Chapter 1**, of the present study were:-

a) To determine and evaluate the lipid class, fatty acids and bis-allylic content

b) To establish and compare oxidative stability between Glyceroglycolipids (GL) and Triacylglycerol (TAG)
c) To analyze volatile compounds formed throughout the oxidation process
d) To assess the efficacy of Glyceroglycolipids (GL) with other compounds as a source of potential for halal ingredients in food

Chapter 2 reviews some background of the underlying studies pertaining to cell membranes, photosynthesis, thylakoids, chloroplast, glyceroglycolipids, oxidation and a brief understanding of the global 'Halal' scenario and basic of between Kosher and 'Halal' diet.

Chapter 3 is on the oxidative stability of glyceroglycolipids (GLs) namely monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) from Spinach and Akamoku including Linseed triacylglycerol (TAG) were compared after being oxidized at 50 ^{o}C under the dark, in the first setting of oxidation analysis. Results from the analysis of oxygen consumption and the effect of polyunsaturated fatty acid (PUFA) composition, demonstrated that the Spinach DGDG had the highest oxidative stability followed by Akamoku DGDG, Akamoku MGDG, Spinach MGDG and Linseed TAG. The results however, were in dispute with the expected average number of bis-allylic positions of each GLs and TAG. DGDG constituents of GLs from both Spinach and Akamoku were more stable than their MGDG constituents. It was predicted that the stereochemistry configuration and assembly of the molecules might be most probably the main reason of the high in stability.

Chapter 4 utilizes the static headspace methods which is found to be an effective method in volatile analysis. This analysis was done in accordance to the same type of sample with was investigated in the previous chapter, chapter 3. Since the analysis involved 2 constituents of GL; both Spinach and Akamoku MGDG and DGDG, although in the previous chapter shows that DGDG is more stable as compared to the MGDG constituents, the volatiles formed in this chapter was in contrary much higher in the DGDG. All samples was especially analysed for the 5 major volatiles; 1) acrolein, 2) propanal, 3) pentane, 4) 1-penten-3-ol, 5) hexanal. Some distinct features was

highlighted as all constituents of GL revealed to be very low and much reduced amount of acrolein as compared to Linseed TAG. Also especially in DGDG, some un-identified compounds were found which to be predicted as either impurities that might cause the sample to form a high amount of hexanal or this could either form as a result of the reaction of sugar breakdown (sugar moieties of GL) throughout the incubation. Overlapping peaks between acetone and propanal was also observed for all GL samples but a clear separation was observed in Linseed TAG. Volatile compounds was managed to be quantified but yet with many incomprehensible involve with the much complexity that DGDG is having.

Chapter 5 deals with a second setting of oxidation analysis, 6 types of lipids (only with Spinach MGDG and Linseed TAG with some samples mixed to medium chain fatty acid (MCT) in varying concentrations were compared under the same condition mentioned. Oxidative stability were compared with regard to bulk oil system and during the presence of spread and mixed molecules. Spinach MGDG with MCT 92 wt % managed to delay the oxidation process the most, followed by Spinach with MCT 67 wt %. Same goes to samples of Linseed TAG when added with MCT 92 % managed to delay oxidation process the most, followed by Linseed TAG when added with MCT 67 %. Oxidative stability of both GL and TAG in mix samples were shown to be much more stable as compared separately to their bulk oils system. By understanding the physical nature of how bulk oil influence lipid oxidation reactions, more effective ways in applying existing antioxidant ingredients and to new development of antioxidant technologies could be achieved.

Chapter 6 was also done in accordance to the setting of oxidation analysis from the previous chapter; Chapter 5. With still using the static headspace method in volatile analysis. Analysis involved only one constituents of GL; Spinach MGDG (variation of mixed samples with MCT and Linseed TAG (also in the same mix ratio). The volatiles formed in this chapter was in accordance to the oxidative stability; where Linseed TAG was found to have much volatiles forming. All samples was again analysed for the 5 major volatiles; 1) acrolein, 2) propanal, 3) pentane, 4) 1-penten-3-ol, 5) hexanal. All samples variation of Spinach MGDG retained its distinct features by having low and

much reduced amount of acrolein as compared to Linseed TAG. Overlapping peaks between acetone and propanal was still observed for all Spinach MGDG samples and again a clear separation was observed in Linseed TAG. Volatile compounds was managed to be quantified with much ease unlike the complexity involved with DGDG. Another unique behavior of MGDG when mixed with considerable amount of MCT, was there could probably be some aggregates depending on the dilution factor (ratio) between them.

Chapter 7 finally access the effect of α-tocopherol in different concentration to the oxidative stability of both Spinach MGDG and DGDG and Linseed TAG. 9 samples was prepared; 3 variation mixed from Spinach MGDG, 3 variation mixed from Spinach DGDG and 3 variation mixed from Linseed TAG. 3 sets of result were evaluated as both GLs and the TAG have different response on the efficacy of α-tocopherol being an antioxidant and prooxidant. With having a low amount of α-tocopherol mixed to Spinach DGDG, resulted in elevated oxidative stability. On the other hand, with more α-tocopherol mixed to it, they exert antagonistic effect. Spinach MGDG slightly differ in the response. While both less and more amount of α-tocopherol mixed resulted in synergistic effect, the sample containing the least α-tocopherol has the most stability among this particular constituents. Last but not least, both much and less α-tocopherol when mixed with Linseed TAG, showed a higher in oxidative stability and it favors much α-tocopherol in order to exert the stability.

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