Oxidative Stability of Blackberry, Black Raspberry and Blueberry Seed Oils as Affected by their Structural Features, Minor Components, and Storage Conditions

by

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ABSTRACT

Cold-pressed blackberry, black raspberry, and blueberry seed oils were evaluated for their fatty acid composition, positional distribution of fatty acids within triacylglycerols, triacylglycerol (TAG) profile, total phenolic content (TPC), tocol profile, and oxidative stability (in the dark at 60°C and under fluorescent light at 27 °C), before and after the removal of their minor components. All three berry seed oils were stripped of their minor components using a silicic acid column and a hexane/methanol solvent partitioning system, respectively. Accelerated oxidations under Schaal oven and photooxidative conditions demonstrated that the oxidative stability of non-stripped, and column-stripped oils was different. Tocopherols and tocotrienols, collectively known as tocols, were determined by high performance liquid chromatography, and found to affect the stability of oils by donating hydrogen atoms in the dark at 60°C. Pigments, including carotenoids and chlorophylls, were also measured by UV-visible spectrophotometry and were found to influence the stability of the oils. The stability of the oils was dictated by their fatty acid composition, TAG profile, and the type and quantity of their total tocols and pigments present.

All tested seed oils contained significant levels of α-linolenic acid, ranging from 13.4 to 33.7 g per 100 g of oil, along with a low ratio of *n*−6/*n*−3 fatty acids (1.49−3.86). Six triacylglycerols, namely LnLnLn, LnLLn, LLLn, LLL, OLL and OLLn were detected by high-

performance liquid chromatography in berry seed oils tested, where O, L and Ln, are oleoyl, linoleoyl, and linonenoyl fatty acids, respectively. Total tocol contents were 286.3−1302.9 ppm, and included α-, γ-, and δ-tocopherols as well as δ-tocotrienol. The highest TPC of 0.48 mg gallic acid equivalents per gram of oil was observed in the black raspberry seed oil. Oxidative stability of the three berry seed oils tested was affected by their triacylglycerol profiles, positional distribution of fatty acids, storage conditions and minor components. These data suggests that the cold-pressed berry seed oils may also serve as potential dietary sources for tocols, α-linolenic acid (ALA) and natural antioxidants. These oils must be well protected from oxidative deterioration by proper storage in the dark and in the absence of oxygen and with minimal processing in order to retain their integrity and health promoting minor components.

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LIST OF ABBREVIATIONS

AOCS	American Oil Chemists' Society
APCI	Atmospheric pressure chemical ionization
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CD	Conjugated dienes
DAD	Diode array detection
EFA	Essential fatty acid
FAMES	Fatty acid methyl esters
GAE	Gallic acid equivalents
GC	Gas chromatography
HPLC	High performance liquid chromatography
MDA	Malondialdehyde
MS	Mass spectrometry
MBI	Methylene bridge index
n-3	Omega-3
n-6	Omega-6
ORAC	Oxygen radical absorbance capacity
PV	Peroxide value
PUFA	Polyunsaturated fatty acids

ROO•	Peroxyl radical
Sen	Photosensitizer
TAG	Triacylglycerol
TBA	2- Thiobarbituric acid
TBARS	2-Thiobarbituric acid reactive substances
TLC	Thin layer chromatography
TPC	Total phenolic contents
UV	Ultraviolet
v/v	Volume / volume
w/w	Weight / weight

CHAPTER 1

INTRODUCTION

Edible oils are essential macronutrients in food and a condensed source of energy. Edible oils could also benefit human health by serving as a source of essential fatty acids, fat-soluble vitamins and other nutrients and non-nutrients. However, lipids are prone to oxidation during processing, packaging and storage. Lipid oxidation can alter the flavor and nutritional quality of foods, and even form toxic compounds. Thus, it is necessary to understand the mechanism of oxidation in oils to prevent or slow down its occurrence.

Berry seed oils are considered specialty oils and are used in different products by an increasing number of consumers. In this study, berry seed oils, namely those of blackberry, black raspberry, and blueberry seed, all commercially available in the market, were used. These berry seed oils are abundant in oleic acid (O), linoleic acid (LA), and α-linolenic acid (ALA), and thus are a rich source of essential polyunsaturated fatty acids (PUFA), which cannot be synthesized in the human body. This potential health benefit has led to a rapid commercial development of these oils in a variety of products for human consumption, animal feed and cosmetics (Parry *et al.,* 2005).

Edible oils are primarily composed of triacylglycerols (TAGs), around 95%, and minor amounts of other components, including diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, tocopherols, sterols, chlorophylls and carotenoids, among others

(Hamilton, 1994; Shahidi and Shukla, 1996). The fatty acids incorporated into TAGs are saturated, monounsaturated, or polyunsaturated, and the degree of unsaturation dictates the oxidative stability of the oils. The oxidation is also influenced by the positional distribution of these fatty acids in the TAG as well as the type and content of minor components and storage conditions of the products.

The minor components, including tocopherols and tocotrienols, exhibit antioxidant activity to protect oils from oxidation. On the other hand, some of the minor components could be pro-oxidant or antioxidant, depending on the storage conditions. For example, chlorophylls are great antioxidants in the dark, but act as prooxidants under light. Therefore, all those factors could contribute to the oxidative stability of edible oils.

To monitor the quality of the berry seed oils, chemical, instrumental and sensory techniques could be applied based on the characteristics of oxidation products. For the primary oxidation products (lipid hydroperoxides), peroxide value (PV), and conjugated dienes (CD) are commonly assessed. For the secondary oxidation products (decomposition products of hydroperoxides), thiobarbituric acid reactive substances (TBARS) and headspace volatiles are often used as indicators (Shahidi and Zhong, 2005).

Cold-pressed berry seed oils contain a higher amount of natural antioxidants than oils extracted using conventional processing because cold-pressing does not involve hexane or heat in the process. Antioxidants are associated with lowering the risk of aging-

related diseases, including cancer and heart disease; therefore, they have potential to promote human health (Yu *et al.*, 2002a; Yu *et al*., 2002b). The mechanisms of action of antioxidants in retarding oxidation have been well-studied (Wanasundara and Shahidi, 2005). The mechanisms could be free radical quenching, forming chelation complexes with transition metals, reducing peroxides, and stimulation of antioxidative defense enzymes in the body (Parker *et al*., 2003).

Oxidative stability of cold-pressed blackberry, black raspberry and blueberry seed oils has not been thoroughly studied, nor have the antioxidant/prooxidant effects of minor components in those berry seed oils. Also there is no literature about the triacylglycerol profiles of cold-pressed berry seed oils or their impact on oxidative stability. Therefore, the objectives of this study were (1) to assess the effects of minor components by comparing the oxidative stability of oils (under autoxidation and photooxidation), before and after the removal of minor components; (2) to characterize the cold-pressed berry seed oils by measuring their fatty acid composition, positional distribution of fatty acids, TAG profile and their minor components (tocols, total phenolics and pigments).

CHAPTER 2

LITERATURE REVIEW

2.1 Lipids composition and health benefits

Fats and oils are essential dietary nutrients for humans and may be categorized by the variation in their origins and their physical features: fats are edible lipids, mostly from animal origin, which are generally solid at room temperature, while oils are commonly from vegetables or plants, and are liquid at room temperature.

Lipids support multiple biological functions in the body. They serve as the structural building material of all membranes of cells and organelles. Lipids are the most efficient fuel for living organisms containing two and a quarter times the energy content when compared with the same weight of carbohydrate or protein. Lipids and their derivatives also serve as signaling molecules that facilitate a variety of physiological functions. In addition, lipids are recognized as important biomarkers of disease and are involved in several pathological conditions. The cellular activities in tissues and organs are to some extent a result of biological actions of fatty acids. These actions are mediated by changes in the membrane bilayer structure and impact the processes of membrane-associated receptors and signal transduction systems and ion channels. Recent literature also demonstrates a specific role for fatty acids in gene modulation and protein expression that lower the risk of chronic disease.

Lipids, generally speaking, are mainly esters of fatty acids and glycerol, and include a variety of compounds, such as triacylglycerols (TAGs), diacylglyerols (DAGs), monoacylglycerols (MAGs), glycolipids (GL), phospholipids (PL), fatty acids (FA), long chain alcohols, sterols, hydrocarbons, pigments and fat-soluble vitamins, among others. Due to their properties, all the above-mentioned compounds can be classified as fats or oils (based on physical properties), essential or unessential, depending on the role they play. TAGs, DAGs and MAGs, each containing three, two and one ester bonds, respectively, make up 95% of lipids in vegetable oils. TAGs have commercial and dietary importance, because they are the most abundant constituent in fats and oils and also serve as an energy source and transporter of dietary lipids.

2.2 Essential fatty acids and nutritional value

Natural fats and oils usually consist of even-numbered straight chain fatty acids, while odd-numbered, branched-chain and cyclic acids are also found in some natural and processed lipid-based products. Generally, animal fats have a high content of saturated fatty acids, particularly stearic acid (S; C18:0), while vegetable or plant oils contain mainly palmitic acid (P; C16:0), oleic acid (O; C18:1n-9), linoleic acid (LA; C18:2 n-6) and alpha-linolenic acid (ALA; C18:3 n-3).

Among fatty acids, saturated and monounsaturated fatty acids can be synthesized within the human body, while polyunsaturated fatty acids (PUFA) have to be provided

from diet and/or supplements. Since the human body cannot synthesize PUFA, these fatty acids are also known as essential fatty acids (EFAs). EFAs are building blocks for structural lipids and precursors of biochemicals which regulate body functions. EFAs, including omega-6 and omega-3 PUFA, respectively, are derived from LA and ALA via elongation and desaturation. Plants can provide EFAs to animals as dietary sources by synthesizing and interconverting these EFAs. Omega-3 oils have several health impacts on human, such as anti-inflammatory, cardioprotective and anticancer activities (Shahidi and Miraliakbari, 2004; Shahidi and Miraliakbari, 2005; Shahidi and Miraliakbari, 2006).

2.3 The market needs for berry seed oils

Today's grocery shopping focuses on the improvement of the quality of life by choosing food products with health benefits. Consumers prefer food products that can prevent disease or promote general health, which leads to a new trend in identifying and characterizing health benefits in food products or primary food ingredients. Edible oils, as one of the most common products in the kitchen, have attracted much attention by the developments and discoveries in the characterization of oils, their oxidation and potential health benefits. An increasing number of consumers prefer vegetable oils to animal fats, due to better health effects of vegetable oils.

The history of extracting oils from edible seeds could be traced back to ancient times. Currently, several crops, including canola, sunflower, corn, soybean, and flax are grown

exclusively, or in major part, for the oil produced in their seeds. Specialty oils, such as berry seed oils have been shown to have unique fatty acid profiles and contain beneficial minor components, including natural antioxidants and phytosterols. The health effects of minor components present in seed oils has become a subject of renewed interest in the recent past (Shahidi, 2000).

In the fat and oils industry, growing consumer awareness is reflected in a trend toward trans-free and more (poly)unsaturated fatty acids. Specialty oils, like berry seed oils, have a unique fatty acid profile and possess interesting minor components, such as powerful antioxidants. These oils are produced from waste streams in juice production, which makes them unique from an economical point of view, as the waste can then be utilized. These oils, despite their nutritionally favorable fatty acid profiles and rich tocol contents, have low oxidative stability, which requires careful packaging and storage. The characteristic chemical properties of berry seed oils are likely to have an important role in ensuring the authenticity of these specialty products as well.

2.4 Overview of berry seed oils

Most berries are grown for their fruit (flesh) to produce juice, wine, jam and soup, while their seeds are discarded as waste by-products or used as a cheap source of animal feed component. During the berry juice or wine production, the liquid is separated from a waste paste-like stream including peels and seeds. The seeds can be further separated

from this mixed paste and used for oil extraction, mainly by cold-pressing which retains the minor bioactive components. These seed oils are particularly rich in antioxidants and essential fatty acids with a favorable low n-6/n-3 ratio (Parry *et al*., 2005).

According to Van Hoed *et al.* (2009), more than 14 thousand tons of raspberries were produced in Canada in 2005, and those raspberries can yield about 10 % (by weight) of seeds with 23% oil. Many studies have been conducted to determine the characteristics of seed oils from black raspberry, red raspberry, blueberry, marionberry, evergreen blackberry, boysenberry, cranberry and strawberry (Oomah *et al.*, 2000; Wang and Lin, 2000; Parker *et al.,* 2003; Bushman *et al.,* 2004; Parry and Yu, 2004; Parry *et al.,* 2005).

The high ratio of n-6 to n-3 in many oils can increase the risk of cancers, heart disease, hypertension and autoimmune disorders (Connor, 2000; Hung *et al.,* 2000; Aronson *et al.,* 2001). The n-6/n-3 ratio in Western diet is between 10 and 25 to 1 (Parry *et al.,* 2005; Bawa, 2008), whereas the favorable ratio is estimated to be 4 to 1 (Parry *et al.,* 2005). This could be due to the emphasis on the intake of vegetable and their seed oils, as well as lack of fish or fish oils consumption. In this case, berry seed oils stand out because of their desirable n-6/n-3 FA ratio in comparison with some other vegetable oils (Parker *et al*., 2003; Parry *et al*., 2006). In addition, these berry seed oils are rich in various antioxidants, which are related to a protective effect against cardiovascular diseases (Wang and Jiao, 2000).

The practical application of berry seed oils in cosmetics and pharmaceutical products based on their anti-inflammatory activity, notably for the prevention of gingivitis, rash, eczema, and other skin lesions, has been patented (Pourrat and Pourrat, 1973). The anti-inflammatory activity of raspberry seed oil had been reported to be superior to other well-known oils such as virgin avocado oil, grape seed oil, hazelnut oil, and wheat germ oil (Pourrat and Pourrat, 1973). According to this finding, berry seed oils can potentially be used as a sun screen, cream for prevention of skin irritation, bath oil, aftershave cream, antiperspirant, shampoo, toothpaste, and lipstick.

2.4.1 Berry seed oil extraction

Conventional oil processing generally uses hexane and heat, following grinding of seeds, while cold pressing does not use these conditions; and hence it is regarded as a more desirable method. Among several seed oil extraction methods, cold-pressing has occasionally been used in the food industry. Cold-pressing procedures apply a conventional screw pressing without heat.

A typical cold press machine is screw driven and removes oil by applying grinding pressure to the seeds. The residue remaining after extraction is the flour or cake. Another type of cold-pressing applies pressure directly to seeds in a barrel with slits down the side that allows oil to run out. Cold-pressing is believed to be a better technique for retaining beneficial value-added components in seed oils that might be

lost through evaporation or chemically modified using conventional solvent extraction methods.

The extent of lipid oxidation depends on a number of factors including the fatty acid composition, the presence of other chemicals that may inhibit or accelerate lipid peroxidation, and the storage conditions (Yu *et al.* 2002b). Several synthetic antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been used to aid in inhibiting oxidation of edible oils. However, recent awareness and utilization of natural antioxidants, and consumer concern about the long-term safety of these synthetic antioxidants has served as a driving force to move away from using the synthetic compounds. Cold-pressed oils may retain higher levels of natural antioxidants that may be removed during conventional oil refining procedures, and exhibit acceptable shelf stability and improved safety without added synthetic antioxidants. These natural antioxidants may also provide additional health benefits to consumers in disease risk reduction and health promotion (Yu *et al*., 2002c).

2.4.2 Black raspberry seed oil

Black raspberry (Rubus occidentalis L.), growing on a leafy cane, is a member of the genus Rubus from the Roseacea family. Black raspberry, also known as caneberry, is grown primarily for its juice. The processing of black raspberry for juice discards the seed as a by product. The majority of black raspberry crops are predominately grown in

temperate regions throughout Canada and the Northwestern United States, especially Oregon. In 2004, 1000 metric tons were harvested in the United States. USDA (2002) reported that more than 99% of the total harvested black raspberries were processed, while less than 1% of the black raspberries were consumed fresh. This data led the development of value-added utilization of black raspberry seeds, which increased the profit margin of black raspberries production.

Cold-pressed black raspberry seed oil has been shown to serve as a rich source of unsaturated fatty acids, especially n-3 fatty acids, and has demonstrated strong antioxidant activity (Bushman *et al*., 2004). The primary fatty acid in cold-pressed black raspberry seed oil was linoleic acid (53.5%) followed by α-linolenic acid at 31.2% (Bushman *et al*., 2004). Therefore, the ratios of n-6 to n-3 fatty acids were very low, approximately at 1.7:1. Other fatty acids, such as oleic (18:1n-9) and palmitic (16:0) acids, were also detected in small amounts in black raspberry seed oil. The unsaturated fatty acids constituted about 98% of total fatty acids. The overall fatty acid composition profile is very similar to red raspberry seed oil (Parry and Yu, 2004).

It has been discovered that black raspberries contain a higher level of natural antioxidants than red raspberries (Wang and Jiao 2000; Wang and Lin 2000). Black raspberry juice also has a stronger oxygen radical absorption capacity (ORAC) and a higher total phenolics content (TPC) than red raspberry and blackberry cultivars (Wang and Lin 2000). Black raspberry juice was found to exert stronger scavenging activity

against superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Wang and Jiao, 2000). This data suggest that cold-pressed black raspberry seed oil can be used as a rich dietary source of 18:3n-3 and essential fatty acids. Antioxidants present in black raspberry seed oil might also serve as a potential functional food ingredient and dietary supplement. Consumption of black raspberry and black raspberry-based products, including the juice, may provide several health benefits that guard against aging-associated ailments, cancer and heart disease.

2.4.3 Blueberry seed oil

Blueberries (Vaccinium corymbosum) are also grown in temperate climates, mainly in the Northern United States and Canada. Parry *et al.* (2005) reported that the cold-pressed blueberry seed oil had a high concentration of linoleic acid (18:2n-6), at 43.5% of the total. Alpha-linolenic acid, the only source of n-3 fatty acids in this product, comprised 25.1% of the total (Parry *et al*., 2005). Therefore, the ratio of n-6 to n-3 fatty acids in blueberry seed oil was 1.7:1, which makes blueberry seed oil a balanced source of these essential fatty acids. This ratio is lower than most vegetable oils, which is around 6:1 (Okuyama *et al*., 2000, Simopoulos, 1999). In the meantime, oleic, palmitic (16:0), and stearic (18:0) acids are also present in blueberry seed oil in fair amounts (5.7 and 2.8%, respectively) (Parry *et al.*, 2005). Like blueberry fruits, the blueberry seed oil also exhibits a higher antioxidant capacity than black raspberry, marionberry, and

cranberry seed oils as evaluated by the ORAC method. Thus, like black raspberry seed oil, blueberry seed oil might also serves as a great dietary source of n-3 fatty acids with good n-6/n-3 ratio and natural antioxidants, and it is particularly rich in γ-tocotrienol. Now commercially prepared edible cold-pressed blueberry seed oil is available for mostly cosmetic and pharmaceutical use.

2.4.4 Blackberry seed oil

The annual global commercial production of Blackberry (Rubus fruticosus) is estimated to be approximately 154,578 tons (Strik, 2007). The primary regions for blackberry production are North America, Europe, Asia, South America, Oceania, Central America, and Africa (in descending order of amounts cultivated) (Strik *et al*., 2008).

Similar to black raspberry seed oil and blueberry seed oil, blackberry seed oil is also rich in PUFA, which make up 78.7% of the total fatty acids. Among PUFA, linoleic acid (C18:2n-6) is most prevalent, followed by α-linolenic acid (C18: 3n-3) with a content of 17.60% and oleic acid (C18:1) at 14.72%. However, unlike the low and favorable ratio of n-6/n-3 found in black raspberry seed oil and blueberry seed oil, blackberry has a higher ratio of n-6/n-3, at 3.48. Nevertheless, this ratio of n-6/n-3 is still considered favorable for humans, because it is significantly lower than the ratio in the average western diet (ranging from 10 to 25).

Blackberry seed oil contains 1.4 g/kg of tocols, mainly γ-tocopherol. The content of total tocols, including tocopherol and tocotrienol, varies depending on the growing environment, processing and storage conditions as well as the other oil constituents, such as TAGs (Boskou and Dimitrios, 2011). Thus, blackberry seed oil also serves a great dietary source of essential fatty acids (EFAs) and natural antioxidants (tocopherols).

2.5 Lipid oxidation and its mechanism of action

Food deterioration could be caused by several factors, the primary one being lipid oxidation. Lipid oxidation brings about undesirable flavor, change of texture, deficiency of bioactives and nutrients to food, and potentially leads to the development of toxic substances. Therefore, prevention of lipid oxidation can help to ensure the quality of lipids or lipid-containing foods. Lipid oxidation in foods may occur during processing, usage and storage. Exposure to oxidizing agents and normal aerobic metabolism could oxidize lipids in living organisms via free radical reactions (Beckman and Ames, 1998). Lipid oxidation products are implicated in the disruption of biological membranes, formation of age-related pigments in damaged membranes, inactivation of enzymes and damage to proteins, oxidative damage of the lungs by atmospheric pollutants, and cancer.

Hydroperoxides were identified as primary products of oxidation (or peroxidation) of unsaturated lipids. Hydroperoxides are unstable and undergo decomposition to

secondary oxidation products. Therefore, systematic studies of lipid oxidation mechanisms and measurement techniques have been carried out. The use of antioxidants (synthetic and natural) for preventing or controlling lipid oxidation and eventually leading to minimizing oxidative deterioration of foods and in the body has been conducted. Antioxidant strategy has been successfully employed in the food industry for quality preservation of food products and in the medicinal industry for risk reduction of numerous oxidative stress-mediated diseases (Shahidi and Zhong, 2010). Oxidative stability of food lipids depends on a number of intrinsic and extrinsic factors, including the degree of unsaturation of their fatty acids, composition of minor components, environment conditions, delivery techniques and use of antioxidants, among others.

As mentioned before (Chapter 2.4), oleic, linoleic and linolenic acids are the main fatty acids in berry seed oils. The degree of fatty acids' unsaturation is associated with the rate of oxidation in a geometric fashion. For instance, the rate of oxidation of linolenic acid (3 double bonds) is 25 times higher than that of oleic acid (1 double bond) and twice as fast as that of linoleic acid (2 double bond) (Labuza, 1971). Lipid oxidation can proceed via autoxidation, photooxidation (photosensitized oxidation), thermal oxidation, hydrolytic oxidation and enzymatic oxidation, depending upon different conditions; though, only autoxidation and photooxidation, which were of interest in this research, will be elaborated here.

2.5.1 Autoxidation

Autoxidation is a spontaneous free radical chain reaction between molecular oxygen and unsaturated fatty acids. This chain reaction involves three steps of initiation, propagation and termination (Frankel, 1964).

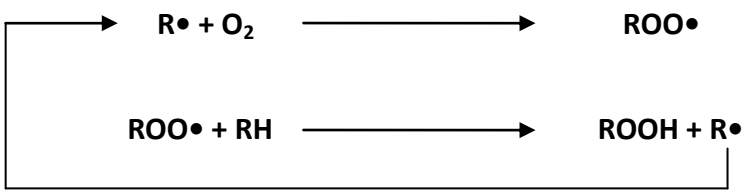
The reaction between atmospheric oxygen and unsaturated lipids (RH) starts with initiation, which requires an initiator, such as a metal catalyst, light, heat, and UV radiation. The activation energy, approximately 35 kcal/mol, is necessary for the formation of free radicals at the phase of initiation. RH loses a hydrogen atom from the allylic or bis-allylic methylenic carbon of an unsaturated fatty acid to form a lipid free radical R•, which reacts with oxygen to afford a peroxyl radical (ROO•). Subsequently, a propagation step is followed, where ROO• abstracts a hydrogen atom from another lipid molecule to produce a hydroperoxide (ROOH) as well as another lipid free radical R•. This free radical chain reaction can be terminated by combination of two radical species to form non-radical products. In this latter stage, for example, R• can react with ROO• to form ROOR. The termination could also be caused by competitive antioxidant, which can react with free radicals before lipid molecules in order to arrest lipid oxidation chain reaction.

Lipid hydroperoxides (ROOH) are known as primary products of autoxidation. These components are colorless and odorless, thus no harm is done to the lipid flavor quality. However, lipid hydroperoxides, are very unstable and tend to decompose to secondary

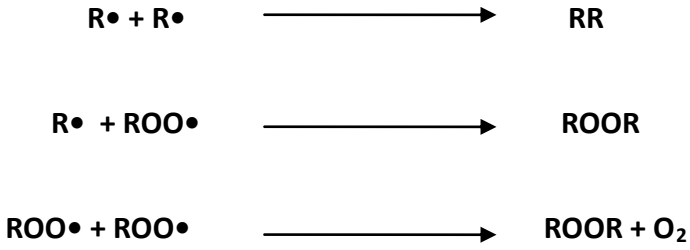
products such as aldehydes, ketones, alcohols, hydrocarbons, esters, volatile organic acids and polymers. These secondary products have undesirable flavors and can adversely affect the quality of lipids. These secondary products are often examined as their content determines the extent of off-odor and off-flavor formation. The scheme for autoxidation of lipids is shown in Figure 2.1.

Initiation:

Propagation:



Termination:



RH refers to any unsaturated fatty ay acid in which the H is hydrogen atom.

Figure 2. 1 Autoxidation mechanism of unsaturated lipid molecules (adapted from Shahidi and Zhong, 2005)

For the mechanism of linolenate autoxidation (shown in Figure 2.4), hydrogen is abstracted from bis-allylic methylene on carbon-11 and carbon-14. Then, two pentadienyl radicals formed, whose end positions are later attacked by oxygen. Thus, the two radicals produce a mixture of 9-, 12-, 13-, and 16-conjugated diene-triene hydroperoxide isomers (Frankel, 1984). By using gas chromatography-mass spectrum (GC-MS) and high performance liquid chromatography (HPLC), it was discovered that external 9-, 16- hydroperoxides were formed more rapidly than internal 12-, 13-hydroperoxides, probably due to the tendency to cyclize (Frankel and Neff, 1977; Chan and Levett, 1977). Similarly, oleate has hydrogen abstracted on carbon-8 and carbon-11 to produce a mixture of 8-, 9-, 10- and 11-hydroperoxides in different proportions (shown in Figure 2.2); linoleate's hydrogen, shown in Figure 2.3 is abstracted from carbon-11 only, and forms an equal amount of conjugated 9- and 13-hydroperoxide isomers (Frankel, 1984).

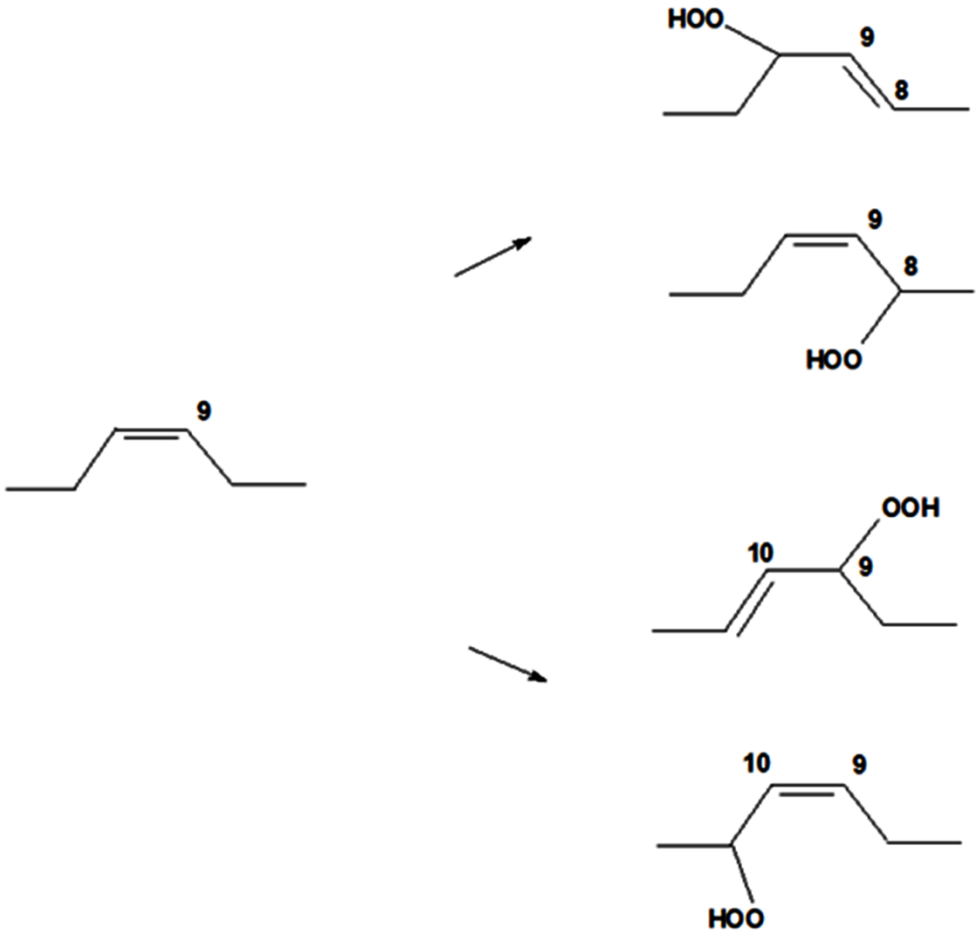


Figure 2. 2 Autoxidation mechanism of oleate (adapted from Frankel, 1984)

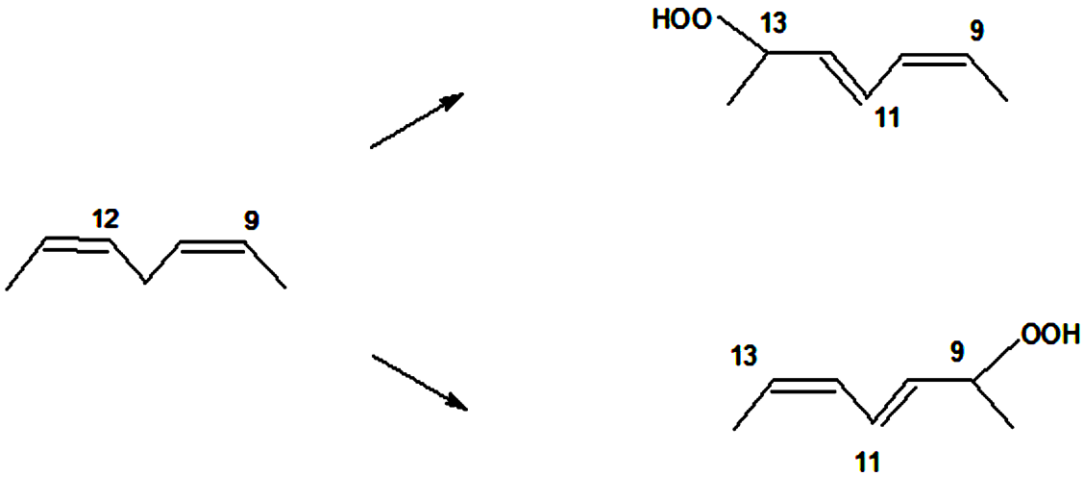


Figure 2. 3 Autoxidation mechanism of linoleate (adapted from Frankel, 1984)

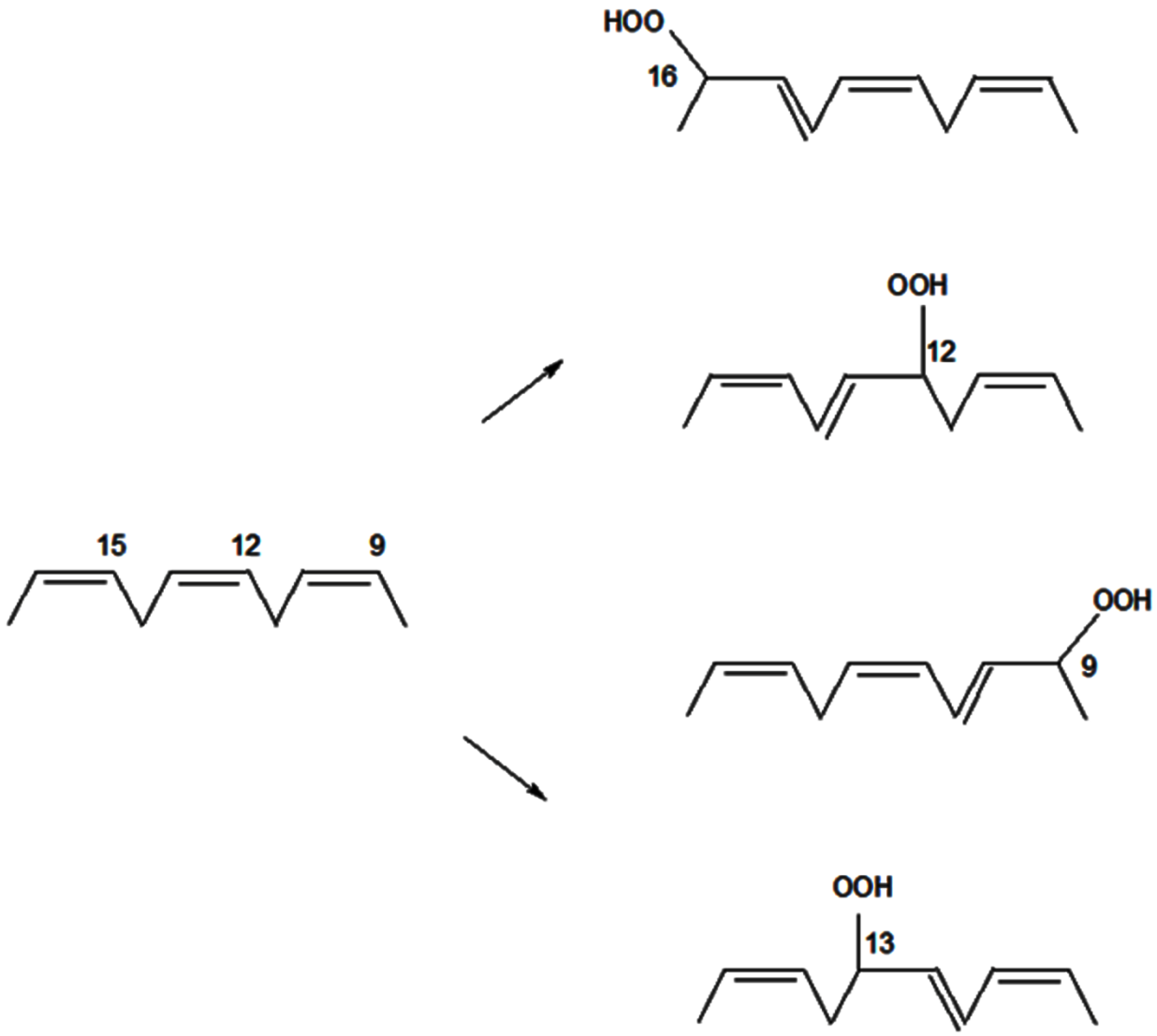


Figure 2. 4 Autoxidation mechanism of linolenate (adapted from Frankel, 1984)

However, all these hydroperoxide isomers are unstable and can decompose into products with off-flavor notes. The decomposition of hydroperoxides starts via homolytic cleavage of oxygen-oxygen bonds, yielding a hydroxyl and an alkoxyl radical. Then, the hydroxyl and an alkoxyl radical decompose by carbon-carbon cleavage to yield aldehydes or hydrocarbons, among others. The alkyl radicals can undergo further reaction to produce ketones or alcohols (Frankel, 1984).

2.5.2 Photooxidation

Photooxidation is another common lipid oxidation process which requires exposure to visible or ultraviolet light and usually proceeds in the presence of a photosensitizer (Sen) and oxygen. The photosensitizer in edible oils is usually chlorophyll, pheophytin, flavin, myoglobin and other heme-containing compounds. Both triplet and singlet oxygen may be involved in this process.

Singlet oxygen ($^1O_2$) is in an excited state, and can react with unsaturated fatty acids directly via non-radical pathways. In a comparison between singlet oxygen and triplet oxygen, singlet oxygen is the primary reactive oxygen species (ROS), which could be 1500 times faster than triplet oxygen in terms of the oxidation reaction (Min and Boff, 2002), as in the case of, linoleate (Rawls and Van Santen, 1970).

Similar to oxyegn, photosensitizers can reach an excited singlet state (^{1}Sen*) by absorbing energy from light, and these excited singlet photosensitizers go back to ground state because of their instability at the excited state.

There are two pathways proposed for photosensitized lipid oxidation. In one pathway (Type 1), exposure to a light-excited singlet photosensitizer can react with lipid molecules directly and then return to ground state. In another pathway (Type 2), an excited photosensitizer can react with triplet oxygen to form singlet oxygen, which then leads to oxidation. These two pathways are shown below:

Type 1:

Type 2:

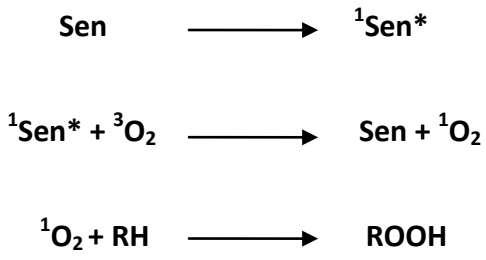


Figure 2. 5 Photooxidation mechanism of unsaturated lipid molecules (adapted from Shahidi and Zhong, 2010)

From the above scheme, it is obvious that Type 1 photooxidation forms lipid hydroperoxide via a free radical or free radical ion route. For Type 2 photooxidation, highly electrophilic singlet oxygen can directly attack the double bonds on unsaturated fatty acids, thus producing lipid hydroperoxides.

During singlet oxygen attack, hydroperoxides are formed at each unsaturated carbon with a shift of the double bonds in the molecules. Therefore, oleate (shown in Figure 2.6) forms 9-, 10- hydroperoxides; linoleate (shown in Figure 2.7) forms 9- and 13- conjugated diene hydroperoxides as well as 10- and 12- unconjugated diene hydroperoxides (which are different from the products of autoxidation); while linolenate (shown in Figure 2.8) produces 9-, 12-, 13- and 16-isomers as well as 10-, 15- isomers (which are different from the products of autoxidation). Then autoxidation may proceed following formation of hydroperoxide by singlet oxygen, producing multiple intermediates and oxidation products, depending on the reaction conditions and time.

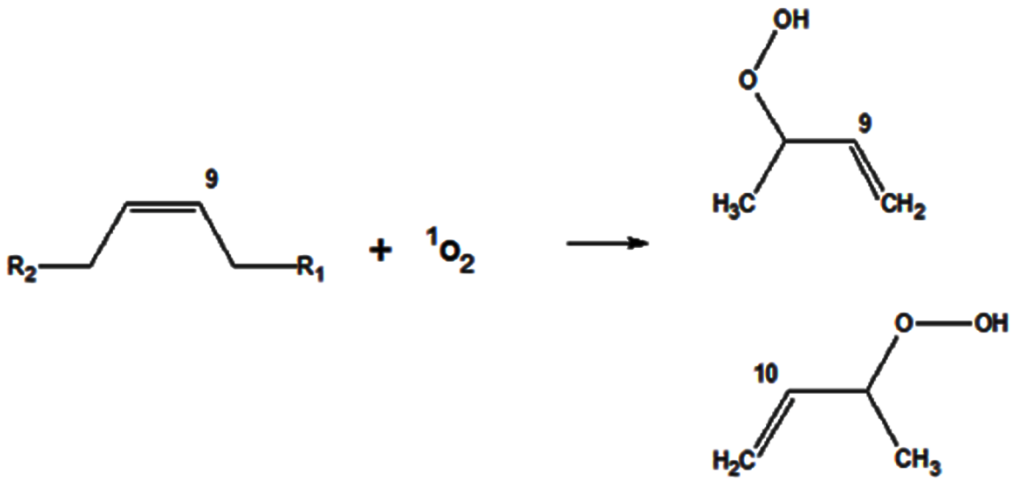


Figure 2. 6 Photooxidation mechanism of oleate (adapted from Frankel, 1984)

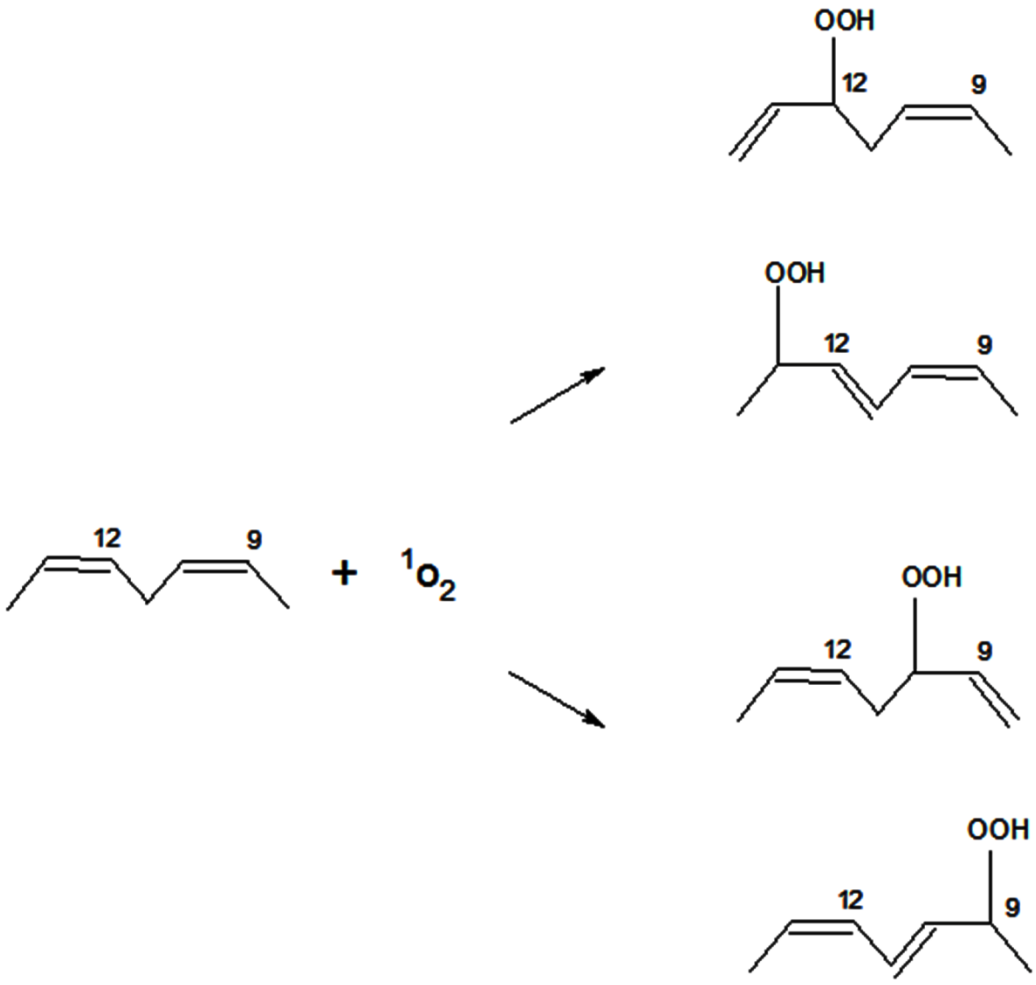


Figure 2. 7 Photooxidation mechanism of linoleate (adapted from Frankel, 1984)

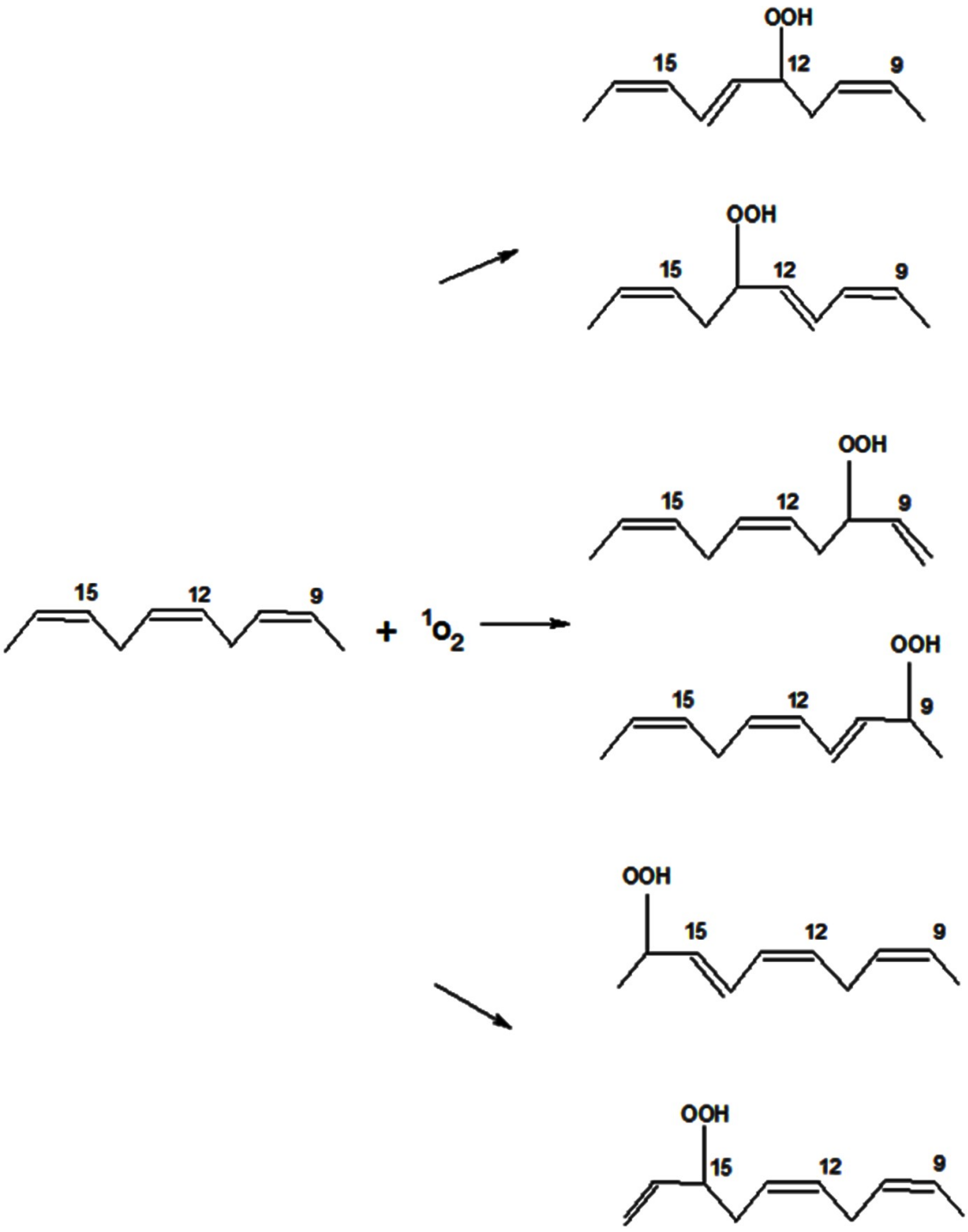


Figure 2. 8 Photooxidation mechanism of linolenate(adapted from Frankel, 1984**)**

The rapid photooxidation could be followed by autoxidation, that might eventually lead to multiple intermediates and oxidation products that change with reaction conditions and time. Therefore, photooxidized hydroperoxides can also decompose, as in autoxidation, to form off-flavor, secondary oxidation products (Frankel, 1984).

Photooxidation has a significant impact on oxidative changes in foods and biological organisms exposed to solar light and UV radiation. For example, liposomes and cell membranes are subjected to photooxidation in numerous skin disorders, including phototoxicity, photoallergy, photosenescence, photoaging and photocarcinogenesis.

Photooxidation could be efficiently inhibited by carotenoids, which are natural quenchers and also found abundantly in some berry seed oils. Carotenoids could absorb light energy before triplet oxygen does, thus preventing the formation of singlet oxygen from triplet oxygen. A large amount of natural antioxidants exist in berry seed oil, and they are able to quench singlet oxygen before it could react with unsaturated lipid molecules. It has been shown that tocopherols can protect and enhance the inhibitory function of carotenoids in vegetable oils (Miyazawa *et al.,* 1995).

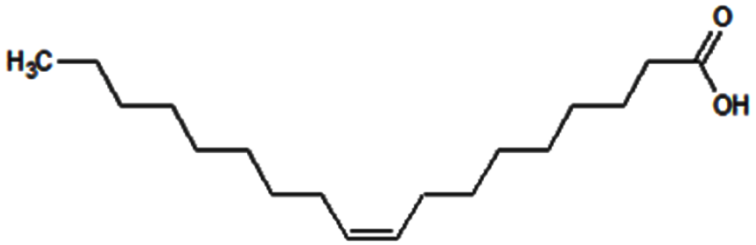
2.6 Factors influencing lipid oxidation

As previously noted, food lipids are primarily composed of triacylglycerols (TAGs), around 95%, and minor amount of other components, including diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, tocopherols, sterols, and carotenoids, among others. The fatty acids incorporated into TAG are saturated, monounsaturated or polyunsaturated, and the degree of unsaturation dictates the oxidative stability of the oils. The oxidation is also influenced by the positional distribution of these fatty acids in the TAG, as well as the type and content of minor components, and storage conditions of the products. The following sections describe the factors responsible for the oxidation of food lipids, their mechanism(s) of action and ways to ensure the preservation of their quality.

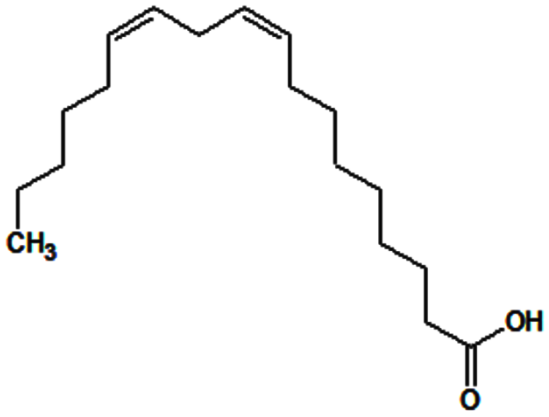
2.6.1 Fatty acid composition

Oxidative susceptibility of lipids depends on their fatty acid profile. Berry seed oils are known for their high content of PUFA; they are low in saturated fatty acids, but rich in oleic (C18:1), linoleic (C18:2n-6) and α-linolenic (C18:3n-3) acids (Figure 2.9). These unsaturated fatty acids are prone to oxidation, even though they are beneficial for human health.

Oleic acid (C18:1 n-9)



Linoleic acid (C 18:2 n-6)



α-Linolenic acid (C18:3 n-3)

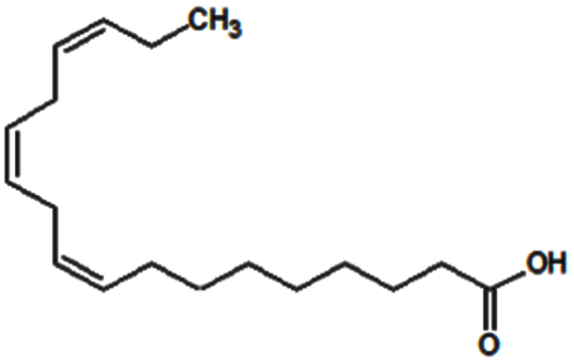


Figure 2. 9 Chemical structure of oleic, linoleic and α-linolenic acids (adapted from Miraliakbari, 2005).

Among factors that can influence lipid oxidation, fatty acid composition has been well studied in the past. It has been found that oils with large proportions of unsaturated fatty acids show compromised oxidative stability. The degree of unsaturation or methylene bridge index (MBI, the mean number of bisallylic methylene positions) has a significant influence on oxidative stability. For instance, PUFA with the highest MBI value are most susceptible to oxidation. Thus, the relative oxidation rate of stearic, oleic, linoleic and linolenic acid is reported to be 1: 100: 1200: 2500 (deMan, 1999). Unsaturated fatty acids as main reactants display significant compositional changes during oxidation, which provide an indirect measure for the extent of oxidation (Senanayake and Shahidi, 2002).

Two different transmethylation methods are commonly used to analyze fatty acids: the sulfuric acid/ methanol method and the boron trifloride (BF_3)/ methanol method (Khan and Shahidi, 2000; Budge and Parish, 2003). Fatty acids are converted to their volatile methyl esters by these methylating agents, following the hydrolysis of fatty acids. Gas chromatography (GC) is generally employed to identify the methyl esters by comparison with standard fatty acid methyl esters. It is necessary to select suitable columns and conditions for GC analysis to identify fatty acid isomers, including trans fatty acids. Other than transmethylation methods, iodine value and saponification value can also be used as indicators of the fatty acids present in oils. Iodine value measures the average number of double bonds in oils. AOCS (1990) Method cd 1-25 can be used to measure iodine value, where iodine value is defined as the number of grams of iodine that can be

added to 100 g of oil. Saponification value, defined as the amount of KOH needed, in mg, to saponify 1 g of oil, measures the alkali-reactive groups in oils. Saponification values of shorter chain fatty acids are higher than those of their longer chain counterparts.

2.6.2 Positional distribution of fatty acids within triacylglycerol (TAG) molecules

The stereospecific positional distribution of fatty acids within the TAG molecules, other than simply the fatty acid profile, also affects the susceptibility of lipids to oxidation. The positional distribution of fatty acids on the glycerol chain brings about differences in melting point, taste quality and other physical properties of oils. Fatty acids are generally more prone to oxidation in the free form than in the glycerol ester form, possibly due to their greater ability to pick up trace metals from the environment (Nawar, 1997) or due to lesser steric effects.

The three carbons of the glycerol moiety of TAG are not chemically equivalent. According to their position and stereochemical numbering conventions, they are known as sn-1, -2, and -3, with the sn-2 position being the secondary hydroxyl in the middle position of the molecule.

Usually, the fatty acids are not randomly distributed among different positions on the glycerol backbone and this varies with oil species and fatty acids. As one of the primary fatty acids found in berry seed oils, linolenic acid is primarily at the sn-2 compared to the sn-1 and sn-3 positions.

The location of PUFA in the sn-2 position of the acylglycerol backbone increases their oxidative stability (Gunstone, 2002). PUFA located at the sn-2 position could increase oxidative stability in natural vegetable oils in comparison to their randomized

counterparts (Wijesundera, 2008). For instance, SDS (1,3-distearoyl-2-docosahexaenoyl glycerol) has a greater oxidative stability than SSD (1,2-distearoyl-3-docosahexaenoyl glycerol), because DHA is more stable against oxidation when located at the sn-2 position of the TAGs instead of the sn-3. (Shen and Wijesundera, 2009; Wang *et al.*, 2010).

Triacylglyclerols can be analyzed by high-performance liquid chromatography, which was employed in this study, as well as gas liquid chromatography for separation and tentative identification of individual triacylglycerol molecules based on their carbon number and the number of double bonds involved.

2.6.3 Minor components

As noted earlier, non-triacylglycerol molecules, known as minor components, include free fatty acids, monoacylglycerols, and diacylglycerols, as well as phospholipids, tocopherols, tocotrienols, flavonoids, sterols, carotenoids, chlorophylls and other coloring matters, and oxidation products, among others. The minor components in fats and oils can play a role in the lipid oxidation process as prooxidants or antioxidants, thus affecting their stability.

Polar lipids, mainly phospholipids, function as antioxidants synergistically with other antioxidative substances, such as tocols (Khan and Shahidi, 2000 b). Tocols (known as vitamin E), including tocopherols and tocotrienols, are important fat-soluble

antioxidants. Tocols enhance antioxidant efficiency with phospholipid synergists, when present (Khan and Shahidi, 2000 b).

The presence of carotenoids and chlorophylls imparts a yellow-red, amber, or green-yellow color to the berry seed oils. Carotenoids are a group of unsaturated tetraterpenes and their oxygenated derivatives. Their strong antioxidant activity against both autoxidation and photooxidation has been well documented (Shahidi and Brown, 1998; Metusalach *et al*., 1999). During processing, carotenoids could degrade to colorless products at temperatures over 150 °C. Chlorophylls impart a green color to most plants and plant products, including oils. Chlorophylls can act as antioxidants in the dark, but they can act as photosensitizers with exposure to light, hence catalyzing lipid photooxidation. Tocols and carotenoids, not removed by deodorization and bleaching during the refining process, can improve the stability of crude oils. Even with the low quantities of α-tocopherol (vitamin E) and carotenoids present in a daily consumption of berry seed oils, their continued ingestion contributes to the overall pool of antioxidants in the human body (Prince *et al*., 1995). Phenolic compounds, other than tocols, can be found in some natural sources (e.g. olive oil), and their presence in vegetable oils might play an important role in the protection against oxidation (Abuzaytoun and Shahidi, 2006).

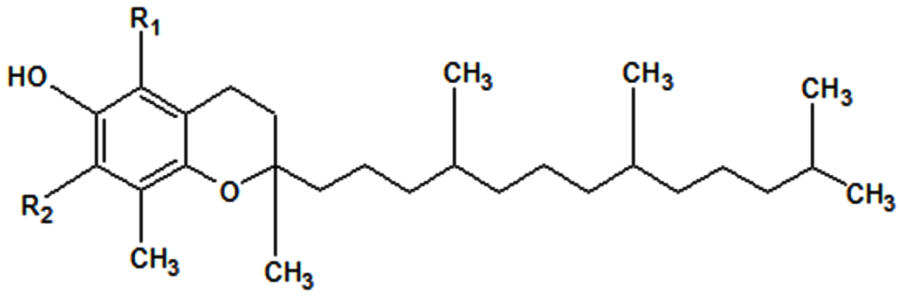
The effect of minor components on the oxidative stability of oils has been investigated by comparing oxidation of stripped and non-stripped natural oils (Khan and Shahidi,

2000a; Waraho *et al*, 2009). In food or biological environments, where the non-lipid components with antioxidant potential are present (e.g. free amino acids and peptides, Maillard reaction products, and/or additives), lipids may show enhanced oxidative stability. On the other hand, other components like trace metals, certain enzymes and heme compounds act as catalysts of oxidation reactions and lead to compromised oxidative stability of the lipids in the environment. Therefore, qualitative and quantitative determination of these minor components is important in quality assurance of lipid- and non-lipid-based products. These minor components play a significant role in lipid oxidation by modulating the oxidation process through various mechanisms.

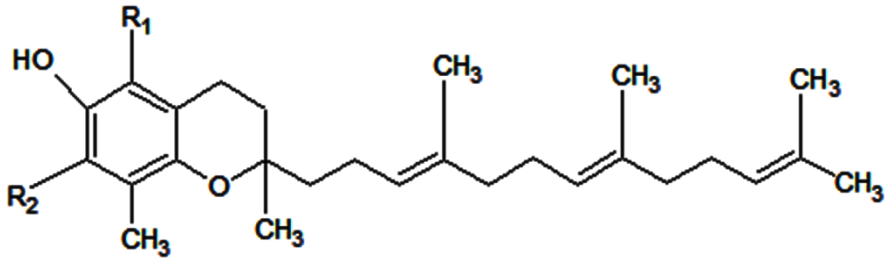
2.6.3.1 Tocols

Tocols, both tocopherols and tocotrienols, are vitamin E compounds. Tocols consist of a chromane head with two rings (one is phenolic and the other is heterocyclic) and a phytyl tail (Figure 2.2). Tocotrienols have a conjugated triene double bond system in the phytyl side chain, while tocopherols do not (Figure 2.10). Both tocopherols and tocotrienols are categorized as α(5,7,8-trimethyl), β(5,7- dimethyl), y(7,8-dimethyl), and δ(8-methyl) based on the number and position of methyl groups on the chromane ring (Shahidi and Shukla, 1996).

Tocopherol



Tocotrienol



Tocopherol or Tocotrienol	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 2. 10 Chemical structures of tocohperols and tocotrienols (adapted from Kim and Min, 2008)

Both tocopherols and tocotrienols are widely distributed in plants, thus they are the primary antioxidants in vegetable oils. Seed oils are mostly dominated by γ- or α-tocopherol, such as linseed. Soybean oil is abundant in γ- and δ- tocopherols, thus soybean oil is commonly used as commercial source of natural antioxidants. Compared to tocopherols, tocotrienols are less common and have not been studied as in depth as tocopherols. Nevertheless, high levels of tocotrienols can be found in rice bran and palm oils (Ko *et al*., 2003; Abidi, 2003), even though they are less common than tocopherols.

Tocols have vitamin E activity, and they also exhibit strong antioxidant effect. Those two properties of tocols are not the same. The vitamin E activity of the tocopherols is generally expressed as:

Vitamin E = α-toco + 0.25 β/γ toco + 0.01 δ-toco (Henon *et al*., 1999)

Therefore, oils with high levels of α-tocopherol, such as sunflower seed, cottonseed oil, particularly have high vitamin E content.

The antioxidant activity of tocopherols is dependent on the temperature. Tocols are soluble in vegetable oils but insoluble in water. They can donate phenolic hydrogen to lipid free radicals during autoxidation reactions, thus terminating free radical chain reactions, as below:

$$ROO\bullet + AH \longrightarrow ROOH + A\bullet$$

The chemical structures of the various tocopherols supports a hydrogen donating power in the order of α>β>y>δ. Therefore, it has been proposed that the relative antioxidant activity of tocopherols and tocotrienols *in vivo* is in the order α>β>y>δ (Dillard *et al*., 1983). The reverse order of activities has been reported for *in vitro* systems, such as bulk fats and oils. Tocopherols are also known to be strong singlet oxygen scavengers and thus are able to prevent or control lipid photooxidation. The effects of α-, y- and δ-tocols, at different concentrations, on chlorophyll photosensitized oxidation of soybean oil have been studied. The results indicate that α-tocopherol exhibits the highest singlet oxygen quenching activity followed by y- and δ- tocopherols (Jung and Min, 1990).

Therefore, tocols are often used in food products deficient in natural antioxidants, such as animal fats, waxes, and butterfat (Smith and Hong-Shum, 2011; Byrd, 2001). Since most vegetable oils naturally contain tocopherols, the addition of this antioxidant may lead to pro-oxidant effects. Tocopherols act synergistically with ascorbic acid, citric acid, and phospholipids.

The highest concentration for tocopherol mixtures acting as antioxidants is approximately 500 ppm. If the concentration of tocopherols goes up (e.g. > 1000ppm), alpha-tocopherol can function as a prooxidant. Generally, vegetable oils contain 200-800ppm of tocols, further additions of them to oils show a limited effect. The tocols are sensitive to oxidation and unstable, as such, hence, they are used in their more stable esterified form.

2.6.3.2 Removal of minor components

In order to quantify the effects of minor components on lipid oxidation, their removal from oils is needed. A dry column stripping technique may be employed for this purpose, in which the column is packed with silicic acid, charcoal, sugar and celite in certain proportions, as proposed for the removal of minor components from soybean oil (Mistry and Min, 1988). The resultant oil after the removal of minor components, known as stripped oil, is odorless, colorless, tasteless, and with no tocols, phospholipids, pigments, free fatty acids, mono- and diacylglycerols, or oxidation products. However, this technique is not always 100% effective. For example, it was reported that stripped rapeseed oil, prepared by the dry column technique, still retained up to 30% α-tocopherol as well as 60% γ-tocopherol (Lampi *et al.*, 1992). However, this technique was improved by using a column packed with activated silicic acid, and charcoal as the middle layer, and the column was conditioned by hexane and with same volume of oil sample passing through it. A water vacuum was also introduced in order to save time (Khan and Shahidi, 2001). A vacuum rotary evaporator was subsequently used to remove the hexane at 37°C, and traces of solvent were eliminated by nitrogen purging. This technique improved the removal of minor components from borage and evening primrose, flax, hemp, soybean and virgin olive oils (Khan and Shahidi, 2001; Abuzaytoun and Shahidi, 2006; Lampi *et al.*, 1992). Tian *et al* (2013) stripped soybean oil by a similar chromatographic column separation process using a lesser amount of two layers of

silicic acid (22.5g/layer compared to 50g/layer) and activated charcoal (5.625g compared to 50g), and by employing hexane as eluent.

2.6.4 Storage conditions

Lipid oxidation can also be affected by environmental factors during processing and storage, in addition to the characteristics of lipids. Though lipids are naturally existing in various sources, procurement of commercial fats and oils generally requires extraction and refining operations. These operations might affect the oxidative stability of the final products through exposure to atmospheric oxygen, high temperature, light and moisture, and possibly contact with rusted metals. Lipids may also undergo oxidative changes during packaging and handling, upon storage of bottled oils under light in display cases, as well as during advanced processing such as roasting and deep frying.

2.7 Measurement of lipid oxidation

To assess the degree of lipid oxidation, it is necessary to choose suitable and adequate methods among a variety of analytical methods that have been employed for evaluation of edible oils. Since lipid oxidation products are a complex, involving both primary and secondary products, a universal or standard method cannot evaluate the overall oxidative status in all food systems. Therefore, it is necessary to employ more than one method, where primary and secondary oxidation products could both be measured.

These analytical methods can be categorized into five groups depending upon what they measure. These include the loss of initial substances, the absorption of oxygen, the formation of free radicals as well as primary and secondary oxidation products (Dobarganes and Velasco, 2002). Chemical, physical and sensory techniques as well as instrumental methods can be employed to measure lipid oxidation parameters, and also predict the shelf life of products. Sensory analyses have been found more accurate, but time-consuming, expensive and sometimes more subjective; hence they are not the primary choice in routine laboratories analysis (Wanasundara *et al*, 1995). Therefore, chemical and physical as well as instrumental analyses are commonly used. These methods of analysis include iodometric titration, peroxide value, and spectrometry for conjugated dienes and trienes; headspace oxygen uptake, specific volatile aldehyde measurement, 2-thiobarbituric acid (TBA) value, and oxidative stability Index (OSI), among others.

2.7.1 Primary products of lipid oxidation

Hydroperoxides are the primary products of lipid oxidation, and they can rapidly decompose to a number of volatile and non-volatile secondary products. At the beginning of initiation stage, the rate of hydroperixde formation exceeds that of their decomposition, and at later stage of initiation, the decomposition rate outruns the formation of hydroperoxides. Therefore, measurement of the amount of

hydroperoxides, when considering the time factor, can be the indicator of initial stages of hydroperoxide formation (Shahidi and Wanasundara, 2002). Futhermore, the measurement of conjugated dienes through ultraviolet (UV) detection is simple, fast, requiring only a small amount of sample and without the need of chemical reagents. Therefore, it is a well-known method to evaluate primary products of lipid oxidation.

The formation of conjugated dienes in lipids is indicated by intense absorption at 230-235 nm in the ultraviolet (UV) region. The increase in UV absorption theoretically describes the production of primary lipid oxidation products. Conjugated dienes are primary products during the formation of hydroperoxides from unsaturated fatty acids and generally show UV absorption peak at 234 nm, while conjugated trienes absorb at 268nm (Shahidi and Wanasundara, 2002).

In comparison with the peroxide value (PV) method, the conjugated dienes (CD) method enjoys less sensitivity and specificity (Antolovich *et al*, 2002). However, good correlations exist between PV and CD (Wanasundara *et al*, 1995). Another disadvantage of conjugated dienes method is that the results might be influenced by other compounds absorbing in the same UV region. For instance, carotenoids are abundant in berry seed oil and absorb at 234nm (Shahidi and Wanasundara, 2002). Therefore, an alternative spectroscopic method is needed to measure conjugatable oxidation products (COPs), to prevent such interferences. Through reduction and subsequent dehydration, hydroperoxides and other decomposition products can be altered to more conjugated

chromophores, such as conjugated trienes and tetraenes, respectively, absorbing at 268 and 301nm (Shahidi and Wanasundara, 2002; Gordon, 2001).

2.7.2 Secondary products of lipid oxidation

Primary products are unstable and susceptible to break down to a complex of mixture which includes aldehydes, ketones, alcohols, hydrocarbons, and volatile organic acids, among others. TBARS method is one of the most commonly employed procedures used for the assessment of lipid oxidation. This test is used to quantify the overall concentration of secondary oxidation products and to report the results as malondialdehyde (MDA) equivalents, which is a secondary oxidation product of PUFA and related compounds.

One molecule of MDA reacts with two molecules of TBA to form a MDA-TBA complex, which has a pink color with absorption maximum at 532 nm (Figure 2.11). The degree of oxidation, or the TBA value is expressed as milligrams (mg) of MDA equivalents per kilogram of sample, or as micromoles (mM) of MDA equivalents per gram of sample. The TBA reagent also reacts with alkenal and alkadienals, other than MDA, hence TBARS has been widely used instead of MDA to express TBA value.

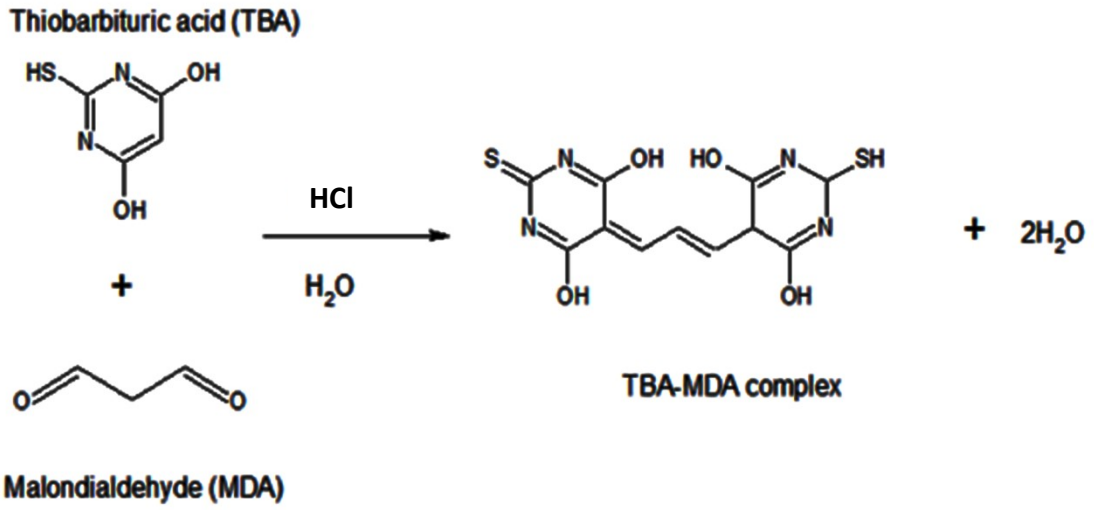


Figure 2. 11 The formation of malondialdehyde-thiobarbituric acid chromogen (adapted from Shahidi and Wanasundara, 2002).

TBA method has its own limitations, such as lack of specificity and sensitivity, since TBA can also react with sugars and oxidized proteins. Nevertheless, edible oils contain little sugars or oxidized proteins, leading to the conclusion that TBA test can commonly be used to measure lipid oxidation in foods. In addition, the presence of barbituric acid impurities in the TBA reagent could develop TBA-MDA-barbituric acid and MDA-barbuturic acid adducts that absorb, respectively, at 513 and 490 nm (Jardine, 2002). This suggests the importance of the purity of barbuturic acid before its use in the TBA test. There have been addition of antioxidants to samples to prevent oxidation during the test (Gomes *et al*, 2003), and decreasing the heating temperature to stabilize the yellow color of aldehyde-TBA complex (Marcuse, 1994), among others.

From the complexes of secondary products, volatile aldehydes such as propanal, hexanal and nonanal which arise from the oxidation of n-3, n-6 and n-9 fatty acids can also be measured to use as an indicator of the oxidation degree. Other aldehydes such as butanal, pentanal, 2-pentenal, octanal, and 2, 4-decadienal, among others, may also be used to evaluate oxidation of lipids. The n-6 PUFA (e.g. linoleate) produces hexanal as the primary volatile during the oxidation, while n-3 PUFA (e.g. linolenate) forms propanal as the main volatile (Frankel, 1993; Shahidi and Wanasundara, 1998). Oxidized lipid samples are heated to vaporize the volatile components, which are decomposition products of hydroperoxides. Then the volatiles in the headspace above the samples are analyzed by gas chromatography. The total peak area of volatiles in this technique increases with the storage period of a sample.

Gas chromatographic analysis of headspace volatiles is simple and rapid, by injecting clean aliquots of volatile compounds from the headspace of samples. The disadvantage of this method is the difficulty of reaching complete equilibrium with viscous and semi-solid samples. In addition, polyunsaturated lipid samples can decompose during the heating period.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Cold-pressed seed oils of blackberry, black raspberry and blueberry were kindly provided by Fruit Smart®, Inc. (Grandview, WA, USA). Folin-Ciocalteu reagent, 2-thiobarbituric acid (2-TBA), gallic acid (3,4,5-trihydroxybenzoic acid), silicic acid powder (mesh size: 100-200, acid-wash) and activated charcoal were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tocopherols and Tocotrienols were kindly provided by Planning & Development Department of Eisai Food & Chemical Co.,Ltd. (Tokyo, Japan). Compressed air, hydrogen, and ultrahigh-purity (UHP) helium were purchased from Canadian Liquid Air Ltd. (St. John's, NL, Canada). Hexane, acetonitrile, methanol, ethanol, sulfuric acid, isooctane, isopropanol, 1-butanol and all other chemicals were obtained from Fisher Scientific Co. (Nepean, ON, Canada), and were used without further purification. All solvents were ACS grade, or better, unless otherwise specified.

3.2 Methods

3.2.1 The removal of minor components from berry seed oils

3.2.1.1 Column chromatographic method

Blackberry, black raspberry and blueberry seed oils were stripped of their minor components, including free fatty acids, according to the method of Abuzaytoun and Shahidi (2005) with minor modifications. A chromatographic column (3.4 cm internal diameter x 40 cm length) was packed sequentially with 45 g of activated silicic acid, followed by 45g of charcoal and another 45g of activated silicic acid. The silicic acid (100 g), before introduction to the solvent, was activated by washing three times with a total of 3 L of distilled water. After each treatment, the silicic acid was left to settle for 30 min, and then liquid was discarded. The silicic acid was then washed with methanol and the supernatant discarded.

Oil (60 g) was diluted with an equal volume of hexane and passed through the chromatographic column. The solvent in the eluent (stripped oils) was evaporated under vacuum at 50 °C, and traces of the solvent were removed by flushing with nitrogen. The column-stripped oils were then transferred into 10 mL glass vials, flushed with nitrogen, and kept at -80 °C for up to one month prior to performing subsequent experiments.

3.2.1.2 Solvent partitioning method

A measured amount of oil sample (20 g) was diluted with hexane (1:10, w/v) and extracted with methanol (10:2, v/v, hexane/methanol) three times at ambient temperature, then the methanol fraction and hexane fraction were each completely removed under vacuum. The stripped oil so obtained was then transferred to 10 mL sample vials and stored under nitrogen at −80 °C for up to one month for use in other experiments.

3.2.2 Analysis of berry seed oils

3.2.2.1 Fatty acid composition

The fatty acid compositions of non-stripped, column stripped and solvent stripped berry seed oils were analyzed by gas chromatography-flame ionization detection (GC-FID). Fatty acids were converted to fatty acid methyl esters (FAMEs) using 6% sulfuric acid in methanol, according to the method of Wanasundara and Shahidi (1997). The transmethylation reagent (2.0 mL), consisting of freshly prepared 6% sulfuric acid in methanol containing 15 mg of hydroquinone as an antioxidant, was introduced to the sample vial, followed by vortexing. The mixture was incubated for 24 h at 60 °C and subsequently cooled to ambient temperature. Distilled water (1.0 mL) was then added to the mixture, after thorough mixing, and hydroquinone was added to each vial to prevent oxidation. The FAMEs were extracted three times, each with 1.5 mL of HPLC

grade hexane. The combined hexane layers were subsequently transferred to the test tube and washed twice with 1.0 mL of distilled water. The hexane was evaporated under a stream of nitrogen, and the extracted FAMEs were then dissolved in 1.0 mL of carbon disulfide and used for subsequent GC analysis.

The resultant FAMEs were analyzed using a Hewlett-Packard 5890 series II gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a fused capillary column (Supelcowax-10, 30 m length, 0.25 mm diameter, 0.25 µm film thickness; Supelco Canada Ltd., Oakville, ON, Canada). The temperatures of the injector and detector (FID) were both set at 250 °C. The oven temperature was programmed to increase from 220 to 240 °C at a rate of 30 °C/min. Ultrahigh-pure (UHP) helium was used as the carrier gas at a flow rate of 15 mL/min. Data were analyzed using a Hewlett-Packard 3365 series II Chem Station software (Agilent, Palo Alto, CA, USA). The FAMEs were identified by comparing their retention times with those of authentic standards (Nu-check, Elysian, MN, USA). Results were expressed as weight percentage of each fatty acid in total fatty acids.

3.2.2.2 Triacylglycerol profiles

The high performance liquid chromatography–photodiode array detection–atmospheric pressure chemical ionization-mass spectrometry (HPLC-DAD-APCI-MS) determination of triacylglycerols (TAG) in tested seed oils was conducted according to the method of Lisa

and Holcapek (2008) with minor modifications. For this purpose, berry seed oils were dissolved in acetonitrile/2-propanol (1:1, v/v) to obtain a 3% (w/v) solution. The chromatographic system consisted of an 1100 Series Agilent Technologies LC-MSD system equipped with a diode array detector (DAD) coupled to a mass spectrometer (quadruple analyzer) equipped with an atmospheric pressure chemical ionization (APCI) interface (Agilent, Palo Alto, CA, USA). The separation was performed on two C-18 columns (4.6mm× 250mm coupled with a guard column, Sigma) using gradient elution with acetonitrile (A) and 2-propanol (B). The elution system was as follows: 0 min, 100% A; 106 min, 31% A; 120 min, 100% A. The UV detection at 215 nm and positive-ion APCI-MS were coupled in series. MS parameters were as follows: vaporizer temperature, 400 °C; drying gas temperature, 350 °C; gas flow (N_2), 3.0 L/min; nebulizer pressure, 60 psi. The instrument was operated in positive ion mode and scanning from m/z 100 to 1200 at a scan rate of 2.0 s/cycle.

3.2.2.3 Positional distribution of fatty acids

3.2.2.3.1 Selective hydrolysis by using pancreatic lipase

The oil samples were hydrolyzed using pancreatic lipase as described by Christie (1982) with minor modifications. The pancreatic lipase is able to selectively hydrolyze and release fatty acids from Sn-1 and Sn-3 positions; in the meantime, leave monoacylglycerol with Sn-2 position esterified (Wanasundara and Shahidi, 1997). The oil

(25 mg) was weighed into a glass tube, and then 5.0 mL of Tris-HCl buffer (1.0 M, pH 8.0), 0.5 mL of calcium chloride (2.2%), and 1.25 mL of sodium taurocholate (0.05%) were added. Porcine pancreatic lipase (5.0 mg; EC 3.11.3) was added into the mixture after it had been kept in a water bath for 5.0 min at 40 °C. The glass tube was subsequently placed in a gyratory water bath shaker at 250 rpm under a blanket of nitrogen for 1 h at 40 °C. The enzymatic reaction was stopped by adding 5.0 mL of ethanol, followed by the addition of 5.0 mL of 6.0 M HCl.

3.2.2.3.2 Extraction and Separation of Hydrolytic Products

Diethyl ether (50 mL in total) was used to extract the hydrolytic products three times, and then the extract was washed twice with distilled water and dried over anhydrous sodium sulfate followed by removal of the solvent under reduced pressure at 30 °C. The hydrolytic products were separated on silica gel TLC plates silica gel thin-layer chromatographic plates (TLC; 20 × 20 cm; 60 Å mean pore diameter, 2-25 µm mean particle size, 500 µm thickness, with dichloroflurescein, from Sigma-Aldrich, St. Louis, MO, USA). The plates were developed using a mixture of hexane/diethyl ether/acetic acid (70:30:1, v/v/v) for 45−55 min. The bands were located by viewing under short (254 nm) wavelength UV light (Spectraline, model ENF-240C, Spectronics Co., Westbury, NY, USA). The free fatty acid bands were scraped off and lipids extracted into diethyl ether,

which were then used for fatty acid analysis as described by Wanasundara and Shahidi (1997).

3.2.2.3.3 Fatty Acid Compositional Analysis of Hydrolytic Products

Fatty acid composition and positional distribution of the products were determined by their conversion to the corresponding methyl esters. The transmethylation reagent (2.0 mL), consisting of freshly prepared 6% sulfuric acid in methanol containing 15 mg of hydroquinone as an antioxidant, was added to the sample vial, followed by vortexing. The mixture was incubated for 24 h at 60 °C and subsequently cooled to ambient temperature. Distilled water (1.0 mL) was then added to the mixture, after thorough mixing, and hydroquinone was added to each vial to prevent oxidation. The FAMEs were extracted three times, each with 1.5 mL of HPLC grade hexane. The combined hexane layers were subsequently transferred to the test tube and washed twice with 1.0 mL of distilled water. The hexane was evaporated under a stream of nitrogen, and the extracted FAMEs were then dissolved in 1.0 mL of carbon disulfide and used for subsequent GC analysis. A Hewlett-Packard 5890 series II gas chromatograph (Agilent, Palo Alto, CA, USA) equipped with a Supelcowax-10 column (30 m length, 0.25 mm diameter, 0.25 µm film thickness; Supelco Canada Ltd., Oakville, ON, Canada) was used to analyze the FAMEs. The oven temperature was first raised to 220 °C and kept there for 10.25 min and then raised to 240 °C at 30 °C/min and held there for 15 min. The

injector and FID temperatures were 250 °C. Ultrahigh-purity helium was used as a carrier gas at a flow rate of 15 mL/min. Hewlett-Packard 3365 series II Chem-Station software (Agilent) was used for data handling and processing. The FAMEs were identified by comparing their retention times with those of a known standard mixture.

Positional distribution of fatty acids at sn-1,3 positions was calculated as (% fatty acid at sn-1,3 positions/% fatty acid in triacylglycerols) × 100.

3.2.2.4 Measurement of minor components and their activities

The methanol fraction, which is obtained from removal of minor components by solvent partitioning method, was completely desolventized under vacuum in order to separate minor components of the seed oils. Then the methanol extracts were flushed with nitrogen, and stored at -80 °C.

3.2.2.4.1 Determination of total phenolic contents in methanol-extracts of berry seed oils

During the solvent partitioning of berry seed oils (Chapter 3.2.1.2 Solvent partitioning method), the methanol fraction was kept at -80 °C for further analysis. The total phenolic content contained in methanol-extracts from berry seed oils was determined according to the procedure explained by Singleton and Rossi (1965) with minor

modifications. One milliliter of Folin-Ciocalteu reagent was added to 50 mL centrifuge tubes containing 1 mL of the extracts (0.2 g/mL) prepared previously. Contents were mixed thoroughly, and 1.5 mL of 20% sodium carbonate was added; the final volume was then made up to 10 mL with distilled water and mixed again. After 2 h of reaction at room temperature, the absorbance of the mixture was read at 765 nm; the value obtained was then used to calculate the phenolic contents using a standard curve prepared with gallic acid. Total extracted phenolics were expressed as milligrams of gallic acid equivalents, a common reference compound, per gram of extract.

3.2.2.4.2 Measurement of pigments (chlorophylls and carotenoids)

Pigments present in the stripped and non-stripped oil samples were determined qualitatively by measuring the absorbance at 430-460 nm for carotenoids and the absorbance at 550-710 for chlorophylls and their derivatives. The oil sample was mixed with hexane (1:1, v/v) and transferred into cuvettes, and the absorbance was read using a 8453A UV-Visible spectrophotometer (Agilent Technologies, Palo Alto, CA, USA) and by recording the absorption spectra between 430 and 710 nm.

3.2.2.4.3 Determination of tocols (tocopherols and tocotrienols)

Tocol contents in blackberry, black raspberry, and blueberry seed oils were determined by reverse phase high performance liquid chromatography-mass spectrometry (HPLC-

MS). The analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a UV-diode array detector (UV-DAD). Separation was achieved on a C-18 column (4.6mm× 250mm coupled with a guard column, Sigma).

Tocols were eluted using a gradient solvent system containing methanol-acetonitrile-isopropanol. Fifty microliters of each tocol standard and oil samples were detected at 295nm. The mobile phase started with methanol/acetonitrile/isoproponal (41:59:0, v/v/v) at a flow rate of 0.8 ml/min and maintained for 15 min. The mobile phase was then gradually changed to methanol/acetonitrile/isoproponal (16.5:23.5:60, v/v/v) from 15 to 25 min, followed by gradual increase to 100% isoproponal from 25 to 35 min, which lasted 10 min. In order to recondition the column, the mobile phase was changed to its initial state, methanol/acetonitrile/isoproponal (41:59:0, v/v/v) in 5 min, and was kept there for 10 min.

All separated fragments were analyzed by mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) using alternating ion atmospheric pressure chemical ionization (APCI). The operating conditions used were 121 V for the fragments, drying temperature of 350°C, APCI temperature of 400°C, nebulizer pressure of 60 psi, drying gas flow of 7 liter/min. Tocopherol and tocotrienol concentrations of samples were calculated based upon standard curve and expressed as ppm (mg/kg).

3.2.3 Oxidative stability of berry seed oils after accelerated oxidation

3.2.3.1 Accelerated oxidations of berry seed oils

The oxidative stabilities of column-stripped and non-stripped seed oils were determined by employing Schaal oven condition and fluorescent conditions. For Schaal oven condition, samples were placed in a forced-air oven set at 60 °C (model 2, Precision Scientific Co., Chicago, IL, USA). For photooxidation under fluorescent lighting, samples were kept in a rectangular polyethylene box (70 cm length × 35 cm width × 25 cm height) equipped with two 40 W cool white fluorescent lights, which were suspended approximately 10 cm above the surface of the oil containers. The fluorescent radiation was at a level of 2650 Lux, and the temperature inside the container was 27±1 °C.

Oil samples were removed from the oven after 1, 3, 5, 7, and 12 days and from the light box after 1, 3, 6, 12, 24, 48, and 72 h, cooled and flushed with nitrogen. The caps of sample containers were all wrapped with Parafilm, and kept at -70 °C for oxidative stability tests within a month.

3.2.3.2 Determination of lipid oxidation

The oxidative stability of column-stripped and non-stripped oils was evaluated by measuring conjugated dienes (CD) for primary and 2-thiobarbituric acid-reactive substances (TBARS) for secondary oxidation products.

3.2.3.2.1 Determination of conjugated dienes (CD)

Conjugated dienes of the oil samples were determined according to the IUPAC (1987) method. Oil samples (0.02-0.04 g) were weighed into 25 mL volumetric flasks, dissolved in isooctane (2,2,4-trimethylpentane), and made up to the mark with the same solvent. The contents were mixed thoroughly, and the absorbance was read at 234 nm in a 10 mm Hellma quartz cell using a 8453A UV-Visible spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Pure isooctane was used as the blank. CD was calculated according to the equation below where A represents absorbance of the solution at 234 nm, C is the concentration of the solution in g/100 mL, and d is the cell length in 1 cm.

$$CD = A/(c \times d)$$

3.2.3.2.2 Determination of 2-thiobarbituric acid-reactive substances (TBARS)

Oil samples (0.05-0.20g) were analyzed for their contents of thiobarbituric acid reactive substances (TBARS) according to the AOCS (1990) method. The samples were accurately weighed into 25 mL volumetric flasks and made up to the mark with 1-butanol. This mixture (5 mL) was transferred into a dry test tube, and then 5 mL of fresh TBA reagent (200 mg of TBA in 100 mL of 1-butanol) were added to it. The contents were mixed and heated in a water bath at 95 °C for 120 minutes. The intensity of the resultant colored complex was measured at 532 nm using 8453A UV-Visible spectrophotometer (Agilent

Technologies, Palo Alto, CA, USA). The TBARS values were reported using a standard line prepared with 1,1,3,3-tetramethoxypropane as precursor of malondialdehyde (MDA).

3.2.4 Statistical analysis and data interpretation

All the experiments were performed in triplicate and the results reported as means ± standard deviation. One way ANOVA (analysis of variance) and Tukey's standardized test were performed at a level of $p<0.05$ using Minitab statistical software version 14 (Minitab Inc., PA, USA) to assess the significance of differences among values.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Removal of minor components from oils using column stripping and solvent partitioning methods

In order to evaluate the antioxidant/ pro-oxidant effects of minor components in the berry seed oils tested, minor components were removed by two methods, namely the column stripping method and solvent partitioning (hexane-methanol extraction). The column chromatographic method was applied by passing oils (dissolved in hexane) through a column packed with activated silicic acid (top and bottom layer) and activated charcoal (middle layer), with hexane used as eluent. The solvent partitioning method, on the other hand, was conducted via dissolving oils in hexane (1: 10, v/v), where oils were then extracted by partitioning into methanol (10:2, v/v, hexane/ methanol) in a separatory funnel. The minor components, which are mostly polar, were extracted into the methanol layer, while non-polar compounds, such as triacylglycerol, remained in the hexane layer. Thus, minor polar components were removed from the oils by solvent partitioning.

The oil yield from solvent partitioning (using hexane-methanol) was 80.72-86.21%, which was higher than that for the column stripping method (62.27-69.85%; Table 4.1). Column chromatographic method resulted in a lower oil recovery, longer processing time and was more expensive than solvent partitioning method, but it afforded a better

Table 4. 1 Recovery rate (g of resultant oil/ 100 g of oil) of stripped berry seed oils using the column stripping and solvent partitioning[1,2]

Seed oil samples	solvent partitioning	column stripping
Blackberry	80.58±2.1[a]	62.21±2.0[b]
Black raspberry	82.00±3.2[a]	67.97±1.3[b]
Blueberry	84.92±3.2[a]	69.23±1.8[b]

[1]Values are mean of triplicate determination ±standard deviation; [2]Values in the same row with different superscripts are significantly different ($p<0.05$).

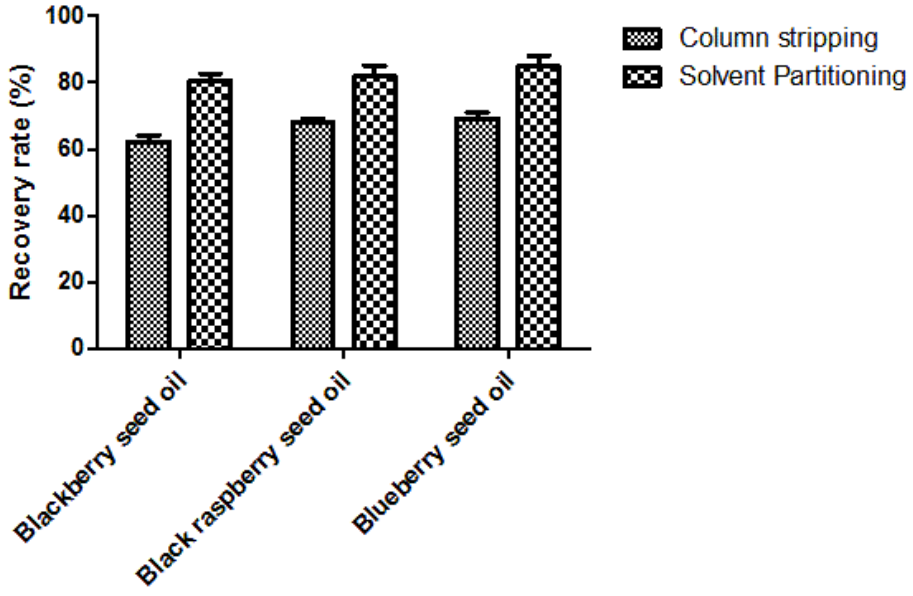


Figure 4. 1 Graphic presentation of recovery rate of stripped berry seed oils as presented in Table 4.1

oil in terms of minor component removal, such as tocols and pigments. Therefore, further experiments in this study were mostly conducted with column stripped berry seed oils and results were compared with non-stripped oils. Figure 4.1 shows the comparison of oil recovery rate using the column stripping and solvent partitioning methods for the berry seed oils examined.

4.2 Characterization of cold-pressed blackberry, black raspberry and blueberry seed oils

The characteristics of edible oils depended on several factors, such as degree of unsaturation, carbon chain length of the fatty acids, presence of geometric isomers of fatty acids, and their positional distribution on the glycerol backbone, the molecular configuration of triacylglycerols (TAGs), and that polymorphic forms. Therefore, this study was conducted to evaluate three berry seed oils by analyzing their fatty acid composition, TAG composition and positional distribution of fatty acids in the TAG molecules.

4.2.1 Fatty acid composition

Palmitic (C16:0), stearic (C18:0), oleic (18:1), linoleic (C18:2n−6), and α-linolenic (C18:3n−3) acids were detected in the non-stripped, column-stripped, and solvent-partitioned cold-pressed seed oils of blackberries, black raspberries and blueberries

(Table 4.2). All berry seed oils tested contained a very high amount of total polyunsaturated fatty acids ranging from 65.8 to 87.2%. Black raspberry seed oil had the highest amount of polyunsaturated fatty acids (87.2%), while blueberry seed oil contained the highest level of monounsaturated (22.2%) and saturated fatty acids (6.5%). Linoleic acid was the most prevalent fatty acid in all tested seed oils, contributing 41.3-63.4% to the total fatty acids. The highest level of linoleic acid was observed in blackberry seed oils including non-stripped, column-stripped and solvent-stripped forms of oils (63.6, 63.3 and 63.4%, respectively). All tested berry seed oils also contained significant amounts of α-linolenic acid, which led to favorable ratios of n−6 to n−3 ratios varying from 4.7 to 1.5. Blueberry seed oil demonstrated the lowest ratio (1.5) among the tested seed oils, whereas blackberry seed oil exhibited the highest ratio of n−6/n−3 fatty acids (3.8); nevertheless, this ratio (blackberry seed oil: 3.8) is still considered as being favorable as it is lower than that for most vegetable oils. Figure 4.2 provides a clear image of major fatty acids found in the tested oils.

Table 4. 2 Fatty acid composition (g/ 100 g of oil) of stripped and non-stripped blackberry, black raspberry, and blueberry seed oil[1,2]

FA(%)	CSBO	SPBO	NBO	CSRO	SPRO	NRO	CSUO	SPUO	NUO
C16:0	3.4^{a}±0.1	3.3^{a}±1.1	3.3^{a}±0.2	1.9^{a}±0.0	1.8^{a}±0.2	2.0^{a}±0.0	4.9^{a}±0.5	5.6^{b}±0.7	5.2^{a}±0.2
C18:0	1.7^{a}±0.2	1.7^{a}±0.9	1.6^{a}±0.3	0.6^{a}±0.0	0.4^{a}±0.0	0.6^{ab}±0.0	1.2^{a}±0.4	1.5^{a}±0.6	1.3^{ac}±0.0
C18:1	15.4^{b}±0.3	14.6^{b}±0.6	14.4^{b}±0.5	10.5^{c}±0.1	9.4^{c}±0.4	9.7^{c}±0.1	22.5^{a}±0.1	24.0^{a}±0.9	22.2^{a}±0.6
C18:2 n-6	63.6^{a}±0.5	63.3^{a}±4.4	63.4^{a}±1.3	54.7^{b}±0.2	53.9^{b}±0.5	53.4^{b}±0.0	43.0^{c}±0.7	41.3^{c}±1.4	41.9^{c}±0.4
C18:3 n-3	13.4^{c}±0.1	16.4^{c}±0.2	16.5^{c}±0.4	30.4^{a}±0.1	33.3^{a}±0.9	33.7^{a}±0.1	27.6^{b}±0.5	24.6^{d}±0.9	28.1^{b}±1.5
∑SFA	5.1	5.0	4.9	2.5	2.2	2.6	6.1	7.1	6.5
∑MUFA	15.4	14.6	14.4	10.5	9.4	9.7	22.5	24.0	22.2
∑PUFA	77.0	79.7	79.9	85.1	87.2	87.1	70.6	65.8	70.0
∑n-3	13.4	16.4	16.5	30.4	33.3	33.7	27.6	24.6	28.1
n-6:n-3	4.7	3.8	3.8	1.8	1.6	1.6	1.6	1.7	1.5

[1]Values are mean of triplicate determination ± standard deviation. Values in the same row with different superscripts are significantly different ($P<0.05$); [2]Abbreviations: NBO, non-stripped blackberry seed oil; CSBO, column-stripped blackberry seed oil; SPBO, solvent-partitioning blackberry seed oil; NRO, non-stripped black raspberry seed oil; CSRO, column-stripped black raspberry seed oil; SPRO, solvent-partitioning black raspberry seed oil; NUO, non-stripped blueberry seed oil; SPUO, column-stripped blueberry seed oil; and SPUO, solvent-partitioning blueberry seed oil.

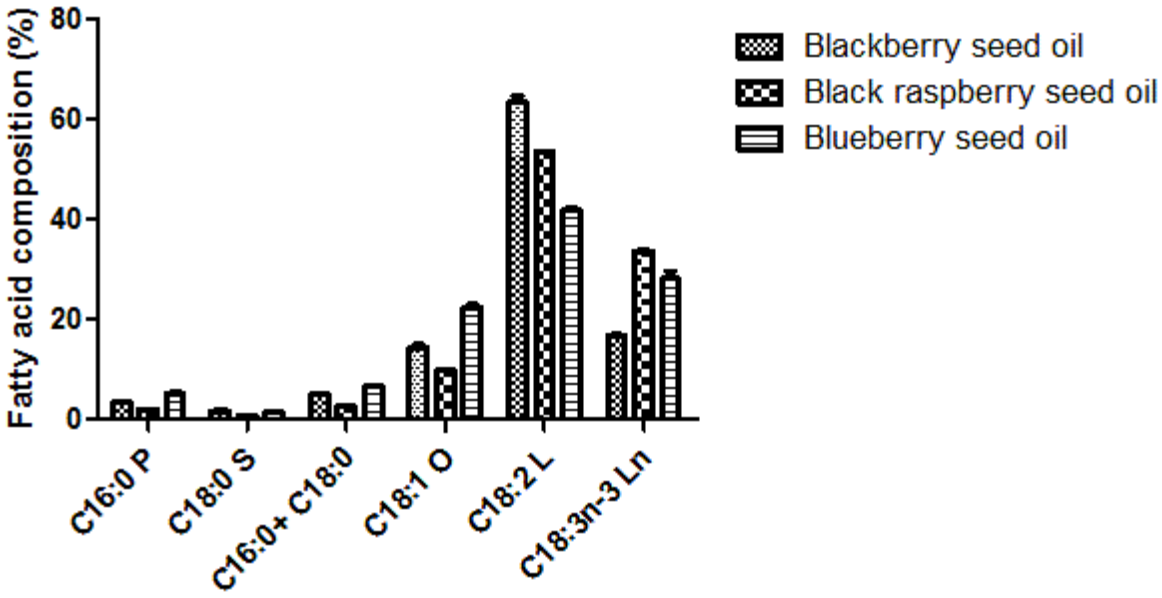


Figure 4. 2 Graphic presentation of major fatty acid composition of non-stripped blackberry, black raspberry, and blueberry seed oils as presented in Table 4.2.

The results presented here correspond favorably with those reported in the literature (Parry *et al.*, 2005; Bushman *et al*., 2004; Van Hoed *et al*., 2009). Thus, blackberry seed oil contained 61.2% linoleic acid, 14.7% oleic acid, and 17.6% linolenic acid, with n-6/n-3 ratio of 3.5. Blueberry seed oil contained 43.5% linoleic acid, 22.9% oleic acid, and 25.1% linolenic acid, with n-6/n-3 ratio of 1.7; and black raspberry seed oil possessed 53.5% linoleic acid, 10.4% oleic acid, and 31.2% linolenic acid, with n-6/n-3 ratio of 1.71 (Van Hoed *et al*., 2009; Parry *et al.*, 2005; Bushman *et al*., 2004). The slight variations between fatty acid profiles presented in this study and those reported in the literature might originate from the existing differences in the source and batch of the oil, process methods, and storage conditions, among others.

A close scrutiny of the fatty acid profiles indicates that the fatty acid composition remained mainly unchanged before and after stripping and removal of minor components (both by column stripping and solvent partitioning). It was demonstrated, however, that blackberry, black raspberry and blueberry seed oils contained very simple but rather unique fatty acids profiles. All tested oils were a good dietary source of essential fatty acids (EFAs) with favorable n-6/n-3 ratios. These berry seed oils could be incorporated into a daily diet to reduce the ratio of n-6 to n-3 fatty acids for humans. Literature has suggested that the reduction in the n-6/n-3 ratio could help prevent or slow down cancer development, reduce the risk of cardiovascular disease, and improve bone health (Aronson *et al*., 2001; Cave, 1991; Hu *et al*., 2002; Narayanan *et al*., 2001; Watkins *et al*., 2000).

4.2.2 Triacylglycerol composition

As mentioned earlier (Chapter 2.5), triacylglycerols (TAG) are the most abundant constituents (more than 95%) in edible oils. Therefore, analysis of TAG species in oils is necessary to understand their physical and chemical features, as fatty acid composition could only offer a general view of the oxidative stability and nutritional quality of oils.

The TAG compositions of cold-pressed blackberry, black raspberry and blueberry seed oils were determined by using high performance liquid chromatography–photodiode array detection–atmospheric pressure chemical ionization-mass spectrometry (HPLC-DAD-APCI-MS). Table 4.3 presents the TAG composition of the tested oils. The most prevalent TAGs detected were linolenoyl-linoleoyl-linolenoylglycerol (LnLLn), followed by dilinoleoyl-linolenoylglycerol (LLLn), trilinoleoylglycerol (LLL), oleoyl-linoleoyl-linolenoylglycerol (OLLn) and oleoyl-dilinoleoylglycerol (OLL). No trisaturated TAG species were detected, and this was possibly because UV detection shows low sensitivity for saturated TAG, thus making their quantification very difficult. It is significant to state that the triacylglycerol profile of cold-pressed blackberry, black raspberry and blueberry has not previously been reported in the literature. In addition, no γ-linolenic acid was found in any of the tested oils, thus Ln in Table 4.3 and elsewhere represents α-linolenic acid only.

The results of the fatty acid composition study (Table 4.3) are in agreement with those of fatty acid composition, since the primary fatty acids present in TAG species were also

Table 4. 3 Triacylglycerol (TAG) composition (relative concentration percentage) of non-stripped blackberry, black raspberry and blueberry seed oils[1,2,3]

TAG (%)	NBO	NRO	NUO
LnLnLn	3.78^{b}±0.16	13.25^{a}±0.15	7.65^{c}±0.22
LnLLn	12.90^{b}±1.23	24.29^{a}±0.12	17.96^{c}±0.44
LLLn	20.16^{b}±2.47	23.83^{a}±0.09	16.81^{c}±0.41
LnOLn	2.95^{c}±0.22	4.98^{b}±0.02	8.18^{a}±0.20
LnLnP	0.97^{b}±0.21	0.60^{b}±0.01	2.58^{a}±0.06
LLL	20.16^{a}±2.17	12.78^{b}±0.09	6.27^{c}±0.15
OLLn	7.83^{b}±0.90	7.23^{b}±0.07	13.24^{a}±0.33
LnLP	2.69^{b}±0.23	1.48^{b}±0.06	4.51^{a}±0.11
SLnLn	0.58^{a}±0.02	nd	0.90^{a}±0.02
OLL	9.27^{a}±0.85	4.31^{c}±0.11	6.09^{b}±0.15
OLnO	1.10^{b}±1.89	nd	2.82^{a}±0.07
LLP	3.65^{a}±0.54	1.28^{c}±0.06	2.37^{b}±0.06
SLLn	2.11^{a}±0.28	0.83^{c}±0.03	1.64^{b}±0.04
LnOP	n.d	nd	1.37±0.04
OLO	1.85^{b}±0.02	0.66^{b}±0.04	2.09^{a}±0.05
SLL	2.64^{a}±0.18	nd	2.10^{a}±0.06
SOLn	n.d	0.42±0.06	n.d
OOO	0.10^{a}±0.21	nd	0.10^{a}±0.01
ALL	0.27±0.01	nd	n.d
SLO	0.54^{a}±0.32	nd	0.45^{a}±0.01
Total	95.92	95.92	97.13

[1]Values are mean of triplicate determination ± standard deviation. [2]Values in the same row with different superscripts are significantly different ($p<0.05$). [3]Abbreviations: NBO, non-stripped blackberry seed oil; NRO, non-stripped black raspberry seed oil; NUO, non-stripped blueberry seed oil. nd, not detected.

oleic, linoleic, and α-linolenic acids. The positional distribution of fatty acids within the TAGs was determined by the mass spectrometry according to the ratio of fragment ions, because the neutral loss of fatty acid from the middle sn-2 position compared to sn-1 and sn-3 positions is less favored, hence providing the fragment ion with lower relative abundance than that expected statistically.

In addition to fatty acid composition's impact on oil oxidation, the oxidative stability of the oil also depends on TAG composition (Neff *et al*., 1997). The TAG composition affects taste and texture quality of products, because they can affect functional properties of foods into which the oil is incorporated, such as melting point range, solid fat index, and crystal structure (Neff *et al*., 1999; Bakowska-Barczak *et al*., 2009). Neff *et al.* (1993, 1994) reported that triacylglycerol types such as LLLn, LnLP, LLP, LnLO, LLS, and PLP decreased oil oxidative stability, while LLO, LOO, LOP, OOO, LOS, and POO increased its stability. Therefore, the results in Table 4.3 demonstrate that all tested berry seed oils are rich in LnLLn, LLLn, LLL, OLLn, and OLL, which are more susceptible to oxidative deterioration than oils rich in oleic acid, such as mango seed kernel oil or Saskatoon berry seed oil (Lakshminarayana *et al.*, 1983; Ali *et al*., 1985; Bakowska-Barczak *et al*., 2009).

4.2.3 Positional distribution of fatty acids

The physical characteristics of edible oils depend on the nature of their TAGs. The characteristics of TAGs depend on the stereospecific distribution of fatty acids on each individual TAG molecule, in addition to the type of fatty acids. Therefore, it is necessary to evaluate their fatty acid distribution patterns within the triacylglycerols to enhance and develop their industrial application. Stereospecific analyses of cold-pressed blackberry, black raspberry and blueberry seed oils were conducted by hydrolysis and thin layer chromatography (TLC).

Figure 4.3 displays the fatty acid distribution pattern within TAG molecules. The compositions of fatty acids in the sn-2 position of the cold-pressed blackberry, black raspberry and blueberry seed oils are shown. Variations in the fatty acids of tested berry seed oils in the sn-2 position were significant ($P < 0.05$). The sn-2 positions were primarily occupied by linoleic acid (ranging from 47.2 to 64.2%), oleic acid (from 11.38 to 29.56%) and α-linolenic acid (from 8.03 to 11.4%). The positional distribution of fatty acids within TAG molecules reported in this study can be considered as reference data for the future studies on cold-pressed blackberry, black raspberry and blueberry seed oils as no such data have previously been reported. For cold-pressed blackberry seed oil, all saturated fatty acids were placed on sn-1 and sn-3 positions, and most oleic and α-linolenic acids were also located on sn-1 and sn-3 positions; while only linoleic acid was relatively evenly distributed on the sn-2 and sum of sn-1, 3 positions (47.2 and 52.8%, respectively). For black raspberry seed oil, similar to blackberry seed oil, most oleic and

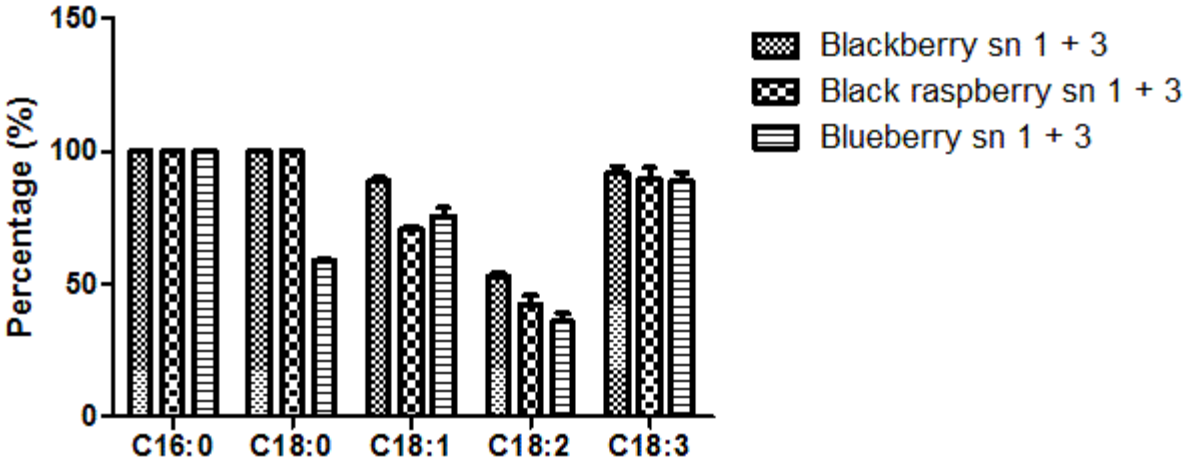


Figure 4. 3 Distribution of fatty acids on the external positions of TAG molecules in non-stripped blackberry, black raspberry and blueberry seed oils

α-linolenic acids were distributed on the external (sn-1 and sn-3) positions of the glycerol, while only 29% of oleic acid and 10% of α-linolenic acid were located on the internal, sn-2, position. All saturated fatty acids detected in black raspberry seed oil were located on the sn-1 and sn-3 positions. For blueberry seed oil, the same pattern was observed and up to 75% of oleic acid and 88% of α-linolenic acid were on the external sn-1 and sn-3 positions; however, stearic acid was an exception and was distributed almost evenly on internal and external positions. No published result about positional distribution of fatty acids on glycerol backbone of the in cold-pressed seed oils tested in this study has previously appeared (Figure 4.3).

In summary, oleic and α-linolenic acids were more distributed on the external sn-1 and sn-3 positions, while linoleic acid was more distributed in the internal position (sn-2). The saturated fatty acids were present in low amounts in tested berry seed oils and these were located preferentially on the external positions than the internal position. Thus, berry seed oils tested are very much prone to oxidation since most unsaturated fatty acids are located on the external positions. However, the minor non-acylglycerol components found in tested berry seed oils play an important antioxidative role. Therefore, the comparison of oxidative stability under accelerated oxidation conditions between original oil and minor components removed oil (by column chromatography) showed the oils' stability was affected by their molecular characteristics as well as their minor components.

4.3 Assessment of minor components in blackberry, black raspberry and blueberry seed oils

4.3.1 Analysis of total phenolic contents in blackberry, black raspberry and blueberry seed oils

Phenolic compounds contribute to the overall antioxidant capacity of oils, thus affecting oils' stability by retarding radical reactions, and hence lipid deterioration (Tovar *et a*l., 2001). Phenolic compounds also have an impact on sensory and nutritional characteristics of oil samples. Tovar *et al.* (2001) reported that oil stability is correlated with total amount of phenolics, and also the type of phenolics present.

Phenolic compounds were extracted from the berry seed oils using hexane/methanol (10:2, v/v). Table 4.4 shows total phenolic compounds in berry seed oils expressed as gallic acid equivalents (GAE). The levels of total phenolics determined in this way are not absolute measurements of the amounts of phenolic materials, but are in fact based on the chemical reducing capacity of gallic acid. The presence of these phenols might contribute to better stability of non-stripped berry seed oils under Schaal oven conditions as compared to those of their stripped counterparts. Table 4.4 shows that blackberry and blueberry seed oil extracts had a similar ability to reduce Folin-Ciocalteu's reagent. Data in this figure demonstrate that black raspberry seed oil extract had a better ability ($p < 0.05$) to reduce the Folin-Ciocalteu's reagent than blackberry and blueberry seed oil extracts. Parry *et al.* (2005) reported the total phenolic contents in

Table 4. 4 Total phenolic compounds (mg/g oil) in berry seed oil extracts[1,2]

Seed oil	Gallic acid equivalents (mg/g of oil)
Blackberry	0.19^{b}±0.004
Black raspberry	0.48^{a}±0.005
Blueberry	0.20^{b}±0.010

[1]Values are mean of triplicate determination ± standard deviation. Values with different superscripts are significantly different (P<0.05).

blueberry extracts, which was more than 1.0 mg GAE/g of oil, yet was only 0.2 mg GAE/g of oil in this study. This discrepancy might reflect the difference in the ratio of extraction solvents, variation among genotypes, and growing environments of berries.

4.3.2 Assessment of tocols in stripped and non-stripped berry seed oils

The concentration of tocols in cold-pressed blackberry, black raspberry and blueberry seed oils and their striped counterparts using column stripping and solvent partitioning methods are shown in Table 4.5. The non-stripped blackberry, black raspberry, and blueberry seed oils differed in their α-, γ-, δ-, and total tocopherols and tocotrienols. The blueberry seed oil, although lowest in its α-tocopherol content, was very rich in γ-tocotrienol (1244.78 ppm). The non-stripped black raspberry seed oil contained the highest concentration of γ -tocopherol (449 mg/kg of oil) as well as total tocopherols which was much higher than the other tested berry seed oils (Table 4.5). However, non-stripped blueberry seed oil exhibited the highest concentration of total tocols, and more than twice the concentration found in the second best sample (non-stripped black raspberry seed oil). Non-stripped blackberry seed oil contained high concentrations of both γ- and δ-tocopherols compared to non-stripped blueberry seed oil (Table 4.5).

Column-stripped blackberry, black raspberry and blueberry seed oils were devoid of any tocopherol or tocotrienol, while solvent-stripped seed oils contained small amounts of tocopherol and tocotrienol. Based on the above results, the column stripping method

was effective in removing tocopherols and tocotrienols while solvent (methanol) stripping only partially removed tocopherols and tocotrienols. Therefore, oxidative stability under accelerated oxidation was conducted only for column-stripped and non-stripped berry seed oils, in order to estimate the effects of minor components on their oxidation.

Table 4. 5 Tocol concentration in original, column stripped, and solvent partitioned blackberry, black raspberry, and blueberry seed oils[1,2].

Tocols (ppm)	Non-stripped	Column-stripped	Solvent-partitioned
Blackberry seed oil			
Alpha-Tocopherol	52.4±5.6	nd	tr
Gamma-Tocopherol	187.38[a]±4.8	nd	98.25[b]±12.0
Delta-Tocopherol	46.51[a]±11.3	nd	6.82[b]±0.2
Total Tocols	286.27[a]±21.7	nd	105.06[b]±12.3
Black raspberry seed oil			
Alpha-Tocopherol	136.4[a]±1.6	nd	16.39[b]±2.1
Gamma-Tocopherol	445.8[a]±8.3	nd	233.14[b]±23.6
Delta-Tocopherol	143.5[a]±1.2	nd	88.18[b]±1.6
Total Tocols	725.72[a]±11.2	nd	321.32[b]±25.2
Blueberry seed oil			
Alpha-Tocopherol	16.47±1.0	nd	tr
Gamma-Tocopherol	39.42[a]±1.9	nd	22.5[a]±2.4
Delta-Tocopherol	2.20±0.9	nd	tr
Delta-Tocopherol	1244.78[a]±25.4	nd	577.3[a]±14.8
Total Tocols	1302.87[a]±29.2	nd	599.82[b]±17.2

[1]Values are mean of triplicate determination ±standard deviation. Values with different superscript in each row are significantly different from each other ($P<0.05$). [2]Abbreviation: nd, not detected; and tr, trace.

4.3.3 Pigments determination in column-stripped and non-stripped berry seed oils

Pigments, including carotenoids and chlorophylls, were detected spectrophotometrically in the range of 430-460 and 550-710 nm, respectively (Blekas *et al*., 1995). The content of carotenoids in non-stripped black raspberry seed oil was much higher than those present in non-stripped blackberry and non-stripped blueberry seed oils. However, chlorophylls existed in higher levels in non-stripped blueberry seed oil than blackberry and black raspberry seed oils (Figure 4.4), which may influence oxidative stability under light. It is noteworthy that column stripped blueberry seed oil did not contain any pigments. By comparing pigments found in non-stripped oils and their counterparts, it is clear that stripped oils had no or less ($p < 0.05$) pigment than their non-stripped counterparts (Figure 4.4) as indicated by the absorbance of pigments at different wavelengths. For example, the absorbance at 430 nm for non-stripped blackberry seed oil was 0.90, which is higher ($p < 0.05$) than that of its stripped counterpart (0.22). Similarly, the absorbance at 460 nm for non-stripped black raspberry seed oil was 1.02, which is much higher ($p < 0.05$) than that of its stripped counterpart (0.28).

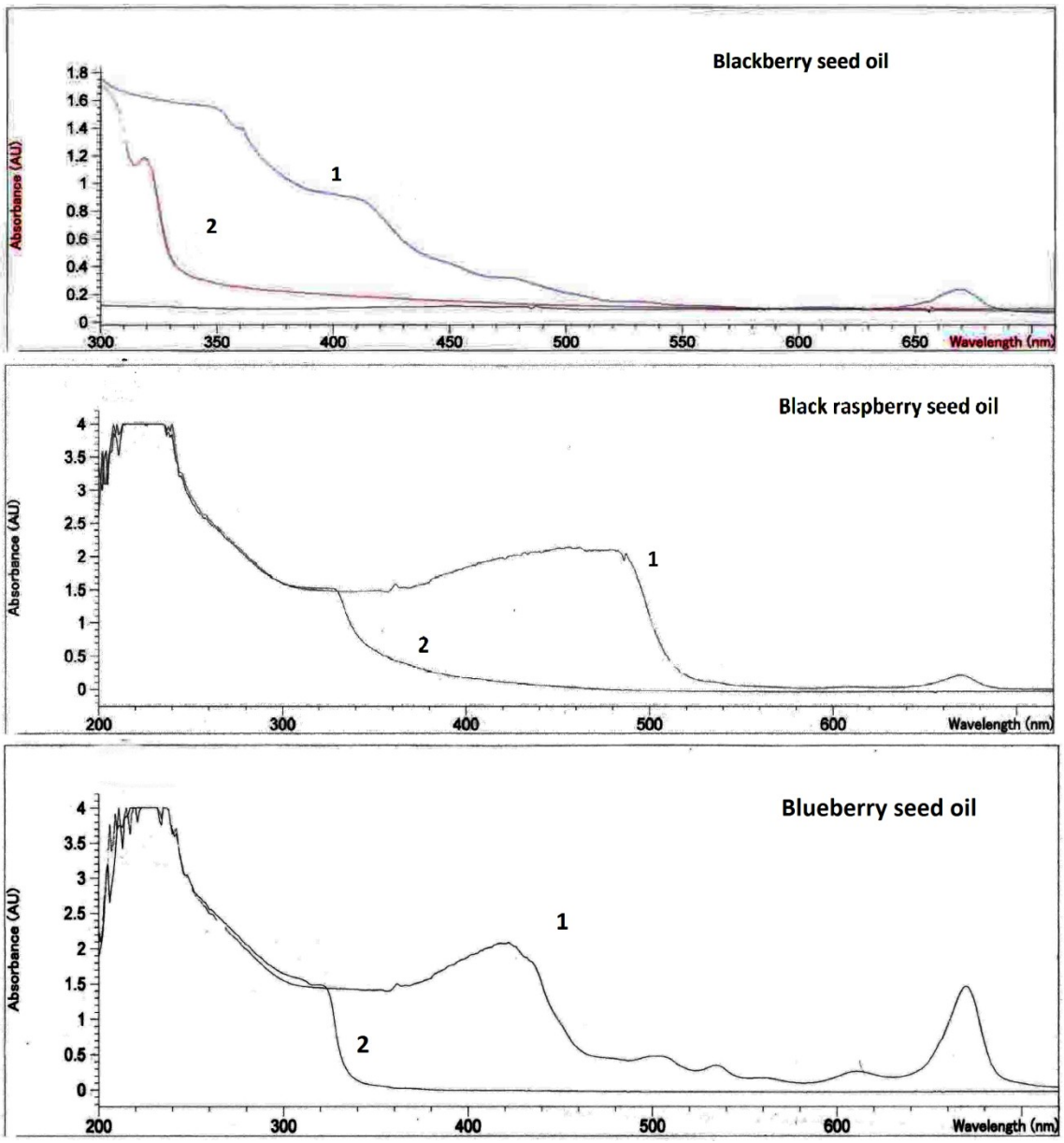


Figure 4. 4 Visible spectra of pigments in berry seed oils before (1) and after (2) column chromatography

4.4 Stability of autoxidized and photooxidized stripped and non-stripped blackberry, black raspberry and blueberry seed oils

The original non-stripped blackberry, black raspberry and blueberry seed oils contained higher amounts of primary oxidation product (conjugated dienes) than their corresponding column-stripped counterparts, but for secondary oxidation products (TBARS), no difference existed between non-stripped and stripped seed oils (Figures 4.5 and 4.6). Thus, column stripping better removed the primary oxidation products than secondary oxidation products; however, perhaps the original oils had a higher content of primary rather than secondary oxidation products during production and storage prior to accelerated oxidation studies.

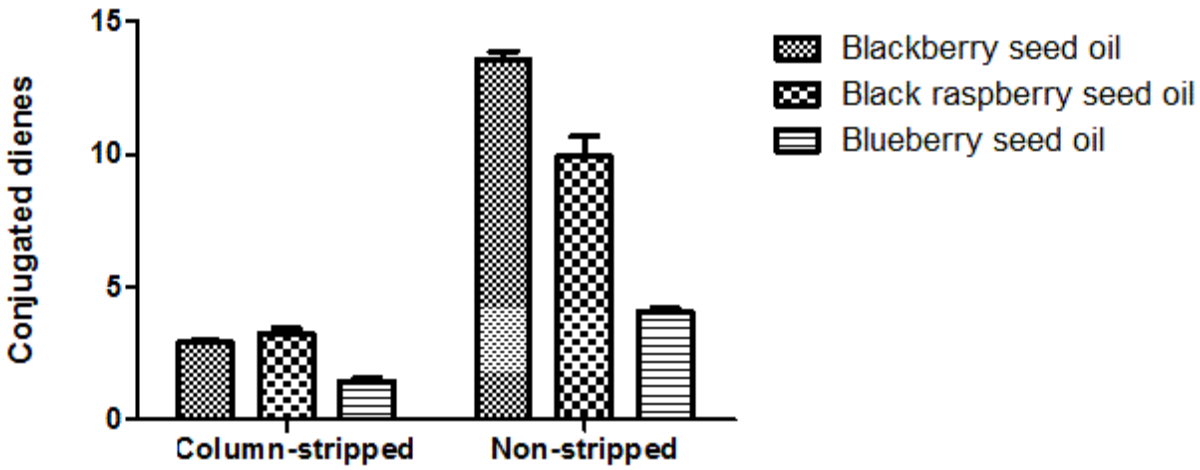


Figure 4. 5 Comparison of CD value in column-stripped and non-stripped blackberry, black raspberry and blueberry seed oils[1]; [1]Values are mean of triplicate determination ±standard deviation.

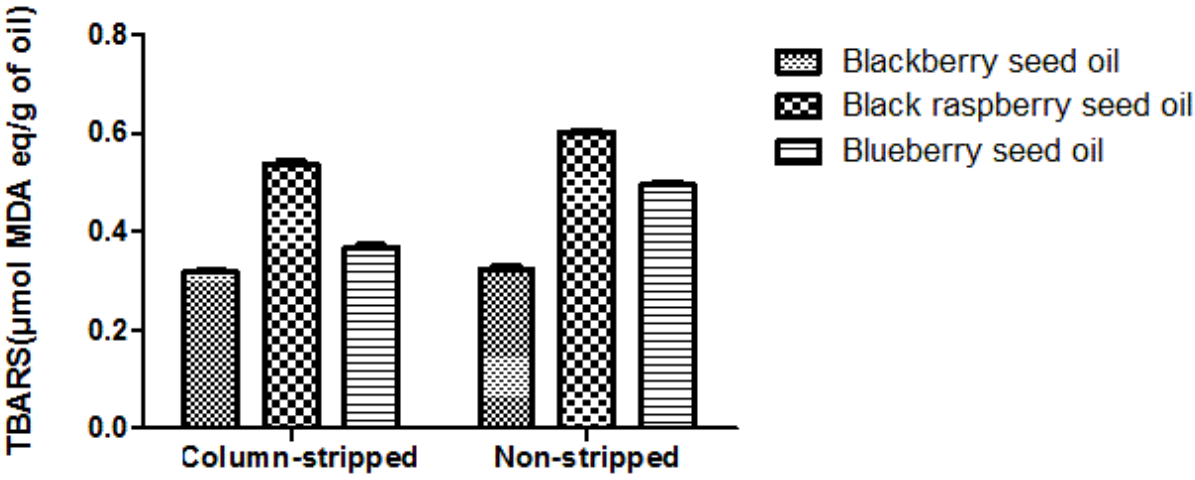


Figure 4. 6 Thiobarbituric acid reactive substances (MDA equivalents), of column-stripped and non-stripped blackberry, black raspberry and blueberry seed oils[1]; [1]Values are mean of triplicate determination ±standard deviation.

4.4.1 Autoxidation of berry seed oils and formation of primary and secondary oxidation products under Schaal oven condition

4.4.1.1 Primary products under Schaal oven condition

Schaal oven condition, as an accelerated oxidation test, is commonly used to examine the oxidative stability of edible oils and eventually predicts their shelf life. During the Schaal oven test, oil samples are placed in a forced air oven and the temperature is maintained between 60 and 70°C (Frankel, 1993a; Malcolmson et al., 1994). Abou-Gharbia *et al*. (1996) reported that 1 day storage under Schaal oven conditions at 65 °C is equivalent to 1 month of storage at room temperature (25°C). Similarly, flavor scores of edible oils at 60°C for 4 days agreed with those stored at ambient temperature for 4 months (Warner *et al.,* 1989).

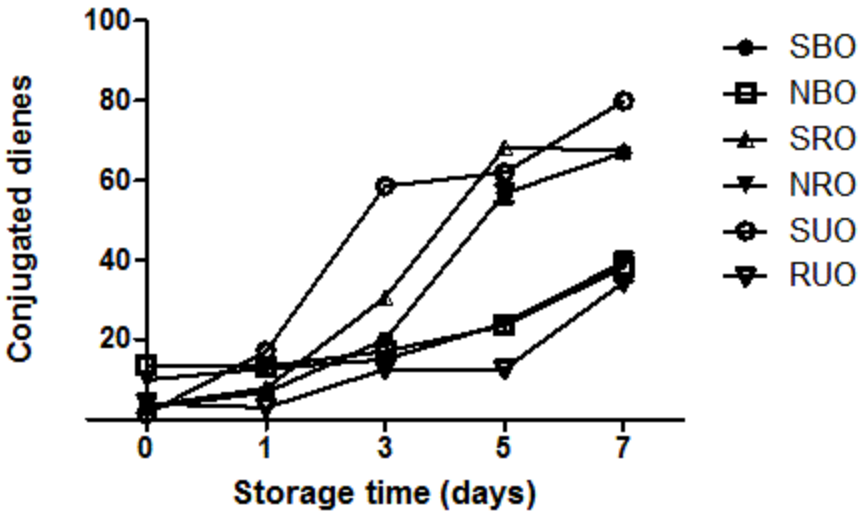


Figure 4. 7 Conjugated dienes of stripped and non-stripped blackberry seed oil, black raspberry seed oil, and blueberry seed oil during a 7-day storage at 60 °C under Schaal oven conditions ($p > 0.05$).

All oil samples were carefully handled to minimize exposure to light during storage and experiments. Figures 4.7 shows that all stripped berry seed oils were less stable than their non-stripped counterparts under Schaal oven condition, and that all stripped oils reflected similar trends as non-stripped oils. This is perhaps due to the removal of minor components that serve as antioxidants against oxidation of polyunsaturated fatty acids. Thus, minor antioxidative components present in tested berry seed oils, such as tocols, carotenoids and chlorophylls, played a significant role in stabilizing the non-stripped oils. Since all tested berry seed oils contain high amounts of unsaturated fatty acids, free radical chain reactions that would occur can be interrupted by the action of tocols, thus terminating the propagation reactions (Rossel, 1994; Porter *et al*., 1995). Tocols are gradually consumed during the induction period in both circumstances, leading to rapid oxidation of the stripped oils (Jung and Min, 1990; Kaitaranta, 1992; Baldioli *et al.*, 1996).

Stripped blueberry seed oil showed the least stability towards autoxidation, followed by stripped black raspberry seed oil and stripped blackberry seed oil. Interestingly, non-stripped blueberry seed oil displayed the best stability during autoxidation, again followed by non-stripped black raspberry seed oil and non-stripped blackberry seed oil.

The results of tocol determination indicated that blueberry seed oil contained the highest amount of tocol (up to 1302 ppm, primarily γ-tocotrienol), which was almost twice that of black raspberry and 5 times higher than tocols in blackberry seed oil. This

explains why non-stripped blueberry was most stable during autoxidation, while it became the least stable tested oil after the removal of its tocols. Thus tocols and other minor components present exert an impact on the quality of edible oils and their shelf lives.

4.4.1.2 Secondary oxidation products under Schaal oven condition

During autoxidation, secondary oxidation products of stripped and non-stripped cold-pressed blackberry, black raspberry and blueberry seed oils were determined by examining TBARS. TBARS test is based on the determination of color intensity of the TBA-MDA at 532 nm and expressing theresults as umol malondialdehyde equivalents per g of oil.

Figure 4.8 shows the trend from the formation of secondary oxidation products in tested berry seed oils under Schaal oven condition. All three pairs of stripped and non-stripped oils shared a similar trend to conjugated dienes formation, which showed that stripped oils were less stable than their non-stripped counterparts. The formation of secondary oxidation products increased rapidly after 3 days. However, upon further storage, changes occurred in the TBARS values, but this was not tested due to solidification of the oils used by their polymerization. It is noteworthy that during the experiments, all samples were polymerized after 12 days, thus making it impossible to test for CD or TBARS.

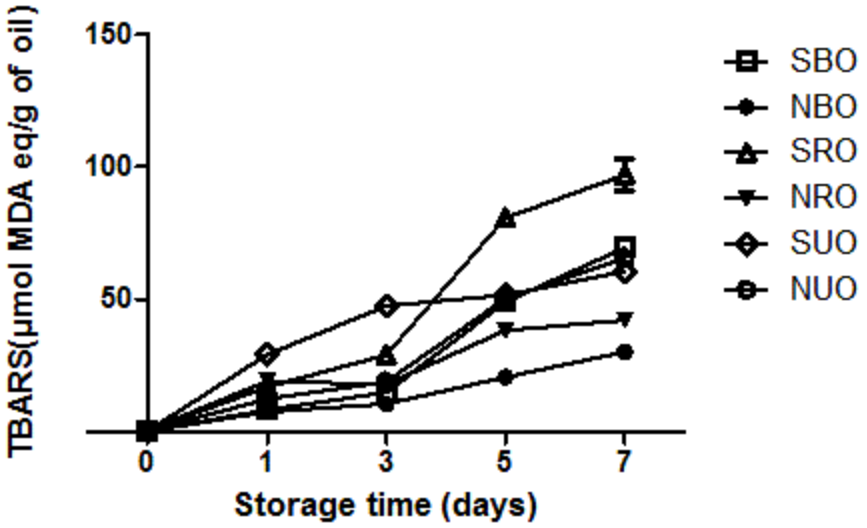


Figure 4. 8 Thiobarbituric acid reactive substances (MDA equivalents) of stripped and non-stripped blackberry, black raspberry, and blueberry seed oils during a 7-day storage at 60°C under Schaal oven condition ($p > 0.05$).

The examination of TBARS conducted during autoxidation showed that stripped blueberry and black raspberry seed oils were less stable than stripped blackberry seed oil. This indicates the importance of minor components on the stability and oxidation of blueberry and black raspberry seed oils. In addition, with a large amount α-linolenic acid located at the sn-1 and sn-3 positions of the triacylglecerol molecules, stripped blueberry and black raspberry were more prone to oxidation. Non-stripped blackberry showed the best oxidative stability among all tested oils (both stripped and non-stripped); this could be due to the depletion of tocols. Under Schaal oven condition (autoxidation), large amounts of tocols in blueberry and black raspberry (which have 5 and 3 times more tocols than blackberry seed oil, respectively), were gradually consumed during the induction period in both cases, leading to a rapid oxidation. Therefore, stabilities of the tested berry seed oils depend largely on the remaining amount of tocol and the location of unsaturated fatty acids in the triacylglycerol molecules.

4.4.2 Assessment of oxidative stability of stripped and non-stripped blackberry, black raspberry and blueberry seed oils under fluorescent light

4.4.2.1 Primary oxidation products under fluorescent light

Figure 4.9 presents the trend for the formation of primary oxidation products during photooxidation by measuring the conjugated dienes (CD) in stripped and non-stripped blackberry, black raspberry and blueberry seed oils. As seen, all stripped berry seed oils were significantly more stable ($p < 0.05$) than their non-stripped counterparts. All three pairs of stripped and non-stripped berry seed oils followed a similar trend in the formation of conjugated dienes, which showed a more rapid and continuous increase for conjugated dienes of non-stripped oils compared with their stripped counterparts. Thus, non-stripped oils were more susceptible to oxidation than stripped ones, which is a reverse of that for the autoxidation results; presence of pigments, mainly chlorophylls and carotenoids, in non-stripped berry seed oils is contemplated as possible reasons. Chlorophylls are photosensitizers which lead oils to photooxidation even in the presence of tocols. Non-stripped blueberry seed oil exhibited the least stability against photooxidation indicated that chlorophylls are responsible for photooxidized oils since blueberry seed oil contained the highest amount of chlorophylls; even though blueberry seed oil had the highest amount of tocols, the presence of chlorophylls still prevail over the antioxidant effect of tocols.

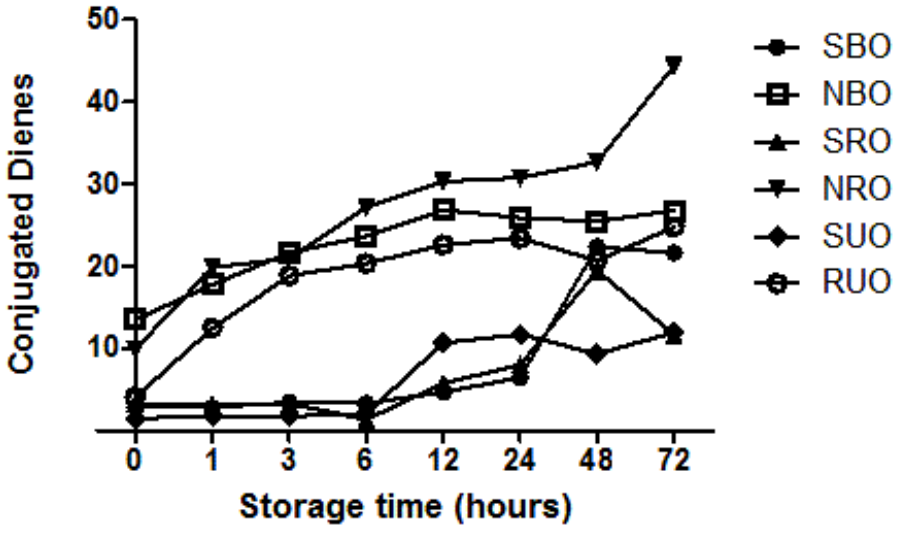


Figure 4. 9 Conjugated dienes of stripped and non-stripped blackberry, black raspberry, and blueberry seed oils during a 72-hour storage under fluorescent light at 27°C (P< 0.05).

The carotenoids and chlorophylls act as photosensitizers which eventually lead to the formation of hydroperoxides that subsequently decompose to volatiles causing rancidity or oxidative deterioration in edible oils (Warner and Frankel, 1987; Hall and Cuppett, 1993). The role of photosensitizers in light-induced oxidation has also been observed in non-stripped borage and evening primrose oils, as well as non-stripped hemp and flax seed oils (Khan and Shahidi, 2002; Abuzaytoun and Shahidi; 2006). However, the pigments detected in tested berry seed oils include chlorophylls and carotenoids, where carotenoids could function as singlet oxygen quenchers during oxidation (Kiritsakis and Dugan, 1985; Rahmani and Csallany, 1991). It is noteworthy that traces of carotenoids might be retained in stripped berry seed oils that could be involved in oxidation. This might be due to the instability of carotenoids under light and heat, especially β-carotene (Subagio and Morita, 2001).

4.4.2.2 Secondary oxidation products under fluorescent light

The secondary oxidation products as reflected in TBARS values of non-stripped and stripped seed oils under fluorescent light are illustrated in Figures 4.10. Generally, stripped berry seed oils and their non-stripped counterparts were not significantly different ($P>0.05$) from each other. The trend of TBARS formation during the photooxidation was not as clear as those for primary oxidation products. Nevertheless, Figure 4.10 indicates that non-stripped blackberry and blueberry seed oils had better

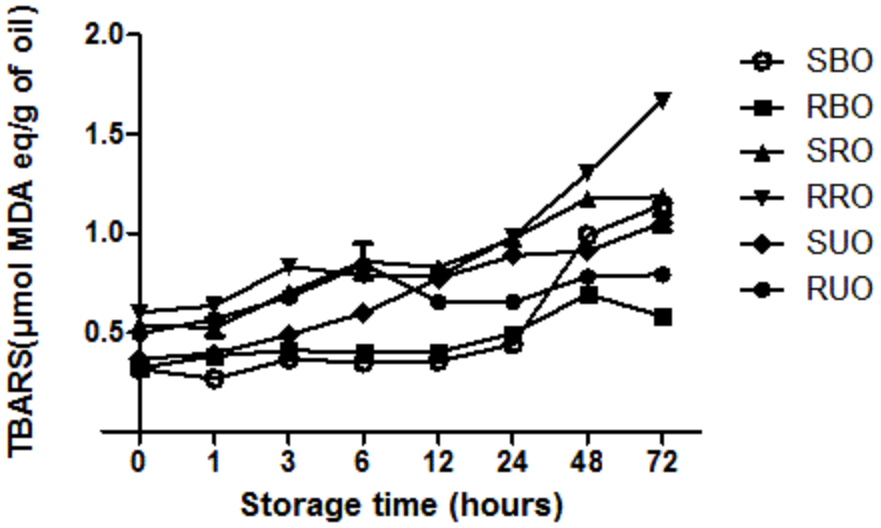


Figure 4. 10 TBARS (MDA equivalents) of stripped and non-stripped blackberry, black raspberry, and blueberry seed oils during a 72-hour storage under fluorescent light at 27°C (P> 0.05).

stability than other oils. According to the data presented, non-stripped and stripped blackberry seed oil had the best stability compared to others during the first 24-hour period. The lesser content of linoleic acid at the sn-1 and sn-3 positions of blackberry seed oil may be contemplated. It is also noteworthy that after 24 hours, polymerization started to develop, thus data retrieved after 24 hours might not be dependable as within the 24-hour period.

CHAPTER 5

SUMMARY AND RECOMMENDATIONS

The quality and oxidative stability of edible oils, depends on their chemical characteristics, such as fatty acid composition, triacylglycerol (TAG) profile, and positional distribution of fatty acids in TAGs, and the endogenous antioxidants, such as tocols, and pigments present. In the meantime, storage condition, such as heat and light, can also impact on the stability of the oils.

Cold-pressed blackberry, black raspberry and blueberry seed oils contain very simple and unique fatty acid and TAG profiles. Oleic, linoleic, and α-linolenic acids were the most prevalent fatty acids in all three berry seed oils examined. LnLLn, LLLn, LLL and LnLnLn were the most abundant TAG molecules in tested oils, with most α-linolenic being located at the sn-1,3 positions and linoleic acid at the sn-2 position. Blackberry, black raspberry and blueberry seed oils may be used as important dietary sources for essential fatty acids with a distinguished and favorable n-6/n-3 fatty acid ratios .

Gamma-tocopherol and gamma-tocotrienol were the predominant tocols detected in blueberry seed oil which contained the highest amount of γ-tocotrienol and total tocols. No significant amount of tocotrienol was found in blackberry or black raspberry seed oils. Blueberry seed oil contained the highest amount of chlorophylls and black raspberry had the most carotenoids. In order to evaluate the antioxidant abilities of these minor components, a comparison of their oxidative stability was made before and

after the removal of minor components. This study compared two techniques for the stripping of minor component, namely column chromatography and solvent extraction by partitioning; the former successfully removed the minor components, but the latter was only partially effective.

The oxidative stability under accelerated oxidation tests was dictated by the presence and contents of (poly)unsaturated fatty acids and their location in the triacylglycerol molecules as well as minor components (including pigments and tocols). During autoxidation, under Schaal oven condition, non-stripped berry seed oils displayed better stability than their stripped counterparts. However, under photooxidation, there was a reverse trend in that stripped berry seed oils exhibited a better stability than their non-stripped counterparts. Thus, tocols worked as antioxidants during autoxidation, while pigments acted dominantly as photosensitizers during photooxidation. Based on the results presented here, it is recommended that blackberry, black raspberry and blueberry seed oils should be stored in small and dark glass containers.

This study has provided detailed information about the chemical characteristics of berry seed oils which can be used to speculate the potential use of these oils in food and non-food products and to confirm product authenticity. Their TAG profile was revealed for first time, which can be used as reference in future studies.

The chemical composition of berry seed oils demonstrates the potential health benefits and applications for the oils. Specialty oils like berry seed oils are the new trends for

product development of the oil-based nutraceuticals and/or supplements which can promote human health. From a nutritional viewpoint, the high unsaturated fatty acid content in the berry seed oils makes it a suitable alternative to highly unsaturated fish oils. In addition, berry seed oils might have similar effects such as cholesterol-lowering activity and prevention of cardiovascular disorders. In order to determine the amount of daily intake that could benefit human health, pre-clinical and clinical studies are necessary. Most berry seeds are by-products from beverage processing, therefore research for other applications may promote the minimization of waste disposal issues, improving the value of the berries and agricultural economy.

Further research is needed in determination of the bioactive components in berry seeds and their oils to develop novel application for optimum human nutrition. In addition, the left over meals, including the peels after oil extraction of the seeds, may be evaluated for the presence of bioactive components and their potential use in value-added applications.

Finally, this study demonstrated that the cold-pressed blackberry, black raspberry, blueberry seed oils contained significant levels of natural antioxidants. Following the growing consumer awareness, novel natural antioxidants with desirable physicochemical properties are highly demanded for food applications to replace synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). Therefore, cold-pressed berry seed oils and their antioxidant

extracts may also have the potential to be further developed as food additives for improving food quality, stability and safety.

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APPENDIX

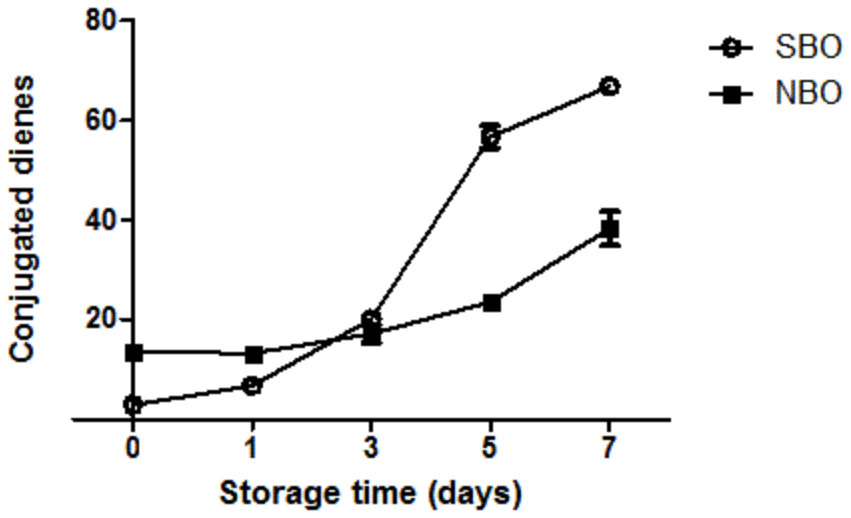

Figure A. 1 Conjugated dienes of stripped (SBO) and non-stripped (NBO) blackberry seed oil during a 7-day storage at 60 °C under Schaal oven condition ($P > 0.05$).

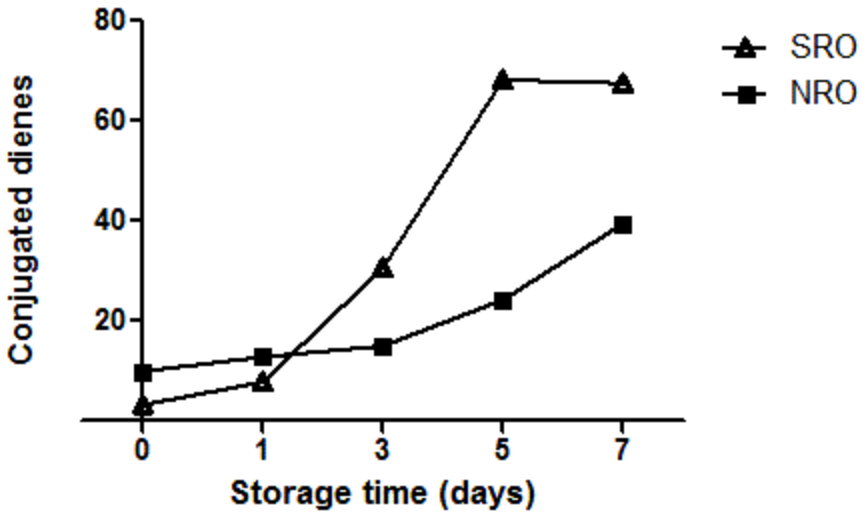

Figure A. 2 Conjugated dienes of stripped (SRO) and non-stripped (NRO) black raspberry seed oil during a 7-day storage at 60 °C under Schaal oven condition (P> 0.05).

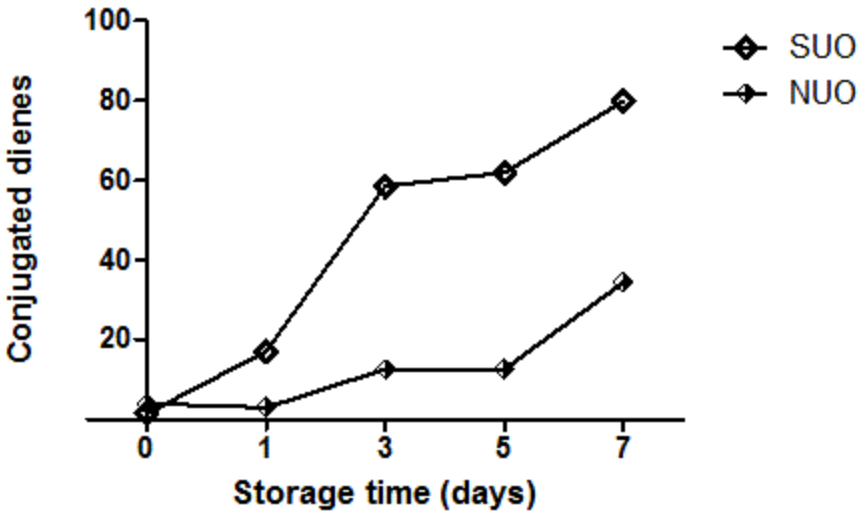

Figure A. 3 Conjugated dienes of stripped (SUO) and non-stripped (NUO) blueberry seed oil during a 7-day storage at 60 °C under Schaal oven condition (P> 0.05).

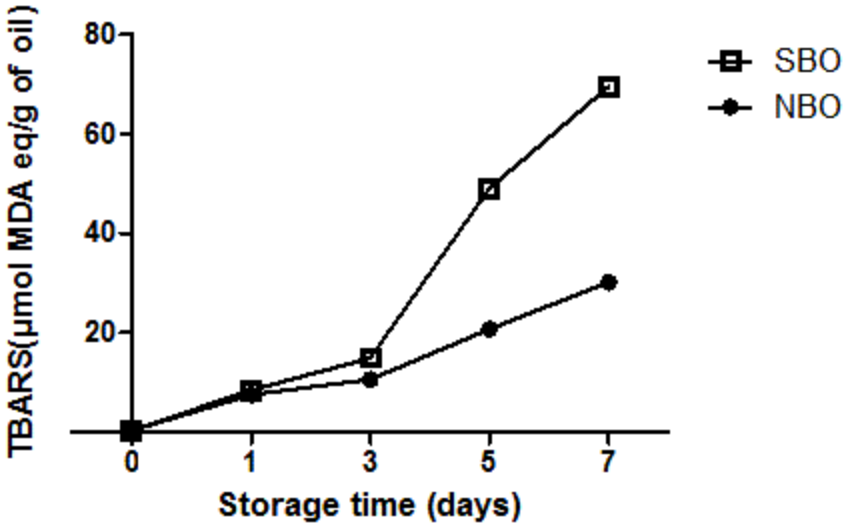

Figure A. 4 Thiobarbituric acid reactive substances (MDA equivalents) of stripped (SBO) and non-stripped (NBO) blackberry seed oil during a 7-day storage at 60°C under Schaal oven condition ($P > 0.05$).

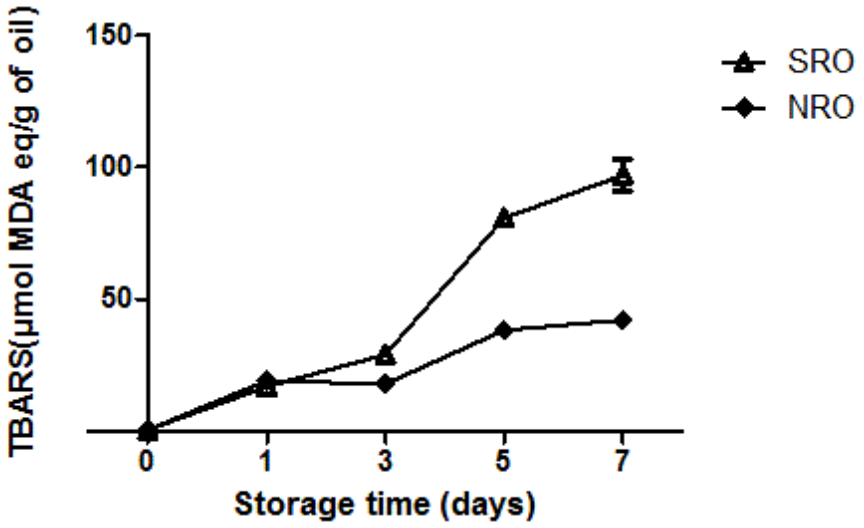

Figure A. 5 Thiobarbituric acid reactive substances (MDA equivalents) of stripped (SRO) and non-stripped (NRO) black raspberry seed oil during a 7-day storage at 60°C under Schaal oven condition ($P > 0.05$).

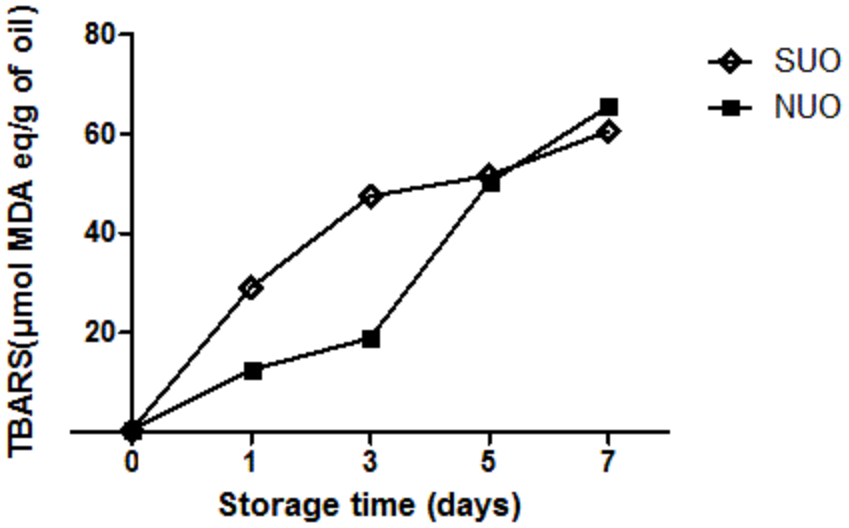

Figure A. 6 Thiobarbituric acid reactive substances (MDA equivalents) of stripped (SUO) and non-stripped (NUO) blueberry seed oil during a 7-day storage at 60°C under Schaal oven condition (P> 0.05).

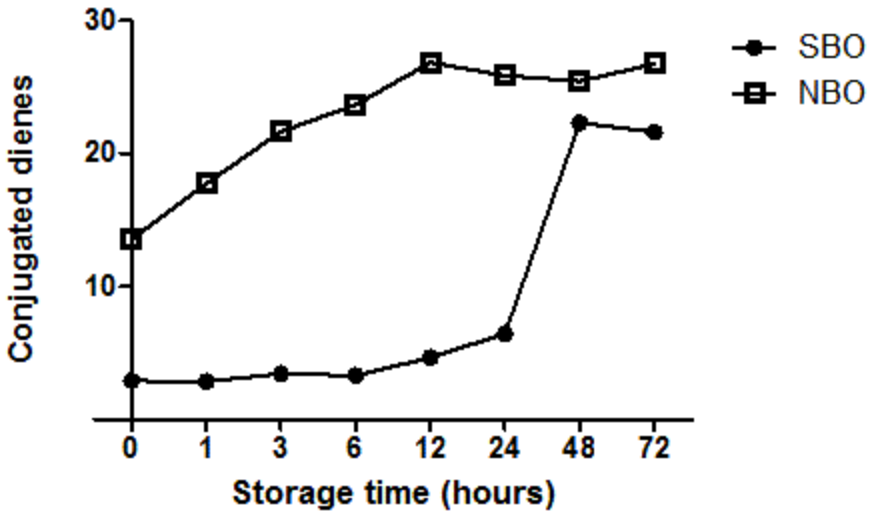

Figure A. 7 Conjugated dienes of stripped (SBO) and non-stripped (NBO) blackberry seed oil during a 72-hour storage under fluorescent light at 27°C ($P < 0.05$).

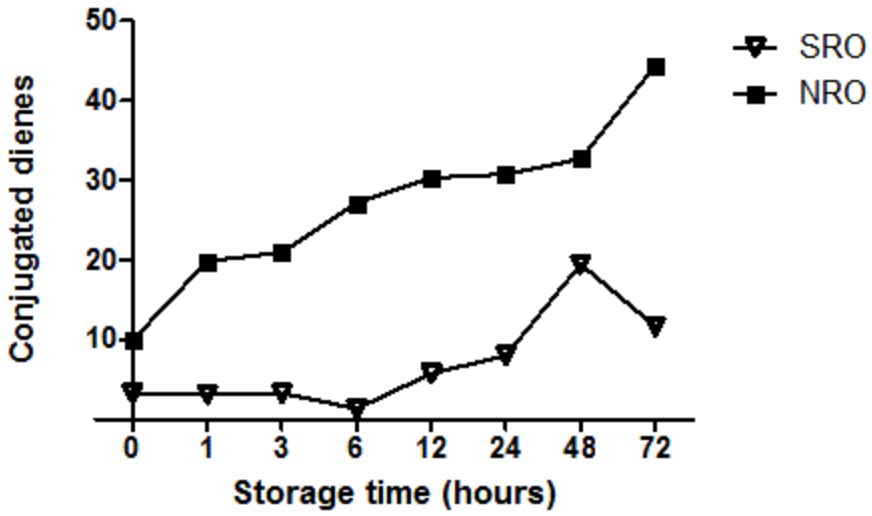

Figure A. 8 Conjugated dienes of stripped (SRO) and non-stripped (NRO) black raspberry seed oil during a 72-hour storage under fluorescent light at 27°C (P< 0.05).

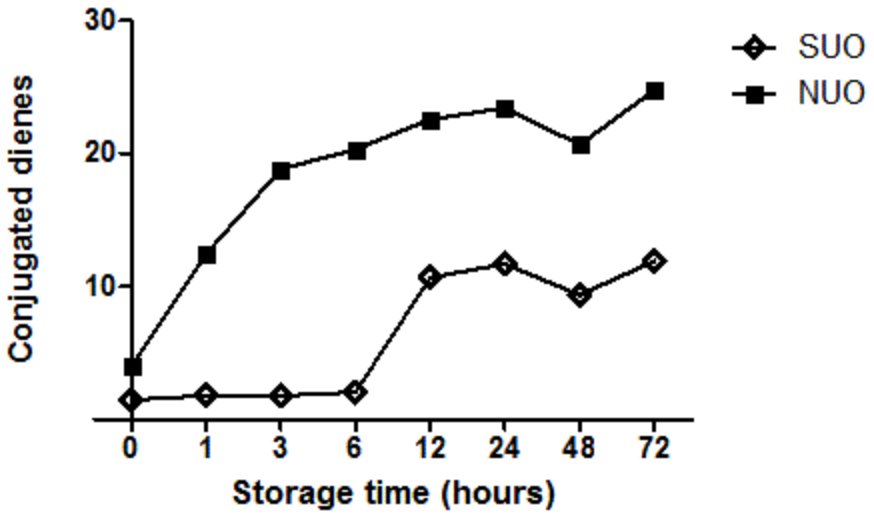

Figure A. 9 Conjugated dienes of stripped (SUO) and non-stripped (NUO) blueberry seed oil during a 72-hour storage under fluorescent light at 27°C ($P < 0.05$).

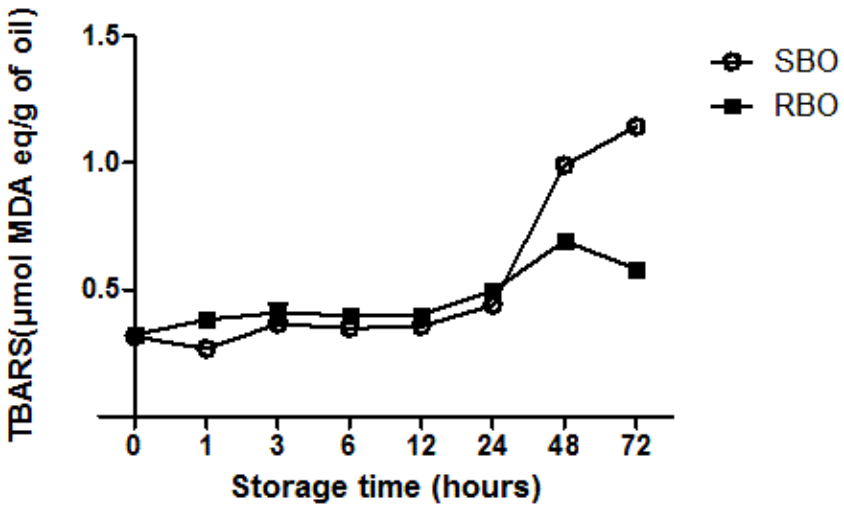

Figure A. 10 Thiobarbituric acid reactive substances (MDA equivalents) of stripped (SBO) and non-stripped (NBO) blackberry seed oil during a 72-hour storage under fluorescent light at 27°C (P> 0.05).

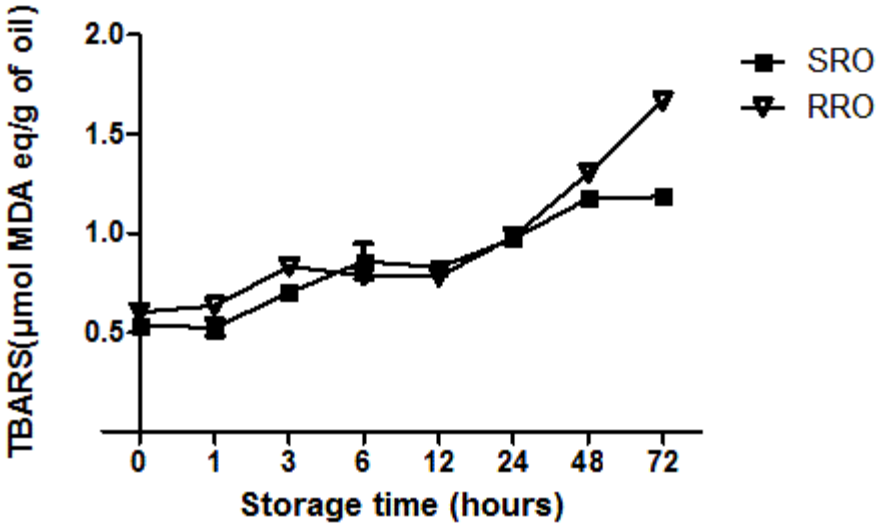

Figure A. 11 Thiobarbituric acid reactive substances (MDA equivalents) of stripped (SRO) and non-stripped (NRO) black raspberry seed oil during a 72-hour storage under fluorescent light at 27°C (P> 0.05).

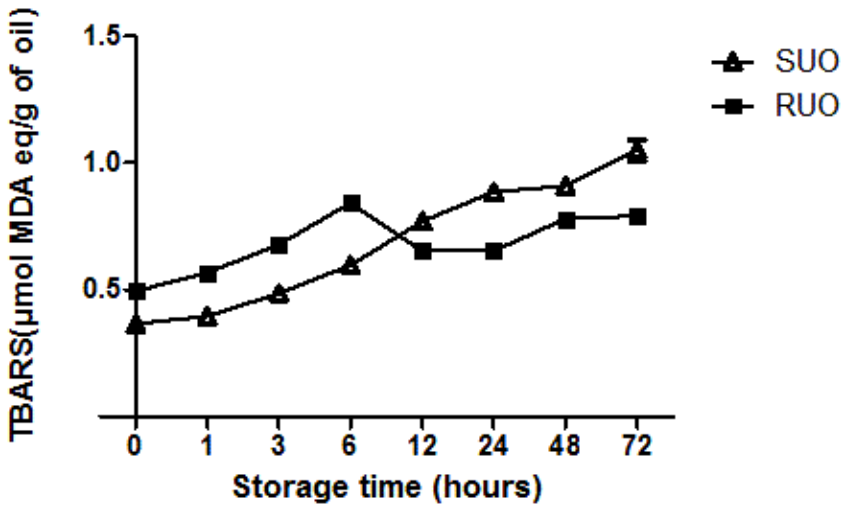

Figure A. 12 Thiobarbituric acid reactive substances (MDA equivalents) of stripped (SUO) and non-stripped (NUO) blueberry seed oil during a 72-hour storage under fluorescent light at 27°C ($P > 0.05$).