Oxidative Stabilities of Docosahexaenoic Acid Oil and Linoleic Acid in an Aqueous System

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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2010

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Abstract

Docosahexaenoic acid (DHA) has been shown to be essential for normal brain and retinal development. Effects of refining, bleaching, winterizing, and deodorizing on the oxidative stability of DHA (22:6 ω-3) oil were studied by a combination of headspace oxygen depletion (HOD) by gas chromatography and total volatile compound formation by SPME-GC. The oxygen content in the refined, bleached, winterized, and deodorized DHA oil samples decreased from 20.9% on Day 0 to 6.7%, 8.3%, 7.8% and 7.8%, respectively on Day 5. The bleached, winterized, and deodorized oils were determined not to be statistically different from each other (p > 0.05), however the refined oil showed significantly more HOD (p <0.05), corresponding to a lower oxidative stability. Refined, bleached, winterized, and deodorized oils showed average volatile compound formations over 5 days of storage of approximately 4,041,000 electronic counts (ec), 482,100 ec, 437,200 ec, and 405,800 ec, respectively. The refined samples again showed significantly (p < 0.05) higher volatile compound formation, corresponding to a lower oxidative stability. The bleached, winterized, and deodorized samples were not significantly different from each other (p > 0.05). Bleaching proved to be the crucial processing step in increasing the oxidative stability of DHA oil.

Ginseng has been shown to be a natural antioxidant, and compounds from its extract are studied here for their individual antioxidant effects. Kaempferol, caffeic acid,
ferulic acid, salicylic acid, and vanillic acid were previously isolated, separated, and identified by the combination of HPLC, NMR and MS. The effects of 100 ppm of each compound on the riboflavin photosensitized oxidation were studied at 4°C for 3 hours. Additionally, the effects of 100 ppm of each compound on the autoxidation of linoleic acid was studied, both with and without added ferrous chloride to give 2 ppm ferrous ion at 37°C in the dark for 10 days (with ferrous ion) or 40 days (without ferrous ion). The HOD of the samples was determined by GC and the peroxide value by spectrometry. Caffeic acid, kaempferol, vanillic acid, salicylic acid, α-tocopherol, and ferulic acid minimized the photosensitized oxidation by 37, 30, 30, 18, 17, and 0%, respectively. The average peroxide value for linoleic acid in aqueous solution without 2 ppm ferrous ion and 100 ppm kaempferol, vanillic acid, ferulic acid, caffeic acid, salicylic acid, α-tocopherol or control during 40 days of storage was 0.2, 0.7, 2.2, 2.3, 2.7, 2.8, or 3.7, respectively. The average peroxide value for samples with 2 ppm ferrous ion and 100 ppm kaempferol, vanillic acid, ferulic acid, caffeic acid, α-tocopherol, salicylic acid, or control during 10 days of storage was 0.3, 1.2, 2.5, 2.8, 5.3, 6.3, or 7.5, respectively. Ginseng compounds did not have antioxidant effects in the photosensitized oxidation of 2% linoleic acid in the aqueous system at p>0.05. Ginseng compounds except salicylic acid had better antioxidant effects than α-tocopherol in the autoxidation of linoleic acid in the aqueous system at p< 0.05. Caffeic acid, ferulic acid, and vanillic acid may act as antioxidants by the combination of donating hydrogen atom and chelating ferrous ion.
Dedication

This document is dedicated to my mother, father and brother, who are unconditionally supportive of everything I do.
Acknowledgments

I would like to express my gratitude to Dr. David Min, my academic advisor, for accepting me into his lab, allowing me the opportunity to work on this project, and trusting me to work independently. It has been a valuable life experience.

A very special thank-you is in order to my lab mate, Hao-Hsung “Andy” Chang, without whom I could never have completed this work.

And finally, thank you to my committee members, Dr. Rodriguez and Dr. Lee for their guidance, and most especially to my friends and colleagues in the Food Science department of The Ohio State University for their support and friendship.
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INTRODUCTION

1. Docosahexaenoic acid and oil processing

Docosahexaenoic acid (DHA, 22:6, n-3) is a long-chain polyunsaturated omega-3 fatty acid that can be derived from algae or fish oils and has been reported to protect against a wide range of diseases, including: atherosclerosis, myocardial infarction, cancer, autism, and sudden death (Lee and others 2003, Mori 2004). Along with other n-3 fatty acids, the effects of DHA have been widely reported as being beneficial to reducing inflammation, lowering blood pressure, raising low-density lipoprotein (LDL) cholesterol, and decreasing plasma triglyceride (TG) (Kasim-Karakas 2001, Mori 2004, Heller and others 2006(b)). DHA is essential for development throughout life, including for normal growth of the central nervous system; a high level of DHA is found in phospholipids of the brain and retina, suggesting an important role in optical development (Innis 1991, Lee and others 2003, Maki and others 2003). One important issue regarding DHA is the environmental implications of the commercial fishing industry, especially as demand continues to increase. Wild salmon and tuna populations have suffered, and some species of tuna are now listed as endangered. Salmon is now being farmed, but with the decreased environmental impact comes the use of antibiotics which may be undesirable for some consumers. Other species have suffered as their natural habitats are depleted from industrial fishing (Heller and others 2006). One
solution to this problem is the use of algal DHA, which can be grown in a controlled environment with no ill effects on the marine ecosystems of the world.

The effects of processing on the oxidative stability of DHA oil have not been reported. Processing steps involved in the production of algal DHA oil include refining, bleaching, winterizing, and deodorizing. These steps are all standard in the oil industry and are used in a variety of unsaturated oils such as soybean and canola (Johnson 2008). Each step is performed for specific purposes in order to produce oil that is acceptable to consumers for use as is, or for addition into other products. Certain prooxidant and volatile compounds are removed during these steps, along with some antioxidant compounds. Tocopherols, phospholipids, metals, chlorophylls, and peroxides are all targeted for removal during the oil production process.

Refining involves the removal of nonglyceride compounds such as proteins, waxes, and free fatty acids. Phosphatides must be removed, as they will react with moisture and precipitate out. The type of refining referred to in this work is alkali refining, which involves the formation of soapstock by reacting free fatty acids with caustic soda (NaOH). Care must be taken so that glycerides do not react with the soda, which results in increased refining loss. This soapstock is then removed as a by-product of the refining process, and the result is a more neutralized oil, which increases oxidative stability (Johnson 2008).

Bleaching is performed to remove naturally occurring pigments, such as chlorophyll. This is not only done for better consumer acceptance, but chlorophyll is also a prooxidant in the presence of light, so bleaching also is done to improve oxidative
The bleached oil then proceeds to the winterization step. Winterization is a chilling step in which compounds with higher molecular weights precipitate and are filtered out. Deodorization follows, which is primarily for the removal of small volatile compounds such as aldehydes and ketones. These volatiles give undesirable flavors and off-odors to the oil. Peroxides are also removed during this step—they are known as powerful prooxidants during the chain reaction of autoxidation (Johnson 2008).

The objective of this work was to study the effects of processing on the oxidative stability of algal DHA oil. Many studies have been performed to investigate processing effects on soybean oil; however no published work exists at this time on the effects of processing steps on DHA oil.

2. Linoleic acid and ginseng

Ginseng (*Panax ginseng* C. A. Meyer) has long been reported to have various health benefits in the Orient (Liu and others 1992; Park 1996; Shin and others 2000). Pharmacological and physiological studies reported that ginseng has beneficial effects on cancer (Yoon 1993; Konoshima and others 1999; Liu and Zhou 2000; Shin and others 2000), hepatic disease (Voces and others 1999), cardiovascular and cerebrovascular diseases (Cicero and others 2003; Huang and others 1999). The medical effects of ginseng have been closely related to ginseng’s antioxidant properties (Kang and others 2006). Caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid were isolated in ginseng, separated by liquid chromatography and identified by the
combination of mass spectrometry and chromatographic retention times by Wi (1989). He also stated that the antioxidant activities of these compounds should be studied.

The importance of natural antioxidants in foods and biological systems has been increasingly recognized in recent years (Lee and others 2004; Wu and others 2004; Choe and others 2005; Nantz and others 2006). Lipid oxidation has negative effects on the flavor stability of foods (Bradley and others 1992; Choe and others 2005), aging (Dillard and others 1973; Lee and others 2004), mutagenesis (Basu and others 1983), carcinogenesis (Diplock and others 1991; Knekt and others 1991), and heart diseases (Jacob 1994). The effects of natural and synthetic antioxidants have been extensively studied in oil systems. Limited studies on the effects of antioxidants in the lipid oxidation of aqueous system were reported by Cillard and others (1980a, 1980b), Chimi and others (1991), Osawa and others (1981), Ramarathnam and others (1988, 1989), Inatani and others (1983) and Rankin and others (1993). Cillard and others (1980a, 1980b) reported that α-tocopherol had prooxidant effects on lipid oxidation in an aqueous system. Osawa and others (1981) reported that some natural compounds showed no antioxidant effects in oil system, but had remarkable antioxidant activity in aqueous system. The objective of this research was to study the antioxidant properties of caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid on the riboflavin photosensitized oxidation and the autoxidation of 2% linoleic acid of aqueous-buffered system.
1. DHA

Omega-3 (n-3) fatty acids first gained attention when a group of nutrition experts began studying the dietary habits of Greenland Eskimos in the early 1970’s (Pique 1986, Heller and others 2006(b)). The team wanted to study the effects of the Eskimos’ high fat, high protein, low fiber, and low Vitamin C diet on the health of the Eskimos. They expected to find adverse health effects and high rates of heart disease, but instead they were surprised to observe remarkable general health and resistance not only to cardiovascular disease, but cancer as well (Pique 1986). In fact, the Eskimo group had a 5.3% mortality rate from heart disease, compared to approximately 40% for the U.S. population at that time (Dyerberg 1981). It was also observed that the Eskimo population had almost no cases of breast cancer. After determining that the differences were not due to genetics and exercise, the team of scientists began focusing on the Eskimos’ marine animal diet, which included oily fish, whale and seal blubber (an estimated ¼ pound of fat per day). Blood analysis showed that the Eskimo population had about sixteen times the amount of eicosapentaenoic acid and approximately four times the amount of docosahexaenoic acid (Pique 1986).

Eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are both long-chain polyunsaturated n-3 fatty acids derived from fish and algae which have been reported to protect against a wide range of diseases, including: atherosclerosis, myocardial infarction, cancer, autism, and sudden death (Lee and others 2003, Mori
Along with other n-3 fatty acids, the effects of EPA and DHA have been widely reported as being beneficial to reducing inflammation, lowering blood pressure, raising low-density lipoprotein (LDL) cholesterol, and decreasing plasma triglyceride (TG) (Kasim-Karakas 2001, Mori 2004, Heller and others 2006(b)). EPA and DHA are essential for development throughout life, including for normal growth of the central nervous system; a high level of DHA is found in phospholipids of the brain and retina, suggesting an important role in optical development (Innis 1991, Lee and others 2003, Maki and others 2003).

DHA is considered essential because it needs to be ingested in the diet. A small amount can be formed inside the body from alpha-linolenic acid; however the conversion efficiency is lower than 5%. This efficiency also decreases with the intake of linoleic acid (18:2 n-6) (Borneo and others 2007). The biochemical synthesis of DHA from alpha-linolenic acid is found in significant amounts only in chloroplasts such as algae and plankton, which are the main essential fatty acid source of marine animals. DHA constitutes approximately 8-20% by weight of fish oil (Watkins and German 2008). The EPA and DHA content of several different fish can be seen in Table 1 (Heller and others 2006). The structure of DHA can be seen in Figure 1.
Table 1: EPA and DHA content of various fish

<table>
<thead>
<tr>
<th>Fish</th>
<th>EPA (g/100g)</th>
<th>DHA (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mackerel, king</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>Herring, Pacific</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Anchovy</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Salmon, Chinook</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Tuna, albacore</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Salmon, pink</td>
<td>0.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Halibut, Greenland</td>
<td>0.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Figure 1: Structure of DHA
2. Health Benefits of DHA

The full spectrum of health benefits provided by regular intake of DHA is broad. Some are well-researched, and some have likely not yet been discovered. Some known benefits of DHA intake include therapy for intensive care and post-operative patients, proper development, regulation of healthy cholesterol levels for coronary health, improved cardiovascular health, and may even be beneficial for cancer patients. Emerging research suggests that DHA may also play a preventative or therapeutic role in autism, Alzheimer’s disease, attention deficit hyperactivity disorder, and some types of depression, though further studies are needed. One of the most important benefits of DHA intake that has been extensively studied and observed in clinical trials is normal development of the fetal cerebral cortex.

2.1 DHA Intake for Development

EPA and DHA are both crucial to normal development. DHA is especially important for neurodevelopment and normal visual function (Innis 1991, Lee and others 2003, Sahena and others 2009). A deficiency in fetal cerebral DHA content has been associated with preterm delivery, and children born preterm have higher risks for autism, ADHD, and schizophrenia. Individuals diagnosed with these disorders exhibit poor gray matter maturation, suggesting that DHA deficiency while in the womb may prove to be a preventable cause of these disorders. Studies have shown that infants given DHA displayed enhanced visual acuity and cognitive abilities (McNamara and Carlson 2006).
This shows that while the consumption of omega-3 fatty acids is essential throughout life, during the development stages it is especially crucial. Formula-fed infants often have low levels of DHA, creating the need for omega-3 supplementation in the formula market (Sahena and others 2009).

Several epidemiological studies have shown that generations of animals that have been fed omega-3 deficient diets have displayed limited learning ability. Normal learning was restored when the diet is changed to be supplemented with DHA. One study sought to determine the effects of DHA on aged mice. Patients diagnosed with Alzheimer’s disease have showed deficiencies in DHA levels in the brain, and there is some evidence that suggests DHA may be associated with reduced risk for the disease. It is thought that DHA may help reduce β-amyloid (aggregated β-protein) toxicity, a major contributing factor in the progression of Alzheimer’s disease. Mice aged 17-19 months old were fed diets of varying DHA content- a control group of 0.09%, a low group of 0.00%, and a high group of 0.60%. Results showed that the high DHA group had a 77% reduction in amyloid level when compared to the low group (p <.05). The researchers concluded that since environmental and genetic factors can impact a patient’s risk for Alzheimer’s disease, the results show simply that DHA intake may be associated with a reduced risk for developing the disease (Lim and others 2005).

2.2 Cardiovascular Health

The beneficial effects of EPA and DHA on cardiovascular health are well documented. Randomized control trials have shown that the intake of omega-3 fatty
acids can not only reduce the risk of cardiovascular disease, but may also be therapeutic for patients already diagnosed. Several mechanisms have been put forth in explanation of the benefits of EPA and DHA, including decreasing inflammation (Calder 2001), decreasing blood pressure (Geleijnse and others 2002), lowering plasma triglycerides (Saks and others 2002), improving vascular activity (Goodfellow and others 2000), and decreasing blood platelet aggregation (Hornstra 2001). Another possible mechanism that is supported by epidemiologic studies is the prevention of arrhythmias. The Gruppo Italiano per lo Studio della Sopravvivenze nell’Infarto Miocardico (GISSI) Prevention trial is the largest randomized controlled trial to date, and dealt with 11,324 patients surviving recent myocardial infarction (within 3 months). Subjects were randomly assigned to take an omega-3 pill, a vitamin E pill, a combination, or no pill (approximately 2830 subjects per group). The groups were compared after 3.5 years. The omega-3 consumption was associated with reductions in cardiac-related mortality, with the strongest benefit in the first 9 months after the initial infarction. There was, however, no reduction in nonfatal myocardial infarction (Marchioli and others 2002). The more immediate benefit combined with the effect on sudden death point to omega-3’s acting to prevent arrhythmias (Breslow 2006).

Evidence supports the claim that for cardiovascular disease risk reduction, 500 mg/day of EPA and DHA is recommended. This can be met with 2 servings per week of fatty fish, or foods that have been enriched with EPA and DHA. For the treatment of existing cardiovascular disease, 1-3 g/day is recommended. When ingesting this amount, supplements are almost certainly needed (Breslow 2006, Gebauer and others 2006).
Future research is aimed toward determining whether the intake of omega-3’s decreases death from cardiovascular events. Researchers are also trying to determine if the benefits are from EPA, DHA, a combination of the two, or the dosage (Breslow 2006).

2.3 Anti-inflammatory Effects

Conditions such as rheumatoid arthritis, ulcerative colitis, inflammatory bowel disorders, and psoriasis are diseases with complex pathophysiology. While the mechanisms of these diseases are slowly becoming understood, treatments are still being researched. Inflammatory reactions can be triggered by microbiological, immunological, and/or toxic agents, working via humoral and/or cellular pathways. Possible consequences of inflammatory disorders in intensive care situations include multiple organ failure, shock, and sepsis (Heller and others 2006(a)). Omega-3 fatty acids, especially EPA and DHA, have been shown to have anti-inflammatory and immunomodulating action. Clinical trials have shown beneficial effects from EPA and DHA on rheumatoid arthritis, psoriasis, inflammatory bowel disorders, and asthma (Mori and Beilin 2004).

Aside from functioning as a barrier system, cellular lipid membranes (arachidonic acid, AA) can also be oxidized into biologically active lipid mediators known as eicosanoids. Some examples of eicosanoid compounds include leukotrienes and prostaglandins. Due to their short half-life, eicosanoids tend to act locally- they are an important part of the onset of inflammation due to their regulatory functions. Some are
pro-inflammatory and some are anti-inflammatory. AA is the basis for all the pro-
inflammatory eicosanoids. It is released from the cellular lipid membrane during
inflammation and metabolized via two major pathways to prostaglandins and
leukotrienes. The two pathways are via cyclooxygenase and lipoxygenase, both enzymes
which catalyze the conversion of AA to eicosanoid compounds. There are two forms of
cyclooxygenase, called COX I and COX II. Over-the-counter non-steroidal anti-
inflammatory drugs (NSAIDS) act as inhibitors of the COX II pathway. Research has
shown that EPA and DHA may promote the formation of eicosanoids with
immunomodulating characteristics. In a state of inflammation, EPA/DHA may compete
with AA for enzymes and allow the production of more anti-inflammatory compounds
(Heller and other 2006). Omega-3 fatty acids down-regulate COX II expression and
therefore may have similar effects as NSAIDs (Heller and Koch 2006). The compounds
formed from EPA/DHA are different from those formed from AA in structure and
biological activity. When EPA/DHA is available, the compounds formed are less pro-
inflammatory (Heller and others 2006).

EPA and DHA may, on the subcellular level, help modulate the acute phase
response and prevent overshooting the overall immune response, resulting in
improvement of the inflammatory disease. However, all the subcellular behaviors of
omega-3 fatty acids are not yet fully understood. It is known that as omega-3’s are
incorporated into the cellular lipid membrane, they replace omega-6 fatty acids, resulting
in improved fluidity of the membrane and also influencing eicosanoid production (Heller
and others 2006).
2.4 Intensive Care and Post-Operative Recovery

Patient nutrition during a stay in the intensive care unit or during post-operative care is extremely important. Often the patient’s health is dependent on enteral nutrition. One such example is in the case of sepsis, a complex condition that results from a combination of infection and systemic inflammatory reaction. It can cause vital organ failure, in which case the condition is known as severe sepsis. If low blood pressure is a complication, the condition is referred to as septic shock. Nutrition therapy, including intake of omega-3 fatty acids, plays an important role in the treatment of sepsis, with the goal to treat each patient in accordance with their individual metabolic demand. Omega-3 treatment for as short a time as three days has been shown to reduce the incidence of sepsis (Heller and Koch 2006). It is thought that formulas containing EPA and DHA may be beneficial to the healing and overall health of intensive care patients. Receiving formulas enriched with EPA and DHA has been proven to raise the EPA and DHA levels to normal in the blood of such patients, improving their fatty acid status (Munakata and others 2009).

Post-operative care is focused on the patient’s recovery without development of malnutrition or infection. Research shows that intake of omega-3 fatty acids can help post-operative patients heal more quickly and with fewer infections. In one study, 661 patients were given short-term infusion doses (at least 5% fish oil) of omega-3 fatty acids after major abdominal surgery were found to improved liver function. The patients also exhibited improved balance between pro- and anti-inflammatory cytokines, helping to prevent hyper-inflammatory complications after surgery. In fact, fish oil significantly
lowered the occurrence of co-morbid infection. Patients receiving the fish oil required fewer antibiotics and were released from the hospital sooner than those not given the infusions, and their liver and pancreatic enzyme levels were more quickly normalized (Heller and Koch 2006).

2.5 DHA Intake as a Potential Treatment for Autism/Attention Deficit Hyperactivity Disorder

Attention deficit hyperactivity disorder (ADHD) is a neurological disorder that affects 1 in 20 children in the United States. It is characterized by periods of hyperactivity or inattention that are significant enough to negatively impact the child’s life at school or at home. Symptoms of ADHD can continue into adolescence and sometimes adulthood (Faraone 2003). A pilot study by Sorgi and others (2007) evaluated the effects of high-dose EPA and DHA on the behavior of children diagnosed with ADHD. The nine Japanese children were supplemented with 16.2 g EPA/DHA concentrates per day. At the four week mark the dosage was adjusted dependent on the ratio of AA to EPA in the plasma phospholipids in order to reach a level normally found in the Japanese population. At the end of the eight-week study, significant increases in EPA/DHA were observed, along with significant reduction of the AA:EPA ratio (p<0.01). A psychiatrist, blind to supplementation or dosage modifications, reported significant behavioral improvements in terms of inattention, hyperactivity, and conduct disorders. Though this pilot study was small, its results suggest that supplementation
with high-dose EPA/DHA may be a potential therapy for children diagnosed with ADHD (Sorgi and others 2007).

Autism is another behavioral disorder that is characterized by three distinct behaviors: inappropriate or inadequate social interactions, impaired language and communication, and repetitive patterns of restricted activities and interests. The cause is unknown and is believed to have both genetic and environmental components (Bui 2009). Recent pilot studies have suggested that EPA/DHA therapy may be a potential treatment for autism. One such study was a double-blind, randomized pilot study that used a placebo control. 13 children aged 5-17 with severe autism symptoms (aggressive behavior, tantrums, self-injuring) were randomly assigned to the placebo or treatment group, which received 1.5 g/day of EPA+DHA. After 6 weeks the children were evaluated using the Aberrant Behavior Checklist. The study observed a significant improvement in behavior in the omega-3 treated group when compared with the control (Amminger and others 2006). While several studies have indicated this beneficial relationship, more clinical studies are needed in order to produce evidence strong enough to support a definitive recommendation for EPA and DHA as treatments for autism (Bui 2009).

2.6 DHA as a Potential Treatment for Depression

Research on the effects of EPA and DHA on depression has yielded inconsistent outcomes, mostly based on the population studied. One study suggests that omega-3 intake may be beneficial to treating childhood depression. 28 children diagnosed with
depression were randomly assigned to a placebo or omega-3 treatment group and evaluated at baseline, 2, 4, 8, 12, and 16 weeks using three established scales measuring childhood depression. Results showed highly significant effects of omega-3 intake versus placebo, suggesting that omega-3 may be therapeutic for childhood depression, but larger clinical trials are needed (Nemets and others 2006). A 2004 study of men ages 50-69 found no conclusive link between omega-3 intake and depression (Hakkarainen 2004).

Post-partum depression is a common occurrence, affecting an estimated 10-15% of mothers. Pilot studies have suggested that there is enough evidence for therapeutic effects of EPA and DHA to warrant a larger, placebo-controlled, randomized clinical trial. One small pilot study showed that any dosage of EPA and DHA treatment from 0.5 g/day to 2.8 g/day significantly reduced depression symptoms on the Edinburg Postnatal Depression Scale and the Hamilton Rating Scale for Depression. No significant differences were seen between the dosages, but all had significant beneficial effects when compared to baseline depression episodes (Freeman and others 2006). Though this trial provides intriguing evidence that omega-3’s may provide therapeutic effects for post-partum depression symptoms, another trial suggested that EPA and DHA may not be sufficient enough treatment to prevent post-partum depression from occurring. Women who suffer post-partum depression during one pregnancy have a 25-50% risk of a recurring depressive episode with subsequent pregnancies. In a study of mothers who had already suffered from post-partum depression in a previous pregnancy, omega-3
treatment during the 34\textsuperscript{th}-36\textsuperscript{th} weeks of pregnancy did not prevent the mothers from experiencing depressive episodes (Marangell and others 2004).

Currently, researchers at Cedars-Sinai Medical Center’s Department of Psychiatry and Behavioral Neurosciences in Los Angeles, CA (in collaboration with Massachusetts General Hospital) are in the 4\textsuperscript{th} year of a large, 5 year clinical trial to examine the effects of EPA and DHA on depression. This is the first study to systematically test EPA and DHA against each other and also against a placebo on a large sample size of patients with severe depression (Cosgrove 2008).

3. Product Development with DHA

Historically, the intakes of omega-3, omega-6, and saturated fatty acids have been approximately equal. Western diets, however, have shifted to emphasize industrially produced vegetable oils (mostly omega-6) and animal fats (heavy in saturated fat), leaving a deficiency in omega-3 fatty acids from marine life (Heller and others 2006). Regular intake of fish and fish oil is essential- in fact a 30 g fish per day serving was associated with a 50\% reduction in death by heart disease over a 20-year period (Kromhout and others 1985, Lee and others 2003). Currently the American Heart Association’s recommendation is at least 2 servings of fish per week, especially those high in EPA and DHA (Harris and Appel 2002).

The food industry has taken note of the increased demand for omega-3 enriched products, and the market has been rapidly growing to include more. Recently products such as enriched margarine spreads, drink mixes, peanut butter, baked goods, and cereals
have emerged. Some complications must be overcome in order to make desirable omega-3 containing products. The major concern is the deleterious effects of lipid oxidation, which increases as the number of double bonds in a fatty acid increases. Lipid oxidation causes off-flavors and aromas to the point which the product is unacceptable to a consumer. Since EPA and DHA are more susceptible to oxidation than vegetable oils or certainly saturated fatty acids, shelf life of enriched products is a concern. The flavor and aroma of fish oil is also considered to be negative in many products, limiting the amount of EPA and DHA that can be added to a product. The food industry is aiming to keep up with consumer demand for omega-3’s with the addition of antioxidants and technologies like microencapsulation, which allows EPA and DHA to be added to a wider variety of products in a more stable, powdered form.

It comes as no surprise, when considering all the evidence showing the many beneficial health effects of omega-3 fatty acids (especially EPA and DHA), that the food industry has been rapidly developing new products supplemented with omega-3’s. Product developers must face certain challenges when enriching food products with EPA and DHA, the most important being quality issues relating to lipid oxidation. The off-flavors and aromas caused by the products of lipid oxidation will cause a food product to be unacceptable to consumers, and will have deleterious effects on the processing and storage of enriched products. Oxidation will also alter the nutrient quality of the fatty acids (Kim and Min 2008). Another issue is that the taste and aroma of fish oil may be undesirable in many products. The food industry has adapted to these issues through technologies such as microencapsulation and oil-in-water emulsions.
In order to achieve maximum shelf life, some type of antioxidant should be used. Mechanisms for antioxidants that are food-product specific include free radical scavenging, inactivation of peroxides and other reactive oxygen species, metal chelating, and quenching of lipid oxidation products. Free radical inactivating antioxidants (also called free radical scavengers) can slow the initiation and propagation stages of triplet oxygen oxidation by competing with the fatty acids for reaction with peroxy radicals. Phenolics, including \( \alpha \)-tocopherol, carotenoids, and butylated hydroxytoluene, often act as antioxidants by this mechanism. Some other antioxidants affect compounds which have an indirect effect on lipid oxidation, for example superoxide anion, peroxides, and photosensitizers. The superoxide anion is produced when an electron is added to molecular oxygen. Complexes of cupric ion with certain amino acids (lysine, tyrosine, histidine) can catalyze the dismutation of superoxide anion (Decker 2008).

Another type of antioxidant reacts with catalytic metal ions. The metal ions accelerate the formation of free radicals, speeding up the overall oxidation process. Certain compounds, for example citric acid or EDTA, will chelate metal ions so they cannot react with lipids or oxidation intermediates (Decker 2008). In the case of an oil-in-water emulsion, there are three different environments: the oil droplet core, the surface of the oil-water emulsion, and the aqueous continuous phase. In such an emulsion, it is believed that the main cause of lipid oxidation is interaction between lipid hydroperoxides at the surface of the oil with metal ions from the aqueous phase. A chelating agent such as EDTA may be used in this case to retard oxidation. EDTA has in face been shown to dramatically reduce salmon oil-in-water emulsion oxidation by
chelating iron ions, preventing interaction between iron and the lipid hydroperoxides. Alamed and others (2005) looked at the effects of heat processing on the ability of EDTA to prevent lipid oxidation in omega-3 containing oil-in-water emulsions. Samples were heated to 50, 70, and 90°C and held for 10 min, then cooled and stored in the dark for 8 days, then evaluated for lipid oxidation rate. The results showed that temperature and process time had no significant effect on the oxidative stability of samples. The sample heated to 90°C did not oxidize at a faster rate than the control sample which was not heated at all. The researchers then added different concentrations of EDTA to the samples, and found that a concentration of 2.5µM EDTA was able to almost completely inhibit oxidation. When calcium was added at a concentration 2x that of EDTA, however, the rate of oxidation increased, possible due to the competition of the calcium ions with iron for interaction with EDTA, increasing the amount of iron catalyst available for interaction with the fatty acids. The overall result of the study was that heat-stable, oxidation-resistant salmon-oil-in-water emulsions were possible with the presence of EDTA. If a product was not high in calcium, this type of emulsion could be used to develop an omega-3 enriched functional food (Alamed and others 2005).

Microencapsulation is a technique that can be used to improve the stability of EPA and DHA for addition to food products. It changes the oil into a dry powder, making it easier to use along with increasing the stability. The material used for microencapsulation protects the oil against oxygen, light, and moisture. The EPA and DHA can then be used in a wider variety of products, including drink mixes and baked goods. The microcapsules themselves are usually no bigger than 1000µm. In the food
industry, spray-drying is the most common technique for microencapsulation, with different materials as coating agents, including starches, milk powder, and plant gums. One study compared methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) as microencapsulating agents for fish oil. Samples were prepared using spray-drying, stored in the dark, and evaluated for oxidative stability by peroxide value. Both materials produced good emulsions, and significantly protected the oil from oxidation compared to a non-encapsulated bulk sample, however fat deterioration did still occur. The MC was slightly better in comparison to the HPMC in terms of oxidative protection, most likely because it acted as a better coating material as it had smaller emulsion particle diameter. The maximum amount of oil content in the microcapsules appeared to be 400 g/kg in order to prevent structural failure of the capsule; however most powders currently used in the food industry contain closer to 300 g/kg. The study concluded that the use of modified celluloses, especially MC, for the production of spray-dried microencapsulated fish oil can produce a more oxidation stable product (Kolanowski and others 2004).

4. Extraction of Marine Fats and Oils

Recovering the oils from marine animals is done by a cooking process known as rendering. There are two methods of rendering- wet and dry rendering, and both are used in the food industry. The product is first crushed or cut into smaller pieces, which are then cooked to evaporate moisture and release the fat. If the wet rendering process is used, the product is then cooked further by steam under pressure. The water and other
solids settle to the bottom, while the fat is allowed to float, as it is less dense than the rest of the material. The water is drained off, and the remaining solids go to a hydraulic or continuous screw press, discharging the fat. The high quality protein of the leftover fish meal puts makes it excellent for poultry diets and other marine animal diets. The discharged fat is centrifuged and/or filtered for purification. Most fish are processed by wet rendering, but dry rendering is a newer, efficient method in which the product is cooked in its own fat. The product is heated in an agitated steam-jacketed vessel for 1.5-4 hours, until all the moisture has evaporated. The product is then passed over a screen, at which point the free fat drains. The remaining product is put through a press, similar to the wet rendering process (Johnson 2008).

The DHA oil used in this study was extracted from an algal source via fermentation in large drums. The fatty acid composition of the oil can be seen in Table 2 (Frankel and others 2002).
Table 2: Approximate fatty acid composition of commercially available RBD DHA oil

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>4.4</td>
</tr>
<tr>
<td>C14:0</td>
<td>15.6</td>
</tr>
<tr>
<td>C16:0</td>
<td>12.8</td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>1.4</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.9</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>21.6</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>0.8</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>42.4</td>
</tr>
<tr>
<td>Other</td>
<td>0.2</td>
</tr>
</tbody>
</table>

5. Principles of DHA Oil Processing Steps

Industrially, oils are then further processed in order to give them the appearance, aroma, taste and stability that consumers expect. In the case of algal DHA, this process includes the steps of alkali refining, bleaching, deodorizing, and winterizing. Each step is performed in order to achieve a specific change in the oil, resulting in a product that is as clear and neutral (in terms of odor and flavor) as possible.

5.1 Alkali Refining

The first step in the processing of DHA is refining, in this case alkali refining, which involves the removal of nonglyceride compounds, such as protein, waxes, or free fatty acids. These compounds act as prooxidants in the oil, therefore removing them will increase the oxidative stability of the product. The oil is mixed with a caustic soda, often NaOH, which reacts with free fatty acids present in the oil. This creates soapstock, which
is separated from the oil by centrifuge and discarded as a byproduct of the refining process. This results in a more neutralized oil, which is usually mixed with hot water and centrifuged again to remove any residual soapstock or other impurities. Care must be taken when mixing the oil and caustic soda to avoid excess refining loss and to prevent emulsions (Johnson 2008).

5.2 Bleaching

The oil is then put through the bleaching process. Bleaching involves the removal of natural pigments, as consumers prefer colorless oil. Some of these pigments, such as chlorophyll, have been shown to be strong prooxidants in oil. Metal ions are also removed during bleaching, known to be catalysts in the initiation phase of autoxidation. Bleaching, therefore, is a crucial step in increasing oxidative stability of oils. Normally bleaching is done using filtering agents such as neutral clays, silica gel, and activated earth. The agent is mixed with the oil under vacuum (to prevent oxidation during the process) to form a slurry. Pigments and metal ions adsorb onto the filtering agent, which is then removed via filtration (Johnson 2008).

5.3 Winterizing

Winterizing follows bleaching, and involves the removal of higher melting point compounds such as saturated fatty acids and triglycerides, for example palmitic (16:0) and myristic (14:0) acids. The oil is chilled in order to crystallize these compounds, which are then removed via filtration. Winterizing also removes some volatile
compounds as a by-product of the process. Oil is passed through chilling heat exchangers to achieve a temperature of approximately 4-7°C, then transferred to an agitation tank, where the oil is slowly agitated to remove latent heat of crystallization. The crystallized portion of the oil containing the saturated fats is then removed, usually by vacuum filtration (Johnson 2008).

5.4 Deodorizing

The final step in the processing of algal DHA is deodorization, in which small volatile compounds responsible for the off odors and flavors associated with lipid oxidation are removed. Examples of such compounds include remaining free fatty acids, aldehydes, ketones, and alcohols. Natural antioxidants such as tocopherols are also removed during deodorization, up to 30% (Johnson 2008).

6. Autoxidation of Oils

The reaction between oxygen and fatty acids such as DHA or linoleic acid is generally a three-step process which is initiated when oxygen reacts with the double bonds in the fatty acid chain. The basic steps of this autoxidation process are initiation, propagation, and termination. During initiation, one of the unsaturated carbons loses a hydrogen to form a radical, and molecular oxygen adds to the double bond to form a double radical as shown below.
Approximately 80 kcal are needed to rupture the C-H bond, with less needed to break the C=H bond, approximately 50 kcal. This energy requirement can be significantly reduced by enzymes (such as lipoxygenase or lipases) or the presence of metal ion catalysts. The propagation stage follows and is known as a free radical chain reaction. Peroxy radicals are formed (hydroperoxides), which readily react with other oxygen molecules. This causes the chain reaction and the continuation of autoxidation, as illustrated below.

Hydrogen atoms in the α position to the double bond are especially susceptible to abstraction by the peroxyl radicals, such that the level of hydroperoxidation increases with the number of double bonds present in the molecule. This explains why DHA, with its 6 double bonds, is so much more susceptible to autoxidation when compared with other animal and vegetable oils.

The reaction between two radicals may cause the formation of a non-radical compound, known as the termination step, shown below (Choe 2008).
7. Effects of Some Minor Components on the Oxidative Stability of Oils

7.1 Tocopherols

Tocopherols can play a large and complicated role in the oxidative stability of oils. At certain concentrations, they have been shown to act as strong antioxidants, while at other concentrations, they may act as prooxidants. The most common forms of tocopherols are α, β, and γ tocopherols, shown below.

![Figure 2: Structure of alpha-tocopherol](image2)

![Figure 3: Structure of beta-tocopherol](image3)

![Figure 4: Structure of gamma-tocopherol](image4)
Various studies have shown that the highest concentration levels of each tocopherol to act as antioxidants in soybean oil are: $\alpha$: 100 ppm, $\beta$: 250-500 ppm, and $\gamma$: 500-1000 ppm. At levels below these, tocopherols are some of the strongest natural antioxidants, however at higher concentrations the tocopherol molecule is believed to accept a hydrogen atom, becoming a radical and propagating lipid oxidation (Kim and others 2007). Tocopherols make good antioxidants at certain concentrations partially due to their phenol groups, which give them the capability to stabilize their radical form via resonance as seen in Figure 5.
Figure 5: Resonance stabilization of antioxidant radicals

\[ \begin{align*}
E^* &= 300-500\text{mv} \\
C(CH_3)_3OCH_3 &\xrightarrow{R\cdot, \ RO\cdot, \ ROO\cdot} RH, ROH, ROOH
\end{align*} \]
7.2 Phospholipids

The effect of phospholipids (such as phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, etc) on the oxidative stability of lipids has been extensively studied. While it has been shown that phospholipids act synergistically with tocopherols as antioxidants, in other studies phospholipids alone acted as prooxidants. In the presence of metals such as iron (at levels of 0.5 to 1.0 ppm), phospholipids act as chelating agents, improving oxidative stability (Stasinopoulos 1982). As with tocopherol, the level of phospholipids is important in determining the effect on oxidative stability, and also on flavor and visual quality of the oil. Levels of phospholipid at 0.1% have long ago been shown to cause an undesirable darkening of the oil and unpleasant odors (Evans and others 1954). During processing, almost all phospholipids are removed during caustic refining (Johnson 2008).

7.3 Metal ions

Metal ions such as iron and copper are present naturally in crude oils at the parts per million and parts per billion levels. The presence of metals reduces the activation energy of the initiation phase of lipid oxidation, thus increasing the overall rate of oxidation. The metals also react directly with lipids to form lipid radicals and also will react with triplet oxygen to produce superoxide anion, further accelerating lipid oxidation (Choe 2008).
8. Linoleic Acid

Linoleic acid is an essential fatty acid and has been associated with various health benefits, such as weight control, cardiovascular health, and improved immune response. Deficiency has been linked to dry hair and nails, hair loss, and poor wound healing. The structure of linoleic acid, 18:2 Ω-6, can be seen below in Figure 6.

![Figure 6: Structure of linoleic acid](image)

Extensive research has been done on the oxidative stability of linoleic acid in oil systems, as it is a major fatty acid component of many commonly used oils such as canola and soybean. However, linoleic acid also often exists in an aqueous system, for example milk (approximately 2.5-3g/100g milk) and some salad dressings, and such systems have not been as thoroughly analyzed for oxidative stability of the linoleic acid present (Palmquist and Jensen 2008).
9. *Panax ginseng*

Korean ginseng (*Panax ginseng*) is widely consumed in the Orient due to its numerous health benefits, including lowering blood glucose, lowering blood pressure, improved recovery from illness, among other uses not as quantifiable, such as improved physical and mental well-being and the easing of menopausal symptoms. Most evidence of these benefits is preliminary, as few large-scale clinical trials have been conducted. (Wiklund and others 1999, Vogler and others 1999). The extract of ginseng contains several antioxidant compounds, which are likely to be responsible for the perceived health effects (Kang and others 2006). These compounds have previously been separated and identified as caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid. The antioxidant capabilities of these compounds were analyzed using 2,2-diphenyl-1-picryl-hydrazyl, and their structures can be seen below in Figures 7-11.

![Figure 7: Structure of caffeic acid](image)
Figure 8: Structure of ferulic acid

Figure 9: Structure of kaempferol

Figure 10: Structure of salicylic acid
The phenolic structure of these compounds aid their antioxidant activity by utilizing free radical resonance as seen in α-tocopherol.

**MATERIALS**

Crude, refined, bleached, winterized and deodorized oils from the same batch were provided by a commercial DHA oil supplier (Columbia, MD). An initial 1100 ppm tocopherol was added during crude oil extraction, however no other additions of antioxidants were performed for research purposes. Oils were stored in the freezer throughout the experimental period to prevent oxidation and other changes in composition. Teflon coated rubber septa, aluminum caps, and serum bottles were purchased from Supelco, Inc. (Bellefonte, PA).

Linoleic acid, caffeic acid, ferulic acid, kampilferol, salicylic acid, vanillic acid, and α-tocopherol were purchased from Sigma Chemical (St. Louis, MO). A spectrophotometric grade ethanol was obtained from Aldrich Co. (Milwaukee, WI). Teflon coated rubber septa, aluminum caps, and serum bottles were purchased from Supelco, Inc. (Bellefonte, PA).
METHODS

1. Gas Chromatographic Analysis of the Oxidative Stability of DHA Oil

1.1. Sample Preparation

Two grams of each oil sample (refined, bleached, winterized, and deodorized oils) were transferred into 10-ml serum bottles which were tightly sealed with Teflon lined rubber septa and aluminum caps. All samples were prepared in triplicate and stored under light at 2500 lux at 25°C for 5 days.

1.2. Determination of headspace oxygen depletion

100 µL of headspace was extracted from each sealed bottle and injected into the gas chromatograph (Hewlett-Packard 5890) equipped with a thermal conductivity detector (Alltech Assoc, Inc, Deerfield, Ill, U.S.A). Molecular oxygen content of the headspace was determined with a 6 ft x 1/8 inch stainless steel column packed with Molecular Sieve 13x and a Nitrogen flow rate of 20 ml/min. Temperatures of the oven, injection port, and detector were 40, 120, and 150 °C, respectively. Sensitivity of the machine was determined by injecting 100 µL atmospheric air into the gas chromatograph and reading the oxygen peak area before each sample. Oxygen content was expressed as electronic counts of peak area of oxygen. All samples were analyzed in triplicate.

1.3. Determination of headspace volatile compounds

Solid phase microextraction (SPME) was used to collect volatile compounds from the headspace of the sample bottles. A polydimethyl sioxlane- divinyl benzene
(PDMS/DVB) fiber was used for maximum adsorption of the compounds. The sample bottle was placed in a 55°C water bath for 30 min to allow volatile compounds in the oil to be adsorbed onto the PDMS/DVB fiber. The fiber was then placed in the injection port of the gas chromatograph: The absorbed volatile compounds by SPME were desorbed at 250°C from the GC injector for 2 min and then separated by gas chromatography (GC-6890) (Wilmington, Del, U.S.A). It was carried out on a SPB-5 column (30X0.25mm, 0.25um film thickness). Oven temperature was programmed from 60 to 120°C at 4°C/min and from 120 to 200°C at 10°C/min.

2. Evaluation of the oxidative stability of 2% linoleic acid in an aqueous system

2.1 Sample preparation for photosensitized oxidation

   The 2% linoleic acid in distilled and deionized water and ethanol (6:4) was prepared by a method of Inatani and others (1983). The aqueous sample was adjusted with phosphate buffer to have pH 7. The 2% linoleic acid aqueous solution having 20 ppm riboflavin and 100 ppm caffeic acid, ferulic acid, kaempferol, salicylic acid, or vanillic acid was prepared. \( \alpha \)-Tocopherol which is the most common and effective natural antioxidant was used as a standard antioxidant to evaluate the relative antioxidant activities of ginseng compounds.

   Ten mL of sample was transferred into a 35 mL serum bottle and the bottle was sealed airtight with a Teflon coated septum and an aluminum cap (Supelco, Bellefonte, PA) (Jung and Min 1990). The sample bottles were stored in a light chamber (70 × 50 × 60 cm) in Figure 1 (Lee and Min 1988; Yang 1994). The inside wall of the light box
were covered with mirrors to provide uniform light intensity to samples. The bottles in a sample holding plate (Figure 1) were rotated at 60 rpm at 3,000 lux. The samples were stored for 180 minutes at 4°C and were analyzed every 30 minutes to study the photosensitized oxidation.

2.2 Sample preparation with and without ferrous ions for autoxidation

The 2% linoleic acid aqueous sample with pH 7.0 having 100 ppm caffeic acid, ferulic acid, kaempferol, salicylic acid, vanillic acid or α-tocopherol was prepared. Ten mL sample was pipetted into a 25 mL bottle and sealed airtight with a Teflon coated septum and an aluminum cap (Supelco, Bellefonte, PA). The sample bottles were stored in a Blue M oven (Blueland, IL) at 37°C under dark for 40 days. The peroxide contents were analyzed every 4 days. To study the effects of catalytic metal ion on the antioxidant activities of ginseng compounds, ferrous chloride was added to 2% linoleic acid in the aqueous solution to have 2 ppm ferrous ion. The samples having 2 ppm ferrous ion were stored in the oven at 37°C in a oven for 10 days. The peroxide values were analyzed everyday to study the effects of ginseng compounds and ferrous ion on the oxidation.

2.3 Chelating antioxidant activities of caffeic acid

To study the possible chelating effect of caffeic acid, the 2% linoleic acid of aqueous system having 0, 25, 50, 100, 500 ppm caffeic acid or 50 ppm α-tocopherol was added with ferric chloride to have 0.4 or 4 ppm ferrous ion. The sample bottles were stored at 55°C for 16 hours in a oven.
2.4 Oxidation evaluation by headspace oxygen and peroxide value

The headspace oxygen of sample bottle was analyzed by Hewlett Packard 5890 with thermal conductivity detector (Lee and Min 1990). The temperatures of injector, column, and detector were 50, 120 and 150°C, respectively. Helium gas was used as a carrier gas and the flow rate was 45 mL/min. The headspace oxygen in a sample bottle was 20.9%. The 20.9% oxygen content in headspace was expressed as 100% headspace oxygen. The peroxide value of 2% linoleic acid in aqueous system was determined by spectrometry using a method of Inatani and others (1983). Thiocyanate was used as coloring agent. The 0.1 mL sample and 0.1 mL of 30% ammonium thiocyanate were added to 9.7 mL of 75% ethanol and the 0.1 mL 0.02 M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture. The absorbance of sample after 3 minutes of reaction was measured at 500 nm. The peroxide value was calculated by multiplying the absorbance at 500 nm by 25. The inhibition rate of oxidation by an antioxidant was calculated by the following equation:

Inhibition rate (%) = (Peroxide value of control - Peroxide value of sample) × 100/

Peroxide value of control

2.5 Statistical analysis

The headspace oxygen and peroxide values of samples were the means of triplicate samples. One-way analysis of variance (ANOVA) and the Fisher’s least
significant differences (LSD) test were used to determine the differences of antioxidants effects on the headspace oxygen and peroxide value of samples during storage at $\alpha = 0.05$ (SAS Institute, Cary, NC).
RESULTS AND DISCUSSION

1. Effects of Processing on the Oxidative Stability of DHA Oil

1.1 Headspace Oxygen Analysis

The effects of processing steps on the headspace oxygen depletion of DHA oil are shown in Figure 12. As expected, oxygen content in the headspace decreased over storage time from 0 to 5 days. This occurs due to the reaction between oxygen in the headspace and DHA in the oil. When the oxygen reacts with the DHA, hydroperoxides and peroxo radicals are formed, which are direct products of lipid oxidation, making headspace oxygen depletion a reliable measurement of lipid oxidation. The oxygen content in the refined DHA oil decreased from 20.9% on Day 0 to 6.7% on Day 5, showing the most headspace oxygen depletion and therefore the lowest oxidative stability of the 4 oils studied. Bleached, winterized, and deodorized oils had headspace oxygen depletions of 20.9% on Day 0 to 8.3%, 7.8% and 7.8%, respectively. The bleached, winterized, and deodorized oils were determined not to be statistically different from each other (p > 0.05), and indeed the final headspace oxygen percentages were very similar. It can be seen in Figure 12 that the second phase of DHA oil processing, the bleaching step, had an important role in increasing the oxidative stability of the DHA oil when noting the difference in the amount of headspace oxygen depletion between refined and bleached oil. The two both started at 20.9%; however the rate of depletion for refined oil was much greater (again ending at 6.7%), showing that refined oil had lower oxidative stability than bleached oil (ending at 8.3%). The removal of photosensitizers
such as chlorophyll along with the removal of metal ions in the bleaching step is most likely responsible for the increased oxidative stability of bleached oil as compared to refined oil.

Focusing on the refined oil sample, a steeper slope can be seen on days 1-2, coinciding with a higher rate of headspace oxygen depletion, with the rate slowing slightly on days 3-5. This may be due to a decrease in the concentration of oxygen in the headspace, thereby causing a decrease in the reaction rate. A similar trend can be seen in the bleached, winterized and deodorized samples.
Figure 12: Effects of oil processing steps on the headspace oxygen depletion of DHA oil during storage under light at 25 C
1.2 Total Volatile Content Analysis

The effects of processing on the total volatile compound formation during storage under light at 25°C can be seen in Figure 13. These volatile compounds are formed via the lipid oxidation reaction between oxygen and DHA in the oil. Hydroperoxides are formed and then broken down into smaller compounds known as volatiles, for example aldehydes and ketones, which are responsible for the off-flavors and rancid odors associated with oil that has been oxidized. Flavor and odor quality of oil is directly related to volatile compound formation- the more volatiles formed, the more rancidity is observed. Certain volatile compounds have been associated with rancid flavors, such as 2, 4 decadienal in soybean oil (Min 1981) and 2, 4 heptadienal in fish oils such as DHA (Karahadian and others 1985). As expected, the total volatile content in DHA oil increased over storage time from Day 0 to Day 5. In refined oil at Day 0, the volatile content was already much higher than the other oils. Volatile content for refined oil at Day 0 was approximately 2,770,500 electronic counts, increasing to approximately 4,325,900 electronic counts on Day 5 for an average of approximately 4,041,000 electronic counts (ec). Refined oil showed the most volatile compound formation and was again determined to have the least oxidative stability of the oils tested. Not only did refined oil start out with the highest volatile content, the rate of volatile compound formation was much higher than that of the other oils. Similar to the trend observed in headspace oxygen depletion, the rate of volatile compound formation was higher on days 1-3, with a decrease in the rate of formation on days 4 and 5. On these days, the decrease can be explained by the decomposition and dimerization of volatile compounds formed
previously into non-volatile compounds not detected in the gas chromatogram. An example of this type of reaction is the formation of a hemiacetal via the reaction between an aldehyde and an alcohol. This same type of trend was not observed in the volatile compound formation in bleached, winterized, and deodorized oils, and the overall formation and rate of formation of volatile compounds in these three oils was much lower than in refined oil.

Bleached, winterized, and deodorized oils showed averages of approximately 482,100 ec, 437,200 ec, and 405,800 ec, respectively. As with the headspace oxygen depletion, the bleached, winterized, and deodorized oils were found to not be statistically different from each other (p > 0.05). Figure 13 shows that bleaching proved to be very important in the removal of volatile compounds when compared with refined oil at Day 0. As with headspace oxygen depletion, the removal of photosensitizers such as chlorophyll and metal ions during bleaching significantly increased the oxidative stability of the DHA oil. Winterizing and deodorizing removed the remaining volatile compounds at Day 0 as can been seen in deodorized oil, which started with 0 ec volatile content. The small decrease at Day 0 in winterized oil as compared to bleached oil, while not statistically significant, is interesting, and can most likely be attributed to the filtration step used to remove the precipitated higher melting point compounds which winterized is designed to eliminate. Some volatiles may also be filtered at this step.

Bleached and winterized oils remained relatively constant in their volatile content during storage; however deodorized oil showed a trend upward, especially at Days 4 and 5. This is believed to be the result of the removal of tocopherols and other natural
antioxidants during the deodorizing process that occurs along with the removal of volatile compounds. The removal of these antioxidant compounds may be causing the deodorized oil to begin losing oxidative stability over time during storage (Tomaino and others 2005).

The correlation coefficients (r) for total volatile compound formation and headspace oxygen depletion for DHA oil during processing were determined. For refined, bleached, winterized, and deodorized oils, r-values were calculated as: -0.92, -0.98, -0.95, and -0.98, respectively. High correlations between these data sets indicate that oxygen was depleted in the headspace as it reacted with DHA to form volatile compounds and hydroperoxides in a gas tight sample vial. These results show that the combination of headspace oxygen depletion and total volatile compound formation can be used as a reliable analytical method for the measurement of lipid oxidation.
Figure 13: Effects of processing on the total volatile content of DHA oil during storage under light at 25°C
2. Effects of ginseng compounds on the oxidative stability of 2% linoleic acid in an aqueous solution

2.1 Reproducibility of headspace oxygen analysis and spectrometry

The coefficients of variations for the analyses of headspace oxygen and the peroxide value of 6 same samples were 1.35% and 1.51%, respectively. The coefficient of variation indicated that the headspace oxygen and peroxide value analyses were very reproducible.

2.2 Photosensitized oxidation

The effects of caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid on the headspace oxygen and peroxide values of 2% linoleic acid in aqueous system for 3 hours under light at 4ºC are shown in Figures 14 and 15, respectively. The average correlation coefficients ($r^2$) between the headspace oxygen and peroxide values for caffeic acid, ferulic acid, kaempferol, salicylic acid, vanillic acid, or α-tocopherol in the riboflavin photosensitize oxidation of linoleic acid was $r^2 = -0.98$. The average peroxide values of samples containing 100 ppm caffeic acid, kaempferol, vanillic acid, salicylic acid, α-tocopherol, and ferulic acid were 3.3, 3.7, 3.7, 4.3, 4.4, and 5.3, respectively. The peroxide value of control sample was 5.3. The combined results of headspace oxygen and peroxide value showed that caffeic acid, kaempferol, vanillic acid, salicylic acid, α-tocopherol and ferulic acid decreased the lipid oxidation by 34, 27, 26, 18, 12, and 0%, respectively. The antioxidant effects of caffeic acid, kaempferol, and vanillic acid in the photosensitized oxidation may be due to the singlet oxygen quencher (Takahama 1985)
and the donation of hydrogen atom to free radicals in the photosensitized oxidation. Salicylic acid, ferulic acid, and \( \alpha \)-tocopherol have many conjugated double bonds in the molecules and are election rich compounds. The electron rich compounds react with electrophilic singlet oxygen as chemical quencher in the photosensitized oxidation (Choe and Min 2006). Salicylic acid, ferulic acid, and \( \alpha \)-tocopherol which are phenolic compounds can donate hydrogen atom to lipid radicals during photosensitized oxidation. However, the antioxidant effects of ginseng compounds were not significant on the photosensitized oxidation in the system of 2\% linoleic acid at p> 0.05.
Figure 14: Headspace oxygen depletion of 2% linoleic acid in an aqueous solution with 100 ppm of different ginseng compounds and tocopherol during storage under light over 3 hours.
Figure 15: Peroxide values for 2% linoleic acid in an aqueous system with 100 ppm of different ginseng compounds and tocopherol during storage under light over 3 hours.
2.3 Autoxidation of linoleic acid without ferrous ion

The coefficients of variations for the determinations of headspace oxygen and peroxide values were 1.35% and 1.51%, respectively. The correlation coefficient \( r^2 \) between the headspace oxygen and the peroxide values was \( r^2 = -0.98 \) in the riboflavin photosensitized oxidation study. The autoxidation study of linoleic acid was evaluated by measuring peroxide values only because of the good coefficient of variations for headspace oxygen and peroxide value analyses and the high correlation coefficient between headspace oxygen and peroxide value. The effects of caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid on the autoxidation of 2% linoleic acid in aqueous system at 37°C in the dark for 40 days are shown in Figure 16. The average peroxide value for samples containing 100 ppm kaempferol, vanillic acid, ferulic acid, caffeic acid, salicylic acid, or \( \alpha \)-tocopherol during 40 days of storage was 0.2, 0.7, 2.2, 2.3, 2.7, or 2.8, respectively. The average peroxide value for the control was 3.7. Kaempferol, vanillic acid, ferulic acid, caffeic acid, salicylic acid, and \( \alpha \)-tocopherol decreased the autoxidation of 2% linoleic acid in aqueous solution by 95, 82, 41, 39, 29 and 24%, respectively. They have significant antioxidant effects on the autoxidation without 2 ppm ferrous ion at \( p < 0.05 \). Kaempferol which is a flavonol acted as the best antioxidant. Caffeic acid, kaempferol, vanillic acid, salicylic acid, ferulic acid, and \( \alpha \)-tocopherol are phenolic compounds and donate hydrogen atom to prevent the free radical chain reaction of linoleic acid (Reische and others, 2002).
Figure 16: Peroxide values of 2% linoleic acid in an aqueous system with 100 ppm of different ginseng compounds and tocopherol during storage in the dark over 40 days.
2.4 Autoxidation of linoleic acid with ferrous ion

The effects of caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid on 2 ppm ferrous ion catalyzed autoxidation of 2% linoleic acid in the aqueous solution for 10 days at 37°C are shown in Figure 17. The peroxide values of 2% linoleic acid in aqueous system without and with 2 ppm ferrous ion for 10 days at 37°C were 1.2 (Figure 16) and 16.8 (Figure 17), respectively. The 2 ppm ferrous ion acted as a strong catalyst for the autoxidation of sample. The average peroxide values for samples containing 100 ppm kaempferol, vanillic acid, ferulic acid, caffeic acid, α-tocopherol or salicylic acid was 0.3, 1.2, 2.5, 2.8, 5.3, or 6.3, respectively. The average peroxide value of the control for 10 days measured daily was 7.5. The 100 ppm kaempferol, vanillic acid, ferulic acid, caffeic acid, α-tocopherol, and salicylic acid reduced 96, 85, 67, 64, 30, and 17% of oxidation, respectively. They have significant effects on the peroxide value at p<0.05. A preliminary study showed that kaempferol had better antioxidant effect than the BHT at the 100 ppm concentration (data not shown). The peroxide values of kaempferol in the samples without and with ferrous ion for 10 days were 0 (Figure 16) and 0.8 (Figure 17), respectively. This indicates that kaempferol effectively quenched free radicals formed by the catalysis of metal ion. The peroxide values of sample having 100 ppm vanillic acid, ferulic acid, and caffeic acid without ferrous ion were 0.2, 0.5, and 0.9 and were 1.5, 4.3, and 4.8, respectively for the sample with ferrous ion for 10 days. The 2 ppm ferrous ion increased the peroxide values of sample containing 100 ppm vanillic acid, ferulic acid, or caffeic acid by 1.3, 3.8 and 3.9, respectively. The peroxide
values of sample having 100 ppm \( \alpha \)-tocopherol without and with 2 ppm ferrous ion for 10 days were 0.9 vs. 13.8, respectively. The addition of ferric chloride to have 2 ppm ferrous ion increased the peroxide value of sample containing 100 ppm \( \alpha \)-tocopherol by 12.9. The ginseng compounds are significant better antioxidants than the \( \alpha \)-tocopherol in the sample of 2 ppm ferrous ion at \( p<0.05 \). Caffeic acid, ferulic acid, or vanillic acid has acidic group which could be ionized into carboxylate anion and proton at pH 7. The carboxylate anion may chelate ferrous ion at pH 7 by forming complex ions. Caffeic acid, ferulic acid and vanillic acid acted as better antioxidants than \( \alpha \)-tocopherol with 2 ppm ferrous ion by possibly chelating ferrous ion. Caffeic acid in oats, canary seeds, and spices has been reported to act as an antioxidant (Pokorny 1987). Pratt (1985) reported that caffeic acid had better antioxidant effect than ferulic acid in the oxidation of soybean oil. The peroxide values of salicylic acid on the peroxide values of sample without and with ferrous ion for 10 days were 0.9 and 14.0, respectively. The results on the effects of salicylic acid on the oxidation of sample with 2 ppm ferrous ion could not be explained logically. Further research is needed on the effects of salicylic on the oxidation of linoleic acid in the aqueous solution at pH 7.
Figure 17: Peroxide values for 2% linoleic acid in an aqueous solution in the presence of 2ppm ferrous ion during storage in the dark over 10 days.
2.5 Possible chelating activities of ginseng compounds

The effects of 0, 25, 50, 100 and 150 ppm caffeic acid or 50 ppm α-tocopherol on the peroxide values of linoleic acid in aqueous solution with 0.4 and 4.0 ppm ferrous ion are shown in Table 3. As the concentration of caffeic acid increased from 0 to 25, 50, 100, and 150 ppm, the oxidation inhibition ratio (%) increased from 0 to 20, 29, 37, and 49% for the sample with 0.4 ppm ferrous ion and from 0 to 49, 52, 64, and 70% for the sample with 4.0 ppm ferrous ion, respectively. The peroxide values for the sample with 50 ppm caffeic acid in 0.4 or 4.0 ppm ferrous ion were 3.6 or 7.4, respectively. The 50 ppm caffeic acid decreased the peroxide values from 5.2 to 3.6 in 0.4 ppm ferrous ion and from 15.5 to 7.4 in 4.0 ppm ferrous ion (Table 3). The 50 ppm α-tocopherol decreased peroxide value from 5.2 to 3.8 in 0.4 ppm ferrous ion and from 15.5 to 12.6 in 4.0 ppm ferrous ion. The 50 ppm caffeic acid and 50 ppm α-tocopherol have similar antioxidant effect in the sample with 0.4 ppm ferrous ion. But, the 50 ppm caffeic acid had better antioxidant effects than the 50 ppm α-tocopherol in the sample with 4.0 ppm ferrous ion. The higher antioxidant effect of 50 ppm caffeic acid than 50 ppm α-tocopherol in the sample with 4 ppm ferrous ion is most likely due to the chelating effect of caffeic acid. The possible formations of Fe²⁺ complex ions with monomer or dimer of caffeic acid are shown in Figure 18. Caffeic acid can be ionized into carboxylate anion and proton in aqueous system. The monoatom anion ligand such as ionized caffeic acid is a unidentate ligand and can donate a single pair of electron to ferrous ion. It has been postulated that the strong bidentate complex ion of two molecules of ionized caffeic acid monodentate
forms strong complex with ferrous ion (Hocking and Intihar 1985; Speier 1986). The phenolic antioxidants such as caffeic acid may be dimerized in the autoxidation of linoleic acid at 37°C in the dark (Takizawa and others 1985; White and Que 1985). The dimerized caffeic acid is a bidentate ligand and can donate two electron pairs to ferrous ion to form complex ions as shown in Figure 18. Carboxylate anions of caffeic acid as monomer or dimmer might effectively decrease the catalytic action of ferrous ion by forming complex ions as shown in Figure 18 and decrease the catalytic effects of ferrous ion in lipid oxidation. \(\alpha\)-Tocopherol does not have functional group which can chelate metal ferrous ion and has not been reported as a metal chelating compound. \(\alpha\)-Tocopherol is not a good antioxidant in the sample having metal ions. It is a better choice to use phenolic antioxidants with carboxylic acid such to prevent the oxidation of foods with free metal ions.
Figure 18: Formations of Fe$^{++}$ complex ions with a monomer or a dimer of caffeic acid
Table 3: The effects of 25, 50, 100, 150 ppm caffeic acid and 50 pm α-tocopherol on the peroxide value of 2% linoleic acid in an aqueous solution of 0.4 or 4.0 ppm FeCl₂ aqueous solution for 16 hours at 55°C.

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>0.4 ppm FeCl₂</th>
<th>4 ppm FeCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxidation Inhibition Ratio (%)</td>
<td>Peroxide Value</td>
</tr>
<tr>
<td>0 ppm</td>
<td>0</td>
<td>5.2</td>
</tr>
<tr>
<td>Caffeic acid 25 ppm</td>
<td>20</td>
<td>4.1</td>
</tr>
<tr>
<td>Caffeic acid 50 ppm</td>
<td>29</td>
<td>3.6</td>
</tr>
<tr>
<td>Caffeic acid 100 ppm</td>
<td>37</td>
<td>3.2</td>
</tr>
<tr>
<td>Caffeic acid 150 ppm</td>
<td>49</td>
<td>2.6</td>
</tr>
<tr>
<td>α-Tocopherol 50 ppm</td>
<td>25</td>
<td>3.8</td>
</tr>
</tbody>
</table>
CONCLUSION

1. Effects of oil processing steps on the oxidative stability of DHA

   Bleaching proved to be the crucial processing step for increasing the oxidative stability of DHA, most likely due to the removal of prooxidant compound such as chlorophyll and metal ions. Winterizing and deodorizing further removed volatile compounds, and deodorized oil showed an initial volatile content of 0 electronic counts. Deodorizing, however, also partially removed natural antioxidants present in the oil, the effects of which can be seen by the higher increase in the rate of volatile compound formation during storage in the deodorized oil as compared with the bleached and winterized oils.

2. Effects of ginseng compounds on the oxidative stability of 2% linoleic acid in an aqueous system

   Preliminary study showed that pH, ferrous ion concentration, and storage temperature of linoleic acid in aqueous system affected the effects of ginseng compounds as were expected. That is, the order of antioxidant effectiveness of caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid shown in this study could be changed according to the pH, storage time, types and content of metal ions, types of singlet or triplet oxygen oxidation, and concentrations of lipids in foods. It is recommended to study preliminary antioxidant activity with ginseng compounds in the specific food systems to choose the most effective antioxidant. Caffeic acid, ferulic acid, kaempferol, salicylic acid, and vanillic acid are good antioxidants on the linoleic acid oxidation in
aqueous system. They are as good as or better antioxidant than α-tocopherol. Caffeic acid, ferulic acid, and vanillic acid acted as antioxidants by the combination of chelating metal ion and donating hydrogen atom to linoleic acid free radicals.
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