EFFECTS OF NATURAL ANTIOXIDANTS ON LIPID OXIDATION OF MENHADEN OIL

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ABSTRACT

Preventing oxidative deterioration of fish oil is a significant challenge for the food industry. Natural antioxidants are widely incorporated into foods and oils to prevent oxidation and extend shelf life. The goal of the study is to investigate the activity of novel antioxidants in menhaden oil and to develop optimum formulations containing mixed tocopherols to control oxidation of menhaden oil. Alpha tocopherol, gamma tocopherol, and delta tocopherol in menhaden oil were found at 0.18mg/g, 0.37mg/g, and 0.14mg/g, respectively, using HPLC analysis. Teng Cha extract effectively delayed oxidation of menhaden oil (MO) when stored at 40℃ for eight days by measuring primary oxidation products and secondary oxidation products. The combinations of Teng Cha extract and rosemary extract and combinations of ascorbyl palmitate, citric acid, Teng Cha extract and rosemary extract more effectively improved stability of MO containing mixed tocopherols than Teng Cha extract alone at 40℃ storage for eight days by measuring primary oxidation products and secondary oxidation products. From this study, Teng Cha extract can be used as a potential natural antioxidant in food industry, especially in combinations with rosemary extract and tocopherols, extending shelf life of menhaden oil.

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LIST OF ABBREVIATIONS OF TERMS

AHA	American Heart Association
AV	Anisidine Value
AOCS	American Oil Chemist's Society
AP	Asocorbyl Palmitate
BHA	Butylated hydroxyanisole
CA	Citric acid
DHA	Docosahexanoic acid
DHM	Dihydromyricetin
ECL	Equivalent chain length
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FO	Fish oil
IUPAC	International Union of Pure and Applied Chemistry
GCMS	Gas chromatography–mass spectrometry
GREX	Grape Seed Extract
GTEX	Green Tea Extract

HPLC	High-performance liquid chromatography
LC -PUFAs	Long-chain polyunsaturated fatty acids
MO	Menhaden oil
MT	Mixed Tocopherols
n-3 PUFAs	Omega 3 Polyunsaturated fatty acids
PV	Peroxide Value
QC	Quercetin
RSEX	Rosemary Extract
TEX	Teng Cha Extract

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CHAPTER 1: INTRODUCTION

Fish oil as Neutraceutical and Functional Foods

Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) are associated with promoting good health including protection against cardiovascular disease, immunity improvements, cancer prevention, reduction of depression and inflammation disease (Bulliyya, 2002; Kris-Etherton, Harris, & Appel, 2002; von Schacky & Harris, 2007). Fish oil (FO) is the major dietary source of eicosapentaenoic acid (20:5 n−3, EPA) and docosahexaenoic acid (22:6 n−3, DHA) and there has been tremendous interest in these fatty acids since the late 1970s. Researchers had focused on fish oil supplements and food products modified with fish oil incorporation including eggs, dairy foods, snacks, infant formulas and juice to produce nutraceutical and functional foods that will provide health benefits (Calder, 2006; Cave, Watson, & Mufti, 1996; Lee, 2011; Chung, Chen, & Su, 2008; Peoples & McLennan, 2010; Ruxton, Reed, Simpson, & Millington, 2004).

Susceptibility to Oxidation and Protection of Oxidative Deterioration of Fish Oil

Fish oil has high numbers of unsaturated fatty acids containing 4, 5 and 6 double bonds and is highly susceptible to oxidation in presence of light, oxygen, transition metals and enzyme (Frankel, 1998). Oxidation dramatically decreases the shelf life of fish oil supplementation and fish oil enriched foods. During the oxidation of fish oil, there is a loss of nutrients and fishy and rancid off flavors are developed from decomposition of hydroperoxide (Indrasena & Barrow, 2010; Rossell, 2009).

Control Oxidation of Fish Oil

Preventing oxidative deterioration in foods has been a significant challenge for the food industry. Incorporation of natural antioxidants into foods and oils instead of synthetic antioxidants is of interest to prevent oxidative deterioration, improve stability and extend shelf life. These natural antioxidants are more used because of consumer preference for clean food labels (Brewer, 2011).

Antioxidants can be classified based on their modes of action. Primary antioxidants donate hydrogen to scavenge alkoky and peroxy radicals and form stable antioxidant radical and this breaks the propagation cycle. Secondary antioxidants such as metal chealating agents, singlet oxygen quenchers, oxygen scavengers, and antioxidant regenerators prevent the formation of volatile and toxic compounds and convert hydroperoxides to stable radical and to non-radical products (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010; Decker, Chen, Panya, & Elias, 2010).

Tocopherols, lipid soluble antioxidants, are commonly added to foods, but use of single antioxidant is less effective in the fish oil compared to combinations of different antioxidants (Drusch, Groß, & Schwarz, 2008; Hamilton, Kalu, Prisk, Padley, & Pierce, 1997; Indrasena & Barrow, 2010; Rossell, 2009). Tocopherols have synergistic antioxidant properties when they were added with other antioxidants with fish oil(Indrasena & Barrow, 2010). As a result of the good synergistic antioxidant properties of tocopherols, greater antioxidant effectiveness in fish oil was observed when primary antioxidants and secondary antioxidants were formulated with tocopherols (Drusch, Groß, & Schwarz, 2008; Mette, Jacobsen, & Meyer, 2007; Yi, Andersen, & Skibsted, 2011).

Research Objectives

The aim of study was to investigate the activity of novel antioxidants in menhaden fish oil and to develop optimum formulations containing mixed tocopherols to control oxidation of menhaden oil.

Objective 1: Determine vitamin E contents in menhaden oil using simplified extraction methods and HPLC analysis.

Objective 2: Determine natural antioxidants concentrations that optimally inhibit oxidation of menhaden oil containing mixed tocopherols.

Objective 3: Evaluate oxidative stability of menhaden oil containing mixed tocopherols and mixtures of Teng Cha extract and rosemary extract.

Objective 4: Evaluate oxidative stability of menhaden oil containing mixed tocopherols formulated by blending natural antioxidants ascorbyl palmitate, citric acid, Teng Cha extract and rosemary extract.

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CHAPTER 2: LITERATURE REVIEW

Fish Oil: Omega-3 Polyunsaturated Fatty Acids

Fish oil is rich in long-chain, omega-3 polyunsaturated fatty acids (n-3 PUFAs) mainly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexanoic acid (DHA, 22:6n-3) (Figure 1.1 and 1.2). Polyunsaturated fatty acids (PUFAs) have two or more double bonds in a cis methylene interrupted configuration. In fish oils, 20 carbon non-methylene interrupted dienes are sometimes found at low concentrations (Ackman, 1969). The cis configuration results in a shorter but wider molecules in three dimensions, compared to saturated or trans bonds. Melting point of PUFAs containing more than two double bonds is lower than unsaturated and saturated fatty acids. Saturated fatty acids have higher melting temperatures, in part as a result of weaker van der Waals interaction found in double bonds structures, compared to saturated structures. The omega (ω) system or shorthand preferred by IUPAC "n-" indicates the position of the terminal double bonds position (O'Keefe, 2002). This assumes cis bonds and methylene interrupted structure if polyunsaturated. The double bond positions in n-3 or n-6 PUFAs can be predicted based on the n- position and number of double bonds. The n-3 PUFAs cannot be synthesized in human body because humans lack the desaturase enzymes to create cis double bonds in the positions beyond Δ9. Humans only have Δ3, Δ4, Δ5, and Δ9 desaturases. One would need Δ12 and Δ15 desaturases to add double bonds at the n-6 and n-3 positions, respectively, in an 18-carbon fatty acid. Humans can, however, elongate and desaturate fatty acids in existing n-6 or n-3 families, and this can synthesize DHA, 22:6n-3, if

provided 18:3n-3. For this reason, essential fatty acids are usually considered as families, n-3 and n-6 (Damodaran, Parkin, & Fennema, 2008)

Fish Oil: Health Benefits of n-3 PUFA

For more than thirty years, fish oils have received a great deal of attention because of the positive health effects of EPA and DHA, and marine fish oils are the main dietary sources of these fatty acids. The amount of EPA and DHA recommended for protection against cardiovascular diseases, prevention of sudden death and secondary cardiovascular diseases is around 1g per day (von Schacky & Harris, 2007). Much research supports the consumption of fish oils supplements, which are a major source of EPA and DHA, to help reduce blood pressure, plasma triglycerides levels and to increase high density lipoprotein levels (HDL), factors that help prevent coronary heart disease (Mozaffarian & Wu, 2012; Rajaram, Haddad, Mejia, & Sabaté, 2009). Besides cardiovascular health benefits, studies have reported that EPA and DHA contribute to other health benefits including improvements of immune function, brain development, cancer prevention, fatigue resistance, vision development and even the weight loss (Cave, Watson, & Mufti, 1996; Nkondjock, Shatenstein, Maisonneuve, & Ghadirian, 2003; C. H. S. Ruxton, Reed, Simpson, & Millington, 2004). According to Hung et al. (1999), EPA and DHA promote anti-allergic activity, and Romieu et al. (2007) reported that frequent fish consumption leads to lower risk of eczema and atrophy of offspring during pregnancy in animals (Hung, Kaku, Yunoki, Ohkura, Gu, IKEDA, et al., 1999; Romieu, Torrent, Garcia-Esteban, Ferrer, Ribas-Fitó, Anto, et al., 2007). Intake of fish oil supplements boosts the speed of muscle recovery after intense exercise as a result of EPA and DHA

reducing oxygen consumption in skeletal muscle (Peoples & McLennan, 2010). In addition, DHA's ability to improve the fluidity of cell membranes and establishment of optimal functionality in the brain and retina in the early stages of fetal development have helped EPA and DHA n-3 PUFAs earn the title of "brain food". In fact, "fish is brain food" was often stated long before long chain n-3 fatty acids became the darlings of nutritional research. Studies have shown that infants who lack DHA exhibited poor cognitive and retina functions (C. H. Ruxton, Calder, Reed, & Simpson, 2005). Furthermore, intake of fish oil supplementation has proven its benefits for mental health and problem solving for children and DHA deficiency results in retarded cognitive development, visual impairment or behavior problems as children grow up (Helland, Smith, Saarem, Saugstad, & Drevon, 2003; Jørgensen, Hernell, Hughes, & Michaelsen, 2001). In adults, intake of EPA and DHA supplements is associated with reduced risk of depression, dementia, Alzheimer's disease, and cognitive dysfunction (Donini, De Felice, & Cannella, 2007; Murakami, Miyake, Sasaki, Tanaka, & Arakawa, 2010).

Dietary Recommendation of n-3 PUFAs Consumption

Although intake of fish oil rich in EPA and DHA n-3 PUFAs reduces coronary heart disease rates, and maintains and promotes good health, controlling the consumption fish oil derived n-3 PUFAs is recommended. According to the American Heart Association, the recommended daily intake of fish oil and EPA and DHA n-3 PUFAs from other sources depends on the individual. The AHA recommends that people who have no history of coronary heart diseases should consume fatty fish rich in n-3 PUFAs, oils, plant sources of n-3 PUFAs at least twice a week, or one gram of EPA and DHA

daily from preferable fatty fish or EPA and DHA supplementation under physician consultation. They recommend patients who need to reduce plasma triglycerides should consume 2-4g daily EPA and DHA under physician consultations (Covington, 2004).

Fish Oil: Susceptibility to Oxidation

Much scientific evidence indicates that long chain n-3 PUFAs (EPA and DHA) provide many health benefits. Fish oil contains highly unsaturated n-3 PUFAs' these fatty acids are highly susceptible to oxidative degradation during processing, handling and storage. This oxidation of fish oil leads to undesirable off-flavors and loss of nutritional value. Autoxidation and photooxdiation of fish oil can easily occur under mild conditions.(Frankel, 1998; Indrasena & Barrow, 2010)

Autoxidation

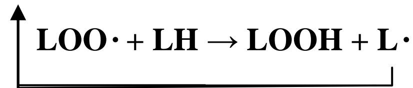
Autoxidation occurs in three stages: initiation, propagation and termination. The initiation stage is the beginning of the oxidation process when free radicals are formed from abstraction of hydrogen in unsaturated fatty acids by initiators such as light, heat, transition metals and enzymes

Initiation: $LH \rightarrow L\cdot + H\cdot$

In the propagation stage, free radicals react with oxygen to produce peroxide free radicals. Peroxide free radicals react with double bonds and abstract hydrogen from another unsaturated fatty acid, forming a primary oxidation product, hydroxperoxide, and new free radical. As soon as a free radical is formed from hydrogen abstraction in the methylene-interrupted group, double bonds rearrangement produces two isomers. (Figure 2.1 modified by Frankel, 1998).The energy required to abstract a H from a bond is lower

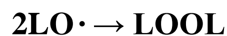
if the H is in an allylic or even lower if in a bis-allylic position 2 (figure 2.2 modified by (Erickson, 2002). This is why the rate of lipid oxidation is higher as the number of double bonds increase. There are 0, 2 and 4 doubly allylic hydrogens in 18:1n-9, 18:2n-6 and 18:3n-3, respectively, and the rates of lipid oxidation is roughly 1:10:20, respectively. The hydroperoxide breaks down to give more free radicals which continue to attack other unsaturated fatty acids with further propagation until there is no hydrogen available, or the free radical reactions are interrupted or slowed down by antioxidants (Indrasena & Barrow, 2010; Rossell, 2009).

Propagation: $L\cdot + O_2 \rightarrow LOO\cdot$



In the termination stage, the peroxy free radicals or free radicals react with each other to form non-radical products. Hydroperoxides easily break down to volatile secondary oxidation products such as aldehydes, alcohols and ketones, which can produce rancid off-flavors in oxidized fats or oils. (Figure 3. modified by Erickson, 2002) Although the autoxidation process can complete one cycle in the termination stage, the autoxidation process normally continues to repeat by re-initiation of free radical formation until all oxidizable substrates are used up (Indrasena & Barrow, 2010; Rossell, 2009).

Termination: $L\cdot + L\cdot \rightarrow L\text{-}L$

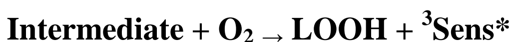


Photooxidation

Photooxidation occurs in the presence of light and photosensitizers such as chlorophyll, phytins, myoglobin, and riboflavin. The photosensitizers in foods and oils absorb UV energy to produce an excited state. Two types of photosensitizers are recognized, Type 1 and Type 2.

A type 1 sensitizer plays a role as a photochemically activated free radical initiator, the triplet state photo sensitizer reacts with fatty acids by abstraction of hydrogen or electron transfer to form radicals, which can react with oxygen. The hydroperoxide is formed from conjugate radical in a similar manner as in free radical autooxidation (Frankel, 1998; Rossell, 2009)

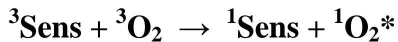
$$\mathbf{^{3}Sens + LH \rightarrow intermediate + O_2}$$



$$\mathbf{Intermediate + O_2 \rightarrow LOOH + {^{3}Sens^{*}}}$$

A type 2 sensitizer absorbs UV energy (hv) to produce singlet oxygen from triplet oxygen. Singlet oxygen is much more reactive than triplet. Singlet oxygen reacts with unsaturated fatty acids to produce peroxyradical which go on to form hydroperoxides. The type 2 photooxidation cannot be inhibited by chain breaking antioxidants since it is completely different from free radical autooxidation. The rate of type 2 photooxidation is much faster than autoxidation, as singlet oxygen reacts much faster than triplet oxygen (Indrasena & Barrow, 2010; Rossell, 2009). The hydroperoxide is produced very quickly as soon as the beginning of photooxidation. Since photooxidation does not have an induction period like autooxidation, fish oil should be stored in darkness without light

exposure (Rossell, 2009).



$$^{3}Sens + {}^{3}O_2 \rightarrow {}^{1}Sens + {}^{1}O_2^*$$

$$^{1}O_2^* + LH \rightarrow LOOH$$

Negative Effects of Lipid Oxidation of n-3 PUFAs

Secondary oxidation products such as aldehydes, ketones, alcohols and hydrocarbons are associated not only with negative effects on the odor, flavor, color and texture but also loss of nutritional qualities such as amino acids, vitamins, polyphenols, phytosterols and squalene (Eder, Ringseis, Decker, Elias, & McClements, 2010). Consumption of oxidized edible oil can lead to toxicity and biological problems such as diarrhea increase in depression and tissue and organ damage due to the oxidation products from unsaturated fatty acids (Ching, 2007). Liver damage was observed in rats that were fed by oxidized oil whereas liver damage was not developed in the rats fed by fresh oil (Totani, Burenjargal, Yawata, & Ojiri, 2008).

Fish Oil: Retarding Lipid Oxidation and Improving Stability of n-3 PUFAs

Minimizing oxidation of EPA and DHA n-3 PUFAs has been a significant challenge to maintaining quality in fish oil and FO fortified foods. Extreme care is necessary to protect against oxidative degradation of fish oil while handling, processing, packaging, transferring, and transporting and storage. Fish oil should not be exposed to high temperature, oxygen and light. Storage of processed n-3 PUFAs must be under darkness or below -20°C and under inert gas (nitrogen or argon) in order to avoid oxidative deterioration. Antioxidants are commonly added to retard oxidation of EPA

and DHA n-3 PUFAs; each antioxidant has various inhibitory characteristics on lipid oxidation of n-3 PUFAs (Indrasena & Barrow, 2010).

Inhibiting Photooxidation

When photosensitizers such as chlorophyll, phaeophytins, myglobins, or riboflavins are present with marine oils, they promote oxidation in the "excited state" after absorbing light. Controlled light is absolutely necessary when the marine oil is processed and the processed oil must be kept in dark containers preventing light exposure to avoid photooxidation (Hamilton, Kalu, Prisk, Padley, & Pierce, 1997). Carotenoids and alpha tocopherol inhibit singlet oxidation by quenching singlet oxygen (Indrasena & Barrow, 2010; Palozza & Krinsky, 1992). Mukai (2005) found that tea catechins may be photoprotective by deactivating singlet oxygen (Mukai, Nagai, & Ohara, 2005).

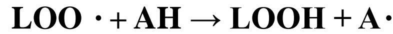
Inhibiting Autoxidation

Antioxidants have various properties that can retard autoxidation by inhibiting formation of free radicals at either initiation or propagation stages. For example, antioxidants can scavenge free radicals, inactivate metal ions, quench singlet oxygen, scavenge oxygen and regenerate antioxidants (Brewer, 2011; Damodaran, Parkin, & Fennema, 2008).

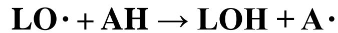
Primary Antioxidants (Chain Breaking Antioxidants)

Primary antioxidants scavenge alkoxy (RO•) and peroxy radicals (ROO•) to form stable radical species by donation of hydrogen from the antioxidants; thus the primary antioxidants produce unreactive, low-energy antioxidant radicala (ROOA) that are unable

to continue propagation during autoxidation (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010).



$$\mathbf{LOO\cdot + AH \rightarrow LOOH + A\cdot}$$



$$\mathbf{LO\cdot + AH \rightarrow LOH + A\cdot}$$

Phenolic antioxidants form as stable intermediate radicals because of electron resonance delocalization of the structures, so the low-energy radicals are stable in part because of the resonant structures possible. The phenolic ring compounds containing electron donating substitutes at ortho- and para- position are more potent and efficient antioxidants than the compounds containing the substitutes at meta positions (Frankel, 1998; Shahidi, Janitha, & Wanasundara, 1992). t-Butyl is one electron donating group that is commonly seen in synthetic antioxidants.

Secondary Antioxidants (Preventive Antioxidants)

Secondary antioxidants include metal chelators, hydroperoxide decomposers, antioxidant regenerators and singlet oxygen scavengers. Metal chelating agents such as citric acid and ethylenediaminetetraaceticacid (EDTA) bind metals to limit the activation caused by metals; mainly, preventing initiation and decomposition of hydroperoxide in the propagation step. The decomposition of hydroperoxides produces a variety of secondary oxidation products such as aldehydes, alkenals, alkenes, alkanes, and other volatile compounds that can contribute to rancid flavors (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010). Citric acid is often added to refined oil after the deodorization step. The food industries commonly use citric acid as both an antioxidant

synergist and a metal inactivator. Another commonly used metal inactivator, EDTA, is known as an effective antioxidant inhibitor retarding oxidation with lower cost than citric acid; however, the food industry has been searching for alternative compounds due to the limited solubility of EDTA and consumers' concern about synthetic additives (Decker, Chen, Panya, & Elias, 2010; Indrasena & Barrow, 2010). Hydroperooxide decomposers such as α-tocopherol and Trolox prevent lipid oxidation by slowing decomposition of hydroperoxides and also they produce either stable or non-radical products (Frankel, 1998). Singlet oxygen scavengers including tocopherols, and carotenoids such as lycopene or β-carotene, quench singlet oxygen to prevent photooxidation (Indrasena & Barrow, 2010).

Natural Antioxidants

Synthetic and natural antioxidants have been commonly used in food industry control oxidation and maintain quality. However, the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylatedhydroxytolune (BHT), propyl gallate (PG), and tertbutylhydroquinone (TBHQ) has been declining in the food industry. Consumers are concerned about potential carcinogenic and health hazard from artificial additives and the FDA has also established limits on the amount of synthetic antioxidants that can be added to food products (Botterweck, Verhagen, Goldbohm, Kleinjans, & Van Den Brandt, 2000; Brewer & Prestat, 2002). Natural phenolics antioxidants derived from plants such as sage, rosemary, tea and grape seeds have received much interest in their incorporation into foods to control oxidation. Researchers continue to study availability, chemical structures and effectiveness of other potential natural antioxidants from botanical, marine

and antimicrobial sources which may also have anticarcinogenic activity and reduce oxygen stress (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010; Brewer, 2011).

Tocopherols

Tocopherols (Vitamin E) are lipid soluble, colorless to light yellow and thick textured oils. Tocopherols are rich in most vegetable oil, but fish oils and other animal fats contain much lower amounts of tocopherols than vegetable oil (Rossell, 2009). Fish oils also generally only contain the α- isomer. Tocopherols are found in eight different forms, as α-, ß-, γ-, and δ-tocopherols and their corresponding tocotrienols. Tocopherols are identified by number and positions of methylation on the phenol ring containing a saturated phytyl side chain (Eitenmiller, Ye, & Landen, 2008). The corresponding tocotrienols have double bonds in this phytyl chain but the methyl substituents are the same.

α-Tocopherol contains trimethyl substitutions on positions (5, 7, 8) (figure 4.11) and ß-tocopherols (figure 4.12) and γ-tocopherols (figure 4.13) have diemethyl group isomers on positions (5,8) and (7,8) of the phenol ring, respectively. δ-tocopherol (figure 4.14) contains one methyl group on position 8 of the phenol ring. Tocopherols react with lipid peroxy radicals by donating a hydrogen atom to form lipid hydroperoxides and produce several tocopheroxy radicals, which are stabilized by resonance. The toxopheroxy radicals are unable to continue propagating the autoxidation chain reactions but produce termination with non-radical products (Damodaran, Parkin, & Fennema, 2008).

Tocopherols can have antioxidant or prooxidant properties depending on their concentrations or temperatures (Indrasena & Barrow, 2010). α-tocopherol can be a prooxidant at high concentrations and form primary oxidation products during early stage of lipid oxidation, whereas α-tocopherol at low concentration (50-100ppm) more effectively inhibited lipid oxidation than α-tocopherol at higher concentrations (E. Kulås & R. G. Ackman, 2001; Zuta, Simpson, Zhao, & Leclerc, 2007). However, γ- and δ-tocopherols have different functions as demonstrated by their delaying formation of hydroperoxides in bulk fish oil (unlike α-tocopherol); γ- and δ- tocopherols at high concentrations more effectively inhibited formation of primary and secondary oxidation products at low concentrations than α-tocopherol. It was observed that δ-tocopherol exhibited more potent antioxidant activity and more effectively inhibited formation of hydroperoxides at a concentration of 1000ppm or above compared to γ-tocopherol. α-tocopherol become prooxidant at this high concentration (Hamilton, Kalu, Prisk, Padley, & Pierce, 1997; E. Kulås & R. Ackman, 2001). α-tocopherol demonstrated more effective antioxidant inhibitory activity on lipid oxidation at low temperature. The order of the most effective antioxidants was α-tocopherol > ß-tocopherol > γ-tocopherol > δ-tocopherol, but the order was reversed at high temperatures (Indrasena & Barrow, 2010; Jung & Min, 1990).

Addition of single antioxidants including tocopherol did not efficiently protect against oxidative deterioration of fish oil or FO supplemented foods during extended storage (Burkow, Vikersveen, & Saarem, 1995; Indrasena & Barrow, 2010). When tocopherols are mixed with other antioxidants in fish oil, the formulated antioxidant

mixtures should be optimized to prevent oxidation. Combinations of tocopherols and other antioxidants possibly exhibit synergism in delaying oxidative deterioration of fish oil. Ascorbic acid or ascorbyl palmitate regenate α-tocopherol from its tocopheryl radical (Wefers & Sies, 1988). Ascorbyl palmitate assists in the prevention of tocopherol loss during storage of fish oil. Ascorbyl pamitate increases the effectiveness of tocopherols by chemically reducing the tocopheryl radical and regenerating tocopherol (Drusch, Groß, & Schwarz, 2008; Indrasena & Barrow, 2010). When tocopherols were added with metal chelating agent such as citric acid in fish oil, the stability of fish oil was improved by synergistic effects (Drusch, Groß, & Schwarz, 2008). Phenolic compounds in rosemary extracts can boost the effectiveness of antioxidant tocopherols, although rosemary extracts have radical scavenging activity alone (Yoshioka, Yamada, & Wada, 2002). Drusch et al (2008) found that the antioxidant mixture formulations created in combination with low levels of alpha tocopherol and high levels of gamma and delta tocopherols, ascorbyl palmitate and citric acid and rosemary extract significantly delayed oxidation and improved stability of fish oil during the storage at ambient temperature (Drusch, Groß, & Schwarz, 2008).

Ascorbic Acid

Ascorbic acid is commonly used in food applications due to its multiple functions; a hydrogen donator to antioxidant radicals to regenerate antioxidants, a metal inactivator, hydroperoxide decomposer and oxygen scavenger in aqueous system(figure 4.21) (Frankel, 1998). Ascorbic acid can be used as an oxygen scavenger or a synergist with tocopherols because of its chemical reducing capacity. According to Frankel and Berdahl,

ascorbic acid prevents degradation of tocopherols because of ascorbate's metal scavenging activity in addition to regeneration of tocopherols from the reduction of tocopheroxy radicals (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010; Frankel, 1998). Asocobyl palmitate is an oil soluble ester derivative of ascorbic acid and exhibits same properties as ascorbic acid. (Figure 4.22) Ascorbyl palmiate is more effective antioxidant in oil emulsion than bulk oil because the lipophilic ascorbyl palmitate is more protective whereas ascorbic acid is more efficient antioxidant in bulk oil system (Decker, Warner, Richards, & Shahidi, 2005)

Rosemary and Other Herb Extracts

Rosemary (*Rosmarinus officinalis* L.) extracts have been popular antioxidants in the United States and Europe since scientific evidence demonstrated good antioxidant activities (Kendrick & Macfarlane, 2003; Keokamnerd, Acton, Han, & Dawson, 2008; Reynhout, 1991). The active phenolic compounds in rosemary extract are both water soluble (rosmarinic acid) and lipid soluble (carnosic and canrosl). Carnosic acid and carnosl scavenges peroxy radical by donating hydrogen to reactive radicals to form stable free radical intermediates, preventing formation of primary oxidation products (figures 4.3) (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010). Carnosic acid and carnosol demonstrated good antioxidant activity inhibiting hydroperoxide formation in corn oil, soybean oil and fish oil bulk oil systems although it was still weaker than TBHQ in fish oil (Wang, Liu, Yang, Zu, Wang, Qu, et al., 2011). Carnosic acid and carnosol antioxidant activities are dependent on the oil substrate and system type, either bulk oil or oil in water emulsions. According to Frankel (1998), the hydrophilic rosemary extract antioxidants such as rosmarinic acid in the bulk oil system is more effective as a

result of protection in the air oil interface (Decker, Warner, Richards, & Shahidi, 2005; Frankel, 1998). Rosemary extracts often have a synergistic effect with other antioxidants. In addition to synergism in mixtures of rosemary extract and tocopherols, rosemary extract increases the antioxidant activity with citric acid in flaxseed oil (Jaswir, Kitts, Che Man, & Hassan, 2004).

Green Tea

The major phenolic compounds in green tea are catechins (flavan-3-ols), (-) epicatechin (EC), (-) epicatechingallate (ECG), (-) epigallocatechin (EGC), (-) epigallocatechingallate (EGCG), (+) catechin (C) and (+) gallocatechin (GC) (Figure 4.4) (Chen & Ho, 1995; Dufresne & Farnworth, 2000). The catechins and other polyphenolic compounds were reported to only have anticarcinogenic effect, but also act as effective antioxidants by acting as free radical scavengers and metal ions chelating agents (Gramza & Korczak, 2005; Komori, Yatsunami, Okabe, Abe, Hara, Suganuma, et al., 1993; Shahidi, Janitha, & Wanasundara, 1992; Yen, Chen, & Peng, 1997). Green tea catechin extracts demonstrated more effective antioxidant effects in lard and soybean oil. These extracts also carried had better antioxidant activity than tocopherols and BHA on fish oil oxidation (Wanasundara & Shahidi, 1998). Although green tea extract containing chlorophyll had a prooxidant effect in marine oil, dechlorophyllized green tea extract inhibited oxidation in refined and deodorized marine oils (Wanasundara & Shahidi, 1998).

Ampelopsis grossedentata (Teng Cha)

Ampelopsis grossedentata grows wild in the south of China and is from the family Vitaceae and genus Ampelopsis Michx (Ye, 2011). The major bioactive flavonoid in

Ampelopsis grossedentata leaves was identified as dihydromyricetin (DHM) (Gao, Liu, Ning, Zhao, Zhang, & Wu, 2009; Yang, Benguo, Liu, & Zhang, 2011) (Figure 4.5 DHM compounds structure adapted from Ye, 2011). For hundreds of years in China, Teng Cha has been used as a medicinal herbal tea. Zhang reported that Teng Cha has favorable health benefit properties such as reduction of risk of high blood pressure, antibacterial and antioxidant activities and analgesic functions (Li, Tan, Li, Xiao, & Dai, 2006; Zhang, Ning, Yang, & Wu, 2003). DHM has been reported as being a potentially potent antioxidant (Reische, Lillard, & Eitenmiller, 2008). Teng Cha extract rich in DHM has similar antioxidant properties as TBHQ (Jianhua, Benguo, Zhengxiang, Ruixiang, Aiyuan, & Qiong, 2009). Moreover, Ye reported effectiveness of DHM as antioxidant is better than BHA, and DHM more effectively inhibited oxidative degradation of soybean oil, as assessed using several primary and secondary oxidation measurements (Ye, 2011).

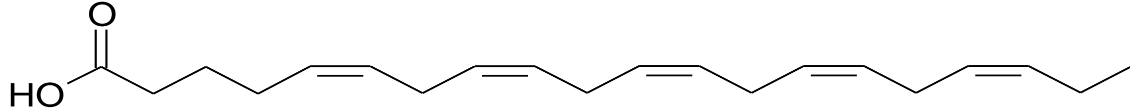


Figure 1.1.Eicosapentanoic acid, EPA, 20:5n-3 (modified by O'Keefe, 2002).

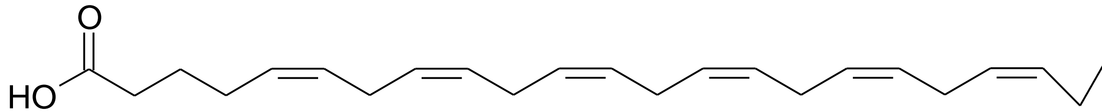
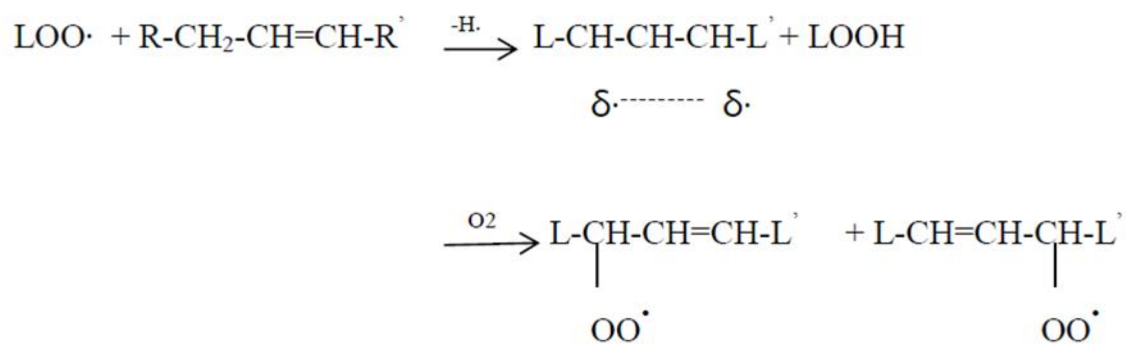


Figure 1.2.Docosahexanoic acid, DHA, 22:6n-3 (modified from O'Keefe, 2002).



Figures 2.1.In propagation stage, two isomers were formed from double bonds rearrangement right after hydrogen abstraction from another unsaturated fatty acids. Primary oxidation products (lipid hydroperoxide) were produced. (Frankel, 1998)

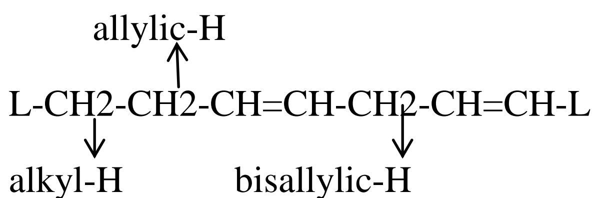


Figure 2.2.Lower bond energy required to abstract biallylic hydrogen bond (75kcal) than allylic hydrogen bond (88kcal) and alkyl hydrogen bond (100kcal) modified from (Erickson, 2002).

Figure 3.Lipid hydroperoxide decomposed to secondary oxidation products which leads to rancid off-flavors modified from (Erickson, 2002).

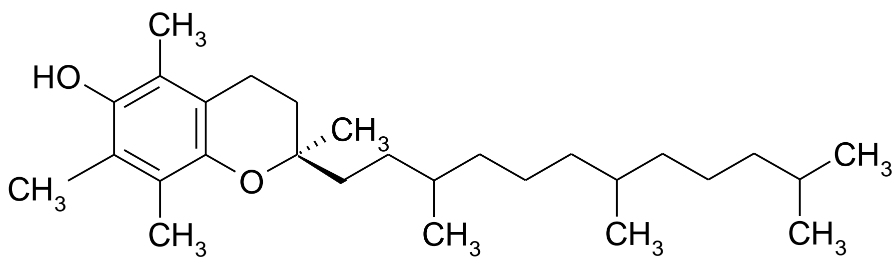


Figure 4.1.α-tocopherol adapted from (Eitenmiller, Ye, & Landen, 2008).

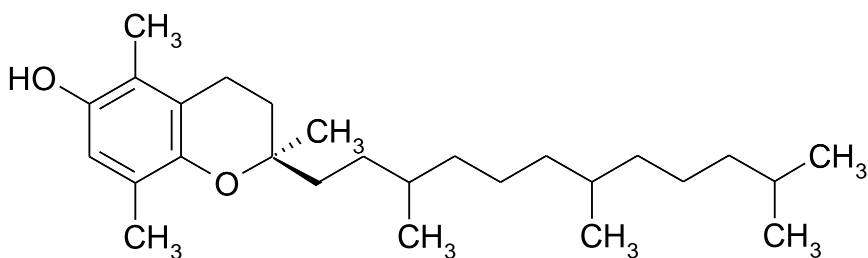


Figure 4.2. ß-tocopherols adapted from (Eitenmiller, Ye, & Landen, 2008).

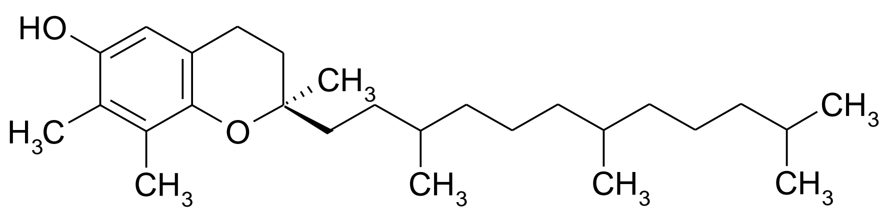


Figure 4.3. γ-tocopherols adapted from (Eitenmiller, Ye, & Landen, 2008).

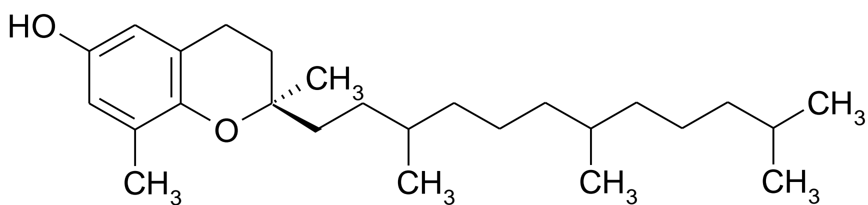


Figure 4.4. δ-tocopherol adapted from (Eitenmiller, Ye, & Landen, 2008).

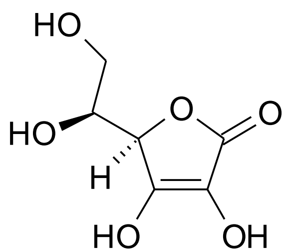


Figure 5.1. Ascorbic acid followed from (Frankel, 1998).

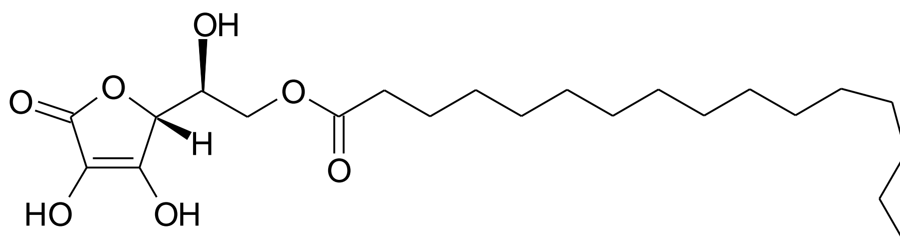
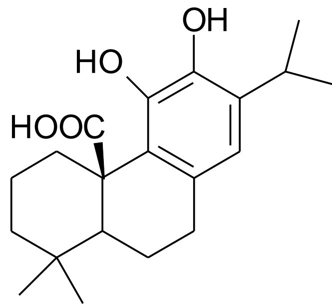
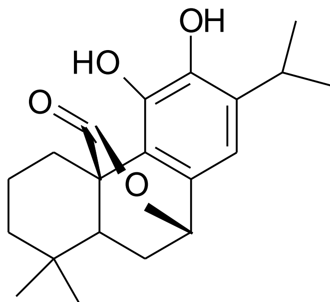


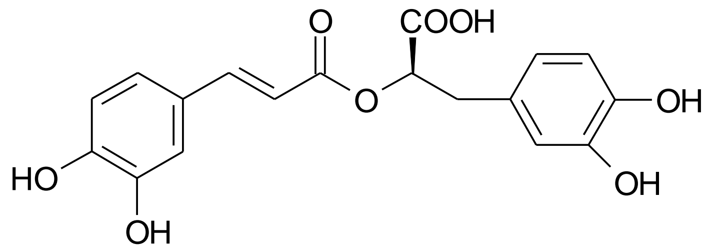
Figure 5.2. Ascorbyl palmitate followed from (Frankel, 1998).



Carnosic acid

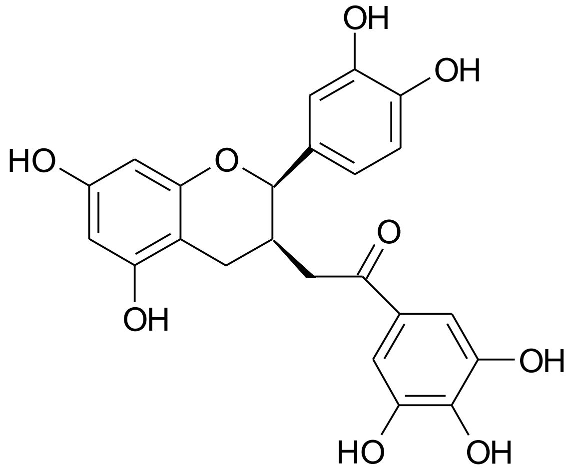


Carnosol

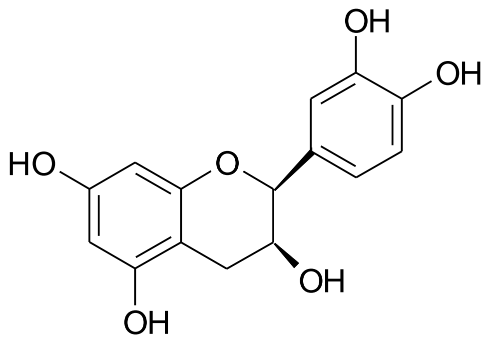


Rosmarinic acid

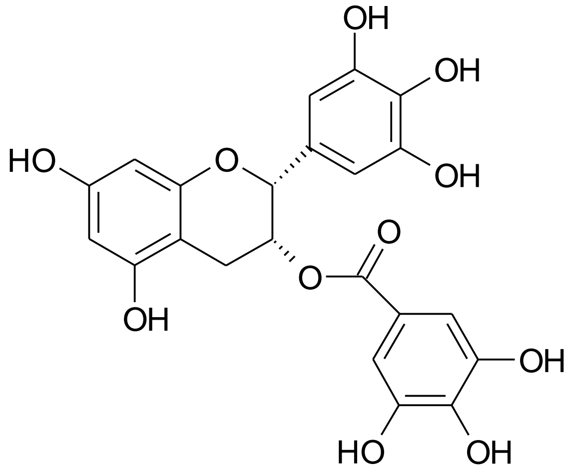
Figure 6. Active phenolic compounds in rosemary extract adapted from (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010).



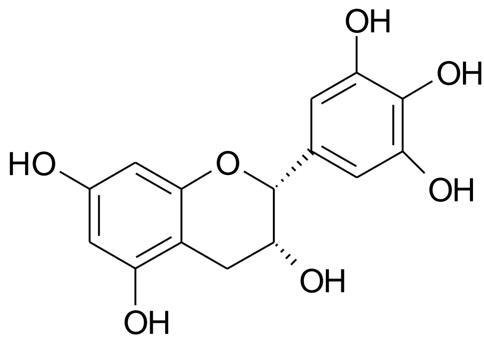
(-)-Epicatechin gallate (EGC)



(-)-Epicatechin gallate (EC)



(-)-Epigallocatechin gallate (EGCG)



(-)-Epigallocatechin (EGC)

Figure 7. Tea catechins compound adapted from (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010).

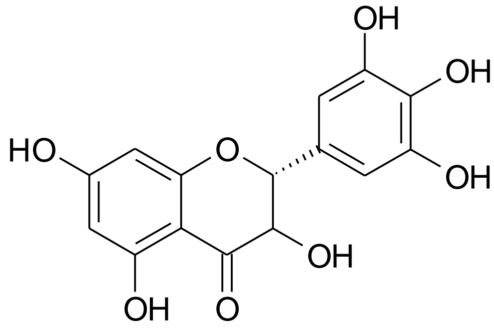


Figure 8. DHM compound structure adapted by (Ye, 2011).

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CHAPTER 3: EFFECTS OF NATURAL ANTIOXIDANTS ON LIPID OXIDATION OF MENHADEN OIL

ABSTRACT

Preventing oxidative deterioration of fish oil is a significant challenge for the food industry. Natural antioxidants are widely incorporated into foods and oils to prevent oxidation and extend shelf life. The goal of the study is to investigate the activity of novel antioxidants in menhaden oil and to develop optimum formulations containing mixed tocopherols to control oxidation of menhaden oil. Alpha tocopherol, gamma tocopherol, and delta tocopherol in menhaden oil were found at 0.18mg/g, 0.37mg/g, and 0.14mg/g, respectively, using HPLC analysis. Teng Cha extract effectively delayed oxidation of menhaden oil (MO) when stored at 40℃ for eight days by measuring primary oxidation products and secondary oxidation products. The combinations of Teng Cha extract and rosemary extract and combinations of ascorbyl palmitate, citric acid, Teng Cha extract and rosemary extract more effectively improved stability of MO containing mixed tocopherols than Teng Cha extract at 40℃ storage for eight days by measuring primary oxidation products and secondary oxidation products. From this study, Teng Cha extract can be used as a potential natural antioxidant in food industry, especially in combinations with rosemary extract and tocopherols, extending shelf life of menhaden oil.

KEYWORDS : Natural antioxidant, menhaden oil, oxidation, tocopherol, Teng Cha extract, rosemary extract, ascorbyl palmitate, citric acid, primary oxidation products, and secondary oxidation products

INTRODUCTION

Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) are associated with promoting good health, including protection against cardiovascular disease, immunity improvements, brain function development, memory and vision improvements, reduction of depression and inflammation diseases and cancer prevention (Gogus & Smith, 2010; Ruxton, Reed, Simpson, & Millington, 2004). Fish oil (FO) is the major dietary source of EPA and DHA and there has been tremendous interest in these fatty acids since the late 1970s, when they were shown to have cardioprotective effects. Research has focused on FO supplements and food products modified with FO incorporation, including eggs, dairy foods, snacks, infant formulas, and juices to produce neutraceutical/functional foods that enhance health (Lee, 2011; Pazos, Alonso, Sánchez, & Medina, 2008). However, fish oils containing highly unsaturated fatty acids with 4, 5 and 6 double bonds, and this results in oils that are highly susceptible to oxidation in the presence of light, oxygen, transition metals and enzymes. Oxidation influences the shelf-life of FO supplements and FO enriched foods. During oxidation of fish oil, fishy and rancid off flavors are developed. There is also a loss of nutrients and toxic compounds may be formed from the decomposition of hydroperoxides (Frankel, 1998; Indrasena & Barrow, 2010).

Preventing oxidative deterioration in foods has been a significant challenge for the food industry. Natural antioxidants are widely incorporated into foods and oils to prevent oxidation, improving stability and shelf life. These natural antioxidants are used due to consumer preferences for clean food labels. Herbs and other spices, especially rosemary

extracts, have become popular additives to improve stability and extend shelf life because of their demonstrated ability to retard oxidation (Keokamnerd, Acton, Han, & Dawson, 2008; Yanishlieva, Marinova, & Pokorný, 2006).

Antioxidants can be classified based on their mode of action. Primary antioxidants donate hydrogen to scavenge alkoxy and preoxy radicals and form poorly-reactive (stable) antioxidant radicals; this breaks the propagateion cycle. Secondary antioxidants, such as metal chelating agents, singlet oxygen quenchers, oxygen scavengers, and antioxidant regenerators prevent the formation of volatile and toxic compounds and convert hydroperoxides to more stable radicals and to non-radical products (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010; Indrasena & Barrow, 2010).

Tocopherols are commonly added to foods (Berdahl, Nahas, Barren, Decker, Elias, & McClements, 2010; Indrasena & Barrow, 2010). However, use of a single antioxidant is less effective FO compared to combinations of antioxidants (Drusch, Groß, & Schwarz, 2008; Hamilton, Kalu, Prisk, Padley, & Pierce, 1997). Tocopherols can have synergistic antioxidant properties when they were added with other antioxidants in fish oil (Indrasena & Barrow, 2010). As a result of the good synergistic antioxidant properties of tocopherols, greater antioxidant effectiveness in fish oil was observed when primary antioxidants and secondary antioxidants were formulated with tocopherols (Drusch, Groß, & Schwarz, 2008; Mette, Jacobsen, & Meyer, 2007; Yi, Andersen, & Skibsted, 2011).

The purpose of this study was to investigate the activity of novel natural antioxidants in menhaden oil and to develop optimum formulations containing mixed

tocopherols to control oxidation of menhaden oil. The specific objectives are: 1) determine Vitamin E content in menhaden oil using simplified extraction and HPLC analysis, 2) determine natural antioxidant concentrations that optimally inhibit oxidation of menhaden oil containing mixed tocopherols, 3) evaluate oxidative stability of menhaden oil containing mixed tocopherols and mixtures of Teng Cha extract and rosemary extract, 4) evaluate oxidative stability of menhaden oil containing mixed tocopherols formulation by blending natural antioxidant combinations of ascorbyl palmitate, citric acid, Teng Cha extract and rosemary extract. Oxidation was determined by measuring primary and secondary oxidation products after storage at 40℃ for eight days. Initial studies were conducted to quantify levels of EPA/DHA and other n-3 PUFAs in menhaden oil using GC-MS. Total phenolic contents of extracts of Teng Cha, green tea and grape seeds were determined using the Folin phenol total phenolic assay. The DHM content of the Teng Cha extract was measured using HPLC.

MATERIALS

Fatty Acid Analysis

HPLC grade isooctane and toluene were purchased from Fisher Scientific (St. Louis, MO). BF_3-Methanol (12%) was purchased from Supelco (Bellefonte, PA). Tricosanoic acid-methyl ester was obtained from Crescent Chemical Company (Islandia, NY). Sodium hydroxide and sodium chloride were purchased from Fischer Scientific. Menhaden fish oil (OmegaPure®) containing natural mixed tocopherols (Covi-ox® T-50) was donated by Omega Protein (Reedville, VA).

Antioxidant Extraction and Analysis

Replicate Teng Cha samples were obtained from a retail tea store in Zhangjiajie, China. Dihydromyrectin (DHM) was purchased from ChromaDex (Irvine, CA). Japanese green tea (Bancha) and grape seed extract were purchased from local natural food stores. Ethanol (95.5% aq) was obtained from Ricca Chemical Company (Arlington, TX). Acetic acid, acetonitrile and methanol were supplied by Fisher Scientific. Gallic acid and 2 N Folin-Ciocalteau's reagent was obtained by Sigma-Aldrich (St. Louis, MO).

Vitamin E Analysis

HPLC grade n-hexane and isopropanol were obtained from Fisher Scientific. Polysorbate 20 (Tween 20), butylated hydroxyanisole (BHA), d,l-α-tocopherol, d,l-γ-tocopherol, d,l-δ-tocopherol and anhydrous magnesium sulfate were obtained from Sigma Aldrich (St. Louis, MO). BHA (50ppm, Fisher Scientific) was added and dissolved in n-hexane to prepare the hexane/BHA solution.

Antioxidant Activity Evaluation

Ascorbyl palmitate was obtained from Spectrum Chemical (Gardena, CA). Quercetin dehydrate (97%) was purchased from Alfa Aesar (Heysham, England). Citric acid, anhydrous, was obtained from Archer Daniels Midland Co. (Decatur, IL). Rosemary extract oil (Herbalox Seasoning, Type HT-25, NS) was obtained from Kalsec (Kalamazoo, MI). Green tea extract was prepared from Japanese green tea (Bancha), grape seed extract was obtained from a commercial preparation, and Teng Cha extract were prepared from Teng Cha tea (Gokoglu & Yerlikaya, 2008; Ye, 2011). Mixed

tocopherols was obtained from Sigma Aldrich. Menhaden oil containing natural mixed tocopherols from Covi-ox® T-50 was donated by Omega Protein (Reedville, VA). Starch indicator (1%) was supplied from Ricca Chemical Company (Arlington, TX). Acetic acid, chloroform, and isooctane of HPLC grade, 0.1 N sodium thiosulfate, and p-anisidine were purchased from Sigma Aldrich (St. Louis, MO). Activated charcoal was purchased from Bio-Rad Laboratories (Richmond, CA).

METHODS

Fatty Acid Analysis

The fatty acid composition of menhaden oil was determined by using the AOCS official method Ce1b-89 (AOCS, 1999) Briefly, the internal standard tricosanoic acid (C23:0) methyl ester was dissolved in toluene (1.0mg/ml). Menhaden oil (25mg±0.1mg) was weighed into a 10 ml Teflon-lined screw cap test tube followed by addition of internal standard (1ml). After addition of 1.5ml of 0.5 N NaOH in methanol, the test tube was flushed by nitrogen, capped tightly and then heated on a boiling water bath for 5 minutes. This step transesterifies the glycerides or partial glycerides to methyl esters. After the tubes were cooled, 12% BF_3 in methanol (2ml) was added, the tubes flushed with nitrogen, capped tightly and then heated on boiling water at 100 °C for 30 minutes. This step creates methyl esters from free fatty acids, which would not be esterified using just the NaOH catalyst. The sample was cooled and then isooctane (1ml) added for extraction of the fatty acid methyl esters (FAME). The sample was mixed vigorously on the vortex for 30 seconds and capped tightly after flushing with nitrogen. A saturated NaCl solution (5 ml) was added to the sample, tubes were nitrogen flushed, and tubes

vortexed. The top isooctane layer was transferred to a clean test tube. The lower layer was extracted a second time with 1ml isooctane. The isooctane extracts was combined in the clean test tube and evaporated using a stream of nitrogen gas to approximately 1 ml. Samples were manually injected (1 µl) into a Shimadzu QP5050GCMS (Kyoto, Japan) equipped with a Zebron ZB-WAX plus capillary column (60m x 0.25mm i.d., 0.25µm film thickness, Phenomenex, Torrance CA) with a split ratio of 1:77. Ultra-pure helium was used as a carrier gas at 30cm/sec linear flow velocity (1.3ml/min column flow). The oven temperature program started at 135℃, was increased to 235℃ by 1℃/min, and was held at 235 ℃ for 20 minutes. The total run time was 120 minutes. Injector temperature was 250℃ and GCMS interface temperature at 230℃. FAMEs were identified by using MS fragmentation patterns and a Wiley 2002 library, and identifications were confirmed by calculation of equivalent chain lengths (ECL) as described by Ackman (1969) and data compared to published values (Schulte 1993). The amounts of EPA and DHA were calculated as mg per gram of menhaden oil. Area percent of the FAME were also calculated. Experiments were conducted in duplicate.

Extraction and Analysis

Teng Cha extract, green tea extract, and grape seed extract were prepared for natural antioxidants' inhibitory activities on lipid oxidation of the menhaden oil. Total phenolic values of each crude extract sample were evaluated using the Folin Phenol method with gallic acid standard solutions using the method described by Spanos and Wrolstad (1990). The phenolic content of the Teng Cha extract sample was compared to commercial DHM. DHM content in Teng Cha extract sample was quantified using

HPLC methods with DHM standard solution (Ye, 2011).

Extraction and Freeze Drying

The extraction and lyophilization of Teng Cha and green tea extracts followed the method described by (Ye, 2011) The commercial grape seed extract was purified using the extraction procedure described by Gokoglu & Yerlikaya (2008).

Teng Cha

Teng Cha (10g) was extracted in 200 ml of 74 % (v/v) aqueous ethanol with shaking at 65 ℃ for 30 minutes at 100 rpm in a water bath (360 Orbital Shaker, Precision Scientific, Chicago, IL). A stomacher bag was used for filtering the extracts to 1000 ml round bottom flasks. For evaporating ethanol in the extract, a Buchi Rotavapor R-3000 (Buchi Laboratory Equipment, Flawil, Switzerland) was operated at 60 ℃ until little ethanol remained in the flask. The extracts in the round bottom flask were transferred to a crystallizing dish (70mm x 50mm), and 50 ml of distilled water was added to the round bottom flask, rinsing remaining extracts. This was combined with the first extract in the crystallizing dish. The extract in the crystallizing dish was covered with parafilm to prevent contamination, the extract was frozen completely at - 50 ℃, and then freeze dried at - 50 ℃ for about 72 hours in a Labconco Freezone18 Freeze Dryer (Labconco Co., Kansas City, MO) with cheesecloth on the top of the crystallizing dishes. The freeze dried extracts were weighed on analytical balance scale and stored at - 50 ℃. Triplicate Teng Cha extracts were prepared.

Bancha Green Tea Extract

Japanese Bancha green tea (10g) was extracted with 200 ml of 70% aqueous

ethanol following the extraction procedure described for Teng Cha (Ye, 2011).

Grape Seed Extract

Commercial grape seed extract (10g) was dissolved completely in 200 ml of 20% (v/v) aqueous ethanol and extracted for four hours by shaking at 40℃ at 160 rpm in a Precision Scientific 360 Orbital Shaker (Precision Scientific Inc., Chicago, IL). The extracts were filtered through Whatman no. 4 paper and transferred to a 1000 ml round bottom flask. The filtered grape seed extract was concentrated until most of ethanol was removed at 60℃ by using a Buchi Rotavapor R-3000 (Buchi Laboratory Equipment, Flawil, Switzerland). The concentrated extract was transferred into a crystallizing dish (70mm x 50mm) and 50 ml of distilled water added to wash residual extracts in the round bottom flask. The extract in the crystalizing dish was frozen at -50 ℃ then freeze dried at -50 ℃ for 72 hours with cheesecloth cover in a Labconco Freezone 18 Freeze Dryer (Labconco Co., Kansas City, MO). The dried extract was weighed on analytical balance and kept frozen at -50 ℃ until use. The grape seed extracts were prepared in triplicate.

DHM Quantification in the Teng Cha Extract by HPLC Analysis

DHM in the Teng Cha extract was quantified by using an Agilent 1260 Infinity Series HPLC (Agilent Technologies, Richmond, VA). An Agilent Porshell 120 EC-C18 column with 2.7 μm particle size (5 cm x 0.46 cm) was operated at ambient temperature. The mobile phase consisted of solvent (A) 90% distilled water containing 0.1% v/v acetic acid and solvent (B) 10% acetonitrile containing 0.1% acetic acid (v/v). The flow rate was 0.5 mL/min, the injection volume was 5.0 μL. The detection wavelength was 290nm and absorbance was recorded from 190-400 nm. A mobile phase gradient was run with

90% A and 10% B from 0-4 min, a linear increase to 70% A and 30 % B from 4-8 min, for an 8 minute run time. The initial solvent conditions were run thereafter for 10 minutes to prepare for the next injection. The triplicate extract samples were prepared in methanol (1mg/ml) and injected into the HPLC. The DHM peak was identified by comparing retention times and UV absorbance spectra with authentic standard. The DHM content in the extract was determined by using an external standard procedure (0-1 mg/ml DHM in methanol).

Total Phenolic Contents

Total phenolic concentration in the Teng Cha extract, green tea extract, grape seed extract and DHM were measured by using the Folin-Ciocalteu reagent with gallic acid (0-0.5 mg/ml) as the phenolic standard (Spanos & Wrolstad, 1990). The reaction mixture contained 100µL of each extract solution (0.5mg/ml) or the gallic acid standard solutions, 900 uL distilled water and 2.5 mL of 0.2 N Folin-Ciocalteau reagent and were mixed on a Fisher Vortex Genie2 mixer (Fisher Scientific, Pittsburgh, PA). Saturated sodium carbonate (2ml) was added to each test tube and mixed on the vortex. After standing for two hours at room temperature, the absorbance was measured at 765nm using a Shimadzu UV-2550 UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). The estimated total phenolic value of each extract was recorded as milligrams of gallic acid equivalents per grams of the extract. Experiments were run in triplicate.

Vitamin E Analysis

The α-, γ- and δ- tocopherol contents of the menhaden fish oil were measured by using a normal phase HPLC analysis originally reported for Vitamin E in margarine and reduced fat products (Ye, Landen Jr, Lee, & Eitenmiller, 1998). The three tocopherols were quantified using an external standard procedure (0-0.1 mg/ml curves). Tocopherol standard solutions were prepared in 25 ml of volumetric flask containing n-hexane and BHA (0.05 mg/ml). The autosampler of the Agilent 1200 Infinity Series HPLC (Agilent Technology, Richmond, VA) was held at 4 ℃ and protected against light.

Sample Preparation, Extraction and Analysis

Menhaden oil (5.0g) was weighed into a 125 Erlenmeyer flask and 40 ml of a hexane/BHA solution was added to dissolve the menhaden oil. Aluminum foil was wrapped outside the flask in order to avoid light exposure during the extraction. The remaining oil residue inside of the flask was rinsed with the n-hexane and BHA solution. Tween 20 (3 drops) and anhydrous magnesium sulfate (3g) were added to the flask, which was flushed with nitrogen and capped tightly. The oil solution was mixed thoroughly and let stand in refrigerator at 3 ℃ for two hours. After two hours, the solution was filtered through Whatman no. 1 filter paper into a 100 ml volumetric flask. The remaining residue solution in the flask was rinsed with 10ml of the hexane/BHA solution, and the 100ml volumetric flask brought to volume. The extracts were prepared in duplicate.

The extracted samples and standard solutions used to determine tocopherol contents in the menhaden fish oil. An Agilent 1200 Infinity Series HPLC was used (Agilent Technology, Richmond, VA) with diode array and fluorescence detectors in

series. A Macherey-Nagel Si (25cm x 0.46cm i.d.) normal phase column was used at ambient temperature. The mobile phase consisted of n-hexane and isopropanol (99.1:0.9 v/v) with a flow rate at 1.0ml/min. The injection volume was 20µl, fluorimeter excitation wavelength was 290 nm and emission wavelength was 330 nm. Total run time was 20 min. The retention times and UV absorbance spectra were used to identify alpha, gamma and delta tocopherol contents in the sample.

Natural Antioxidants Activity on Lipid Oxidation of Menhaden Oil

Menhaden Oil Samples Preparation

The menhaden oil (MO) samples were prepared in each antioxidant treatment at three different concentrations in triplicate. We first focused on evaluating efficacy of each antioxidant in menhaden oil samples because some antioxidants act as prooxidants at high concentration. Twenty seven treatment samples were prepared in triplicate and stored in screw capped glass bottles in an incubator (Blue M, Blue Island, IL) at a temperature of 40℃ for eight days under darkness. Triplicate samples of each treatment were taken randomly from the incubator at each time period and evaluated for peroxide value, headspace oxygen content (primary oxidation products) and anisidine value for the secondary oxidation product.

Single Antioxidant Activity on Oxidation of Menhaden Oil (MO)

Ascorbyl Palmitate (AP)

Three different concentrations (200 ppm, 500ppm, 1000ppm) of AP in MO were prepared were in 10 ml Teflon-lined, screw cap test tubes . Methanol (0.5ml) was used to

help dissolve the AP. The methanol was removed by using a nitrogen stream and then 3g of MO was added to the test tube, the tube was flushed with nitrogen, capped and AP dispersed using vortex mixing for 1 minute. The test tube was then placed in a Fisher FS20 ultrasonic bath (Fisher Scientific, Pittsburgh, PA) for 3 minutes to assist the dissolving of AP. Each test tube containing AP was mixed on the vortex again and put back in the ultrasonic bath. These vortex and ultrasonication processes were repeated three times until AP was dissolved. The mixture of AP and MO in the test tube was transferred to 250 ml Erlenmeyer flaks, and additional amounts of MO were added to prepare 200 ppm, 500ppm, 1000ppm AP final concentration in the MO. The MO and AP samples (6g) were transferred to glass vials and capped, then stored in the incubator at 40℃ for up to eight days.

Citric Acid (CA)

Three different concentrations (50ppm, 100ppm and 150 ppm) of citric acid were prepared in MO. After 0.5 ml of methanol was used to dissolve CA, the methanol solution was removed by nitrogen stream. Similar to preparation of AP samples, about 3 g of MO was added to the test tube to dissolve citric acid in the test tube, which were capped tightly after flushing with nitrogen. The test tube was mixed using a vortex for 1 minute to disperse the citric acid. The test tubes were placed in an ultrasonic bath for about five minutes in order to help dissolve CA in MO. These steps were repeated three times until CA was dissolved. Concentrations of 50ppm, 100ppm and 150ppm CA were prepared. The samples (6g) were placed into glass vials which were capped. Replicate samples were distributed randomly in the incubator for up to eight days storage at 40℃.

Quercetin (QC), Green Tea Extract (GTEX), Grape Seed Extract (GSEX) and Teng Cha Extract (TEX)

Since solubility of QC, GTEX, GSEX and TEX in MO were better than AP and CA in MO, MO (3g) was added to test tubes containing each treatment without the dissolving process. Each antioxidant treatment in the test tubes was dissolved on the vortex for about 1 minute and put in the ultrasonic bath for three minutes. The processes of vortex and ultrasonic bath for dissolving extracts or QC were repeated until the extracts and QC were dissolved in MO. The dissolved extracts were transferred to a 250ml Erlenmeyer flask, and additional MO was added to the flask to prepare each treatment. Flasks were flushed with nitrogen and capped tight. Each flask was then put in the ultrasonic bath for 5 minutes followed by mixing on the Corning stir plate for 10 minutes. The samples (6g) were transferred to glass vials, which were capped and randomly distributed in an incubator at 40°C for up to eight day storage.

Mixed Tocopherols (MT) and Rosemary Extract Oil (RSEX)

MT or RSEX were directly dissolved in MO due to their good solubility. MT (200ppm, 500ppm and 1000ppm) and RSEX (500ppm, 1000ppm, and 2000ppm) in MO were prepared in duplicate. Each treatment (6g) was prepared in glass vials, capped and distributed randomly in an incubator for up to eight day storage at 40 °C.

Preparation of Mixtures of Teng Cha Extract (TEX) and Rosemary Extract (RSEX) in Menhaden Oil

In the evaluation of single antioxidant activity retarding oxidation process, 200ppm, 500ppm and 1000ppm TEX showed good antioxidant inhibitory activity on

lipid oxidation of menhaden oil compared to other antioxidants and control. To examine combined effects, 1000 ppm or 2000ppm RSEX were added to TEX in MO. Nine treatments were prepared in triplicate: 200ppm and 500ppm TEX prepared in 10 ml test tubes. MO (3g) was added to the test tubes, which were flushed with nitrogen. The test tubes were dissolved using an ultrasonic bath for about 3 minutes. RSEX was weighed accurately in 250 mL Erlenmeyer flasks. Accurate amounts of MO were added to the flasks containing RSEX, TEX with stirring to dissolve. The flask containing mixtures was flushed with nitrogen, then stirred vigorously on a Corning stir plate for 10 min, then placed in an ultrasonic bath for 5 min. The dissolving processes were repeated until TEX and RSEX were completely dissolved in MO of the flaks. Each treatment (6g) was transferred to glass vials and capped; samples were prepared in triplicate. They were distributed randomly and stored in the incubator set temperature 40 ℃ for eight days.

Preparation of Mixtures of Ascorbyl Palmiate (AP) and Citric Acid (CA), Rosemary Extract (RSEX) and Teng Cha Extract (TEX) in Menhaden Fish Oil (MFO)

All treatments contained AP (200 ppm) and CA (100 ppm). Two levels of RSEX (1000, 2000 ppm) and two levels of TEX (500 ppm, 1000 ppm) were prepared. All samples (6g) were transferred to glass vials in triplicate and capped. The tubes were distributed randomly in an incubator for up to eight day storage at 40 ℃.

Peroxide Value Evaluation

Peroxide values were determined by the American Oil Chemists' Society methodology (AOCS, 1998). Each sample (5 ± 0.05g) was weighed into a 250ml glass stoppered Erlenmeyer flask. Sample weight was recorded to the nearest 0.01g. Acetic

acid-chloroform (30 ml, 3:2 v/v) was added to the flaks to dissolve the oil. After a saturated potassium iodide solution (0.5 ml aq.) was added to the flask, the mixture in the flask was swirled gently for exact one minute. Distilled water (30ml) was immediately added with vigorous mixing to liberate I_2 from the chloroform layer. Starch indicator solution (1ml) was pipetted to the mixture. Titration was performed using 0.01 N sodium thiosulfate until the blue-gray color in the mixture solution disappeared. The volume of 0.01N sodium thiosulfate volume (ml) used was recorded accurately to two decimal places.

Head Space Oxygen Content

Headspace oxygen contents in MO samples were evaluated using an OxySense 4000B (OxySense, Dallas, TX). Fluorescent O2xyDots® were attached to the inside of glass vials by using a silicon-based translucent adhesive glue (RTV 108, Momentive Performance Materials, Albany, NY). The OxySense 4000B was calibrated by using a 0% oxygen standard containing 1% sodium sulfite and a 21% oxygen standard containing 1ml of distilled water. The samples (5g) of each treatment were added to the vials that already had the fluorescent O2xyDots® on the inside walls. Tubes were capped tightly and distributed in an incubator for up to eight days storage at 40℃. Every two days (0, 2, 4, 6, 8 days), samples were removed from the incubator in order to measure head space oxygen content. The head space oxygen measurement of each sample was performed five times and recorded directly in the excel work sheet of instrument.

Anisidine Value (AV) Evaluation

The AV primarily measures aldehydes, especially unsaturated aldehydes; aldehydes produce off-flavors in foods. The p-anisidine was recrystallized before use. p-anisidine (20g) was dissolved in 500ml of distilled water (75℃) in a1L beaker containing stirring bar, sodium sulfite (1g) and activated carbon (10g) were added with stirring for five minutes. The solution was filtered through Whatman no. 1 filter paper twice. The filtrated solution was stored in the refrigerator 4℃ overnight. The white crystallized p-anisidine was filtered using vacuum filtration and washed with 10ml of cold distilled water. After the crystallized p-anisidine was completely dry, it was transferred to the glass vials and stored under darkness in a refrigerator at 4 ℃. Each MO sample (0.5-0.8g) was weighed in 50ml centrifuge tubes. Isooctane (25ml) was added to dissolve and dilute the oil sample. Isooctane with reagent was used to zero the spectrophotometer. The absorbance of each sample (Ab) was measured at 350nm using a Shimadzu UV-2550 UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). Samples (5ml) and isooctane were added to 1ml of the p-anisidine solution (2.5g/L), which was prepared with acetic acid(IUPAC, 1987). The tubes were capped tight and swirled. After let them stand at room temperature for 10 min, absorbances (As) were measured at 350nm using isooctane blank and and p-anisidine mixture as a reference. The p-anisidine values of each sample were determined by the formula: p-AV = 25 * (1.2 As - Ab)/m where As was the absorbance of the reacted solution, Ab is the absorbance of the diluted menhaden oil sample, m is the weight of the menhaden oil sample (g) (IUPAC, 1987).

STATISTICAL ANALYSIS

The means (n=3) of total phenolic contents (mg GAE/g) and treatments' peroxide values (meq/kg), head space oxygen content (%) and anisidine at 0,2,4,6,8 day were analyzed by one way analysis of variance. The mean values were determined significant differences at $p<0.05$ by using Tukey's test in JMP (Version 9.0.0, 2010, JMP, Cary, NC) for total phenolic contents of the extract treatments and the peroxide value, headspace oxygen contents and anisidine values in SAS (Version 9.1.3, 2003, SAS, Cary, NC).

RESULTS AND DISCUSSION

Fatty Acid Analysis

Concentrations of fatty acids in menhaden oil were determined using an internal standard, tricosanoic acid (C23:0) methyl ester and as area %. Peak areas higher than 1 % were 14:0, 16:0, and 16:1n-7, 18:0, 18:1-9, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3. (Table 1). Moffat & McGill (1993) reported that marine oils contain eight major fatty acids: tetradecanoic acid (14:0, myristic acid), hexadecanoic acid (16:0, palmitic acid), cis-9-hexadecenoic acid (16:1n-7, palmitoleic acid), cis-9-octadecenoic acid (18:1n-9, oleic acid), cis-9-eicosenoic acid (20:1n-11), cis-11-docosenoic acid (22:1n-11), cis-5,8,11,14,17-eicosapentaenoic acid (20:5n-3, EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (22:6n-3, DHA) (Moffat & McGill, 1993). Weight percentages of the fatty acids based on the internal standard showed similar fatty acid profiles to the OmegaPure® E reference data and Moffat & McGill (1993). In most fish oils, the percentage of EPA is usually higher than DHA, but there is an exception with mackerel oil which contains greater amounts of DHA than EPA (McGill & Moffat, 1992; Rossell,

2009)

The typical summed weight percent of EPA and DHA in marine fish fatty acids is around 12 and 34 %, depending fish types, seasons, sex and geographical locations (Hardy & Keay, 1972). The amount of EPA and DHA was 73.4mg/g and 66.8mg/g, respectively (Table 2). The area percent of EPA+DHA (%) was 24.6 %, which was within the range (23-27%) of OmegaPure® E reference data as well as McGill & Moffat (1992). EPA and DHA are important PUFAs and provide protection against cardiovascular diseases and other health concerns (Kris-Etherton, Harris, & Appel, 2003; Wang, Harris, Chung, Lichtenstein, Balk, Kupelnick, et al., 2006).

Extraction and Analysis

Teng Cha extract (3.78g), green tea (3.24g) and grape seed extract (4.20g) were obtained by the extraction and freeze drying procedure (means, n=3). The DHM in Teng Cha tea was 26.2% w/w (dry basis). The Teng Cha extract contained 69.3 % w/w DHM (mean, n=3). This was similar to the DHM content (64.7% w/w, means, n=3) reported by Ye (2011).

Total Phenolic Content (TPC) Determination

Since total phenolic content contributes to overall antioxidant capacity, the total phenolic content of each extract was determined by using a Folin-phenol assay (Spanos and Wrolstad, 1990). The phenolic content means (n=3) were Teng Cha extract (310 mg GAE/ g), commercial DHM (370mg GAE/ g), green tea extract (180 mg GAE/g) and grape seed extract (380 mg GAE/g). The grape seed extract had the highest TPC among the samples. In Table 3, the TPC of the samples were significantly different by using

Tukey's test ($p<0.05$) (Table 3).

Grape seeds, skins and grape pulps have different polyphenol composition depending on the extraction method and use solvents. Use of ethanol as an extraction solvent resulted in a high TPC (Nawaz, Shi, Mittal, & Kakuda, 2006). Dihydromyrectin (DHM) is the major bioactive compound in Teng Cha extract and was reported to have good antioxidant capacity (Reische, Lillard, & Eitenmiller, 2008). The total phenolic content (324 mg GAE /g) reported by Ye (2011) was in agreement with our finding (310mg GAE/g).

The green tea extract was found to have the lowest total phenolic content among the extracts, 180mg GAE/g. The phenolic concentration of the green tea extract expressed higher than 65.8-106.2mg GAE/ g from Khojhar and Magnusdottir (2002) and varied depends on extraction and fraction methods and growing regions.

Vitamin E Analysis

The extraction method with hexane and direct HPLC injection (Ye, Landen Jr, Lee, & Eitenmiller, 1998) was an efficient method to determine tocopherols contents in menhaden oil because the method avoided saponification processes, which often result in low recoveries and tocopherols. Menhaden oil from OmegaPure® is formulated with Covi-ox® T-50, which is natural mixed tocopherols derived from edible vegetable oil. Approximate 1000ppm of mixed tocopherols was added to the menhaden oil for preventing oxidation during processing, handling and shelf-life provided by Cognis Corporation (Cognis., 2006). The typical mixed tocopherols in soybean oil are composed of 14 % of α- tocopherol, 2 % ß- tocopherol, 60% γ- tocopherol, and 24 % δ-tocopherol,

from the Covi-ox® T-50 reference (Cognis., 2006). Alpha tocopherol, gamma tocophol, and delta tocopherol were found at 0.18mg/g, 0.37mg/g, and 0.14mg/g, respectively, on average of duplicate samples by using the external standard procedures (Table 4.). The direct extraction and HPLC analysis used for tocopherol analysis was simple and rapid, although it did require the use of a fluorimeter (Ye, Landen Jr, Lee, & Eitenmiller, 1998). Fluorescence detection for tocopherol analysis provides more sensitivity and specificity compared to UV detection (Meyer, 2010).

Single Antioxidant Activity on Oxidation of Menhaden Oil (MO)

Head Space Oxygen Content

Hydroperoxides of primary oxidation products were evaluated by head space oxygen content. Oxygen levels of MO sample decreased as more oxygen was removed from the headspace through oxidative degradation in the samples. The headspace oxygen contents of each MO sample were determined by day 0, day 2, day 4, day 6 and day 8 (Table 5.1-5.8). At 0 day, headspace oxygen levels of the samples were ~20 %, but oxygen levels of control, AP (200, 500, 1000 ppm), CA (50, 100, 150 ppm), GSEX (200, 500, 1000 ppm), GTEX (500, 1000, 2000 ppm), MT (200, 500, 1000 ppm), QT (200, 500, 1000 ppm) and RSEX (500 and 1000 ppm) were dramatically decreased at 40 ℃ storage for eight days. Although the oxygen contents of 200 ppm, 500 ppm and 1000 ppm of GSEX and TEX, 500, 1000, 2000 ppm of GTEX, 2000 ppm RSEX for four days storage at 40℃ were between 16.3 % and 18.1%, and 200 ppm, 500 ppm and 1000 ppm of TEX and 2000 ppm RSEX were 17.4%, 16.7%, 16.8 % and 15.9 %, respectively, the oxygen levels of other treatments were below 11% for six days storage at 40 ℃. Oxygen levels

of 200 ppm, 500 ppm, 1000 ppm TEX and 2000 ppm RSEX in MO samples were 11.8%, 13.2 %, 14.4 % and 10.4 %, respectively, which contained higher oxygen levels than other treatments at 40°C storage after eight days. 1000 ppm TEX sample had the highest oxygen content, followed by 500 ppm TEX, 200 ppm TEX and 2000 ppm RSEX. The TEX treatments inhibited the formation of primary oxygen products of MFO. These results were in agreement with antioxidant inhibitory effects of DHM and 200 ppm TEX on oxidation of soybean oil at 60° storage for 15 days (Ye, 2011).

Anisidine Values (AV)

Aldehyde levels of the MO samples were evaluated for nonvolatile secondary oxidation products (Table 6.1-6.8). Samples with 200 ppm, 500 ppm and 1000 ppm AP and 200 ppm, 500 ppm and 1000 ppm QC had higher AV than the control. In 200 ppm, 500 ppm and 1000 ppm AP and 200 ppm, 500 ppm and 1000 ppm QC of samples, the head space oxygen contents were low. This shows samples they did not effectively inhibit primary oxidation products and may have acted as prooxidant in MO (Hamilton, Kalu, McNeill, Padley, & Pierce, 1998). TEX, GSEX, GSEX and MT had lower AV than control at eight days. It is important to measure both primary and secondary oxidation products for effectiveness antioxidants in lipid system (Decker, Warner, Richards, & Shahidi, 2005). In some cases, secondary oxidation products measured by AV can be low while primary oxidation products are increased. For example, peroxide values may be high in the presence of tocopherols because tocopherols donate hydrogen to peroxy radicals to form stable peroxides whereas secondary oxidation products maybe formed at low rates in processes of tocopherols (Decker, Warner, Richards, & Shahidi, 2005).

Looking at measurements of both primary and secondary oxidation products, 200 ppm, 500 ppm, and 1000 ppm TEX significantly improved oxidative stability and delayed oxidation of MO at 40 ℃ storage for eight days since the TEX treatments had much higher oxygen contents and lower AV than control and other antioxidant treatments.

Mixtures of Teng Cha Extract (TEX) and Rosemary Extract (RSEX)

Peroxide Value Test

The treatments containing TEX inhibited oxidative degradation of MO at 40℃ storage during eight days (Table 7.1). Mixtures of TEX and RSEX resulted in much lower peroxide values than TEX or RSEX alone. On eight day, peroxide value of control and 1000 ppm RSEX were dramatically increased and exceeded 35 meq/kg, whereas peroxide value of combinations in 500 ppm TEX and 2000 ppm treatment sample (10.7 meq/kg) had the lowest peroxide value in all samples at 40℃ storage during eight days. 200 ppm TEX and 2000 ppm RSEX showed slightly less effective protection against formation of primary oxidation products. The peroxide values of 200 ppm and 500 ppm TEX samples with addition of same concentration of RSEX were not significantly different, but mixture of TEX and RSEX samples with addition of different levels of RSEX concentrations (1000 ppm or 2000 ppm) were significant different in rate of primary oxidation product formation ($p<0.05$). Thus, RSEX can possibly provide additive antioxidant inhibitory effects which assisted in delaying oxidative degradation of MO more efficiently in combination with TEX.

Head Space Oxygen Content

Measuring primary oxidation products of the samples by head space oxygen content test showed similar results as peroxide values. During eight day storage at 40°C, headspace oxygen contents differed significantly ($p<0.05$) (Table 7.2). Mixture of 500 ppm TEX and 2000 ppm RSEX (14.5 %) had highest oxygen percentage followed by mixtures of 500 ppm TEX and 1000 ppm RSEX (14.2 %). The treatments containing TEX had much higher oxygen contents than control or RSEX alone. In combination of TEX and RSEX in MO samples, higher oxygen levels were observed than for TEX alone. RSEX assisted in protection against oxidation, since addition of RSEX increased effectiveness of TEX.

Anisidine Value Test (AV)

The secondary oxidation products which include aldehydes causing rancid food flavors and odors were measured by the p-anisidine test. The AVs of the samples agreed with peroxide values and headspace oxygen content evaluations. The mixture of 500 ppm TEX and 2000 ppm RSEX produced lowest AV (11.2) at 40°C storage for eight days. Combined 500 ppm TEX and RSEX (1000 ppm or 2000 ppm) were significantly better than TEX alone in MO samples. 1000 ppm and 2000 ppm RSEX had improved stability in the mixture formulations. However, the AV of 1000 ppm RSEX alone (24.6) was higher than the control (21.2). Once RSEX, which was stabilized in vegetable oil, started degrading, the rate of oxidative degradation was increased because the effectiveness of RSEX was lost. The results of AV agreed with peroxide value and headspace oxygen contents, since the most effective antioxidant formulation in MO was mixture of 500 ppm TEX and 2000 ppm RSEX with the lowest AV(11.2), the lowest peroxide value (10.7

meq/kg) and highest headspace oxygen content (14.5%) (Table 7.3).

Adding Ascorbyl Palmitate (AP) and Citric Acid (CA) with Mixtures of Teng Cha (TEX) and Rosemary Extract (RSEX)

Peroxide Value

Peroxide values of each mixture treatment and control were determined at 0 day, 2 day, 4 day, 6 day and 8 day at 40℃ storage (Table 8.1). Control (35 meq/kg) and 200 ppm AP + 100 ppm CA (35.3 meq/kg) was had the highest peroxide values among the samples. Like previous peroxide value results, treatments containing TEX had lowest peroxide values. The mixtures of 200 ppm AP + 100 ppm CA + 2000 ppm RSEX and 1000 ppm or 500 ppm had TEX strongly inhibited formation of primary oxidation products.

Head Space Oxygen Content

Oxygen contents of the samples were determined at 0 day, 2 day, 4 day, 6 day and 8 day during storage at 40℃ (Table 8.2). Lower oxygen values of the samples resulted from oxidation, development of hydroperoxides, as more oxygen was absorbed by the oil samples. Oxygen contents of control and 200 ppm AP + 100 ppm CA treatment in MO were significantly decreased whereas mixtures of 200 ppm AP+ 100 ppm CA + 2000 ppm RSEX + 1000 ppm TEX or 500 ppm TEX had the highest oxygen contents after storage at 40℃ for eight days. The head space oxygen content evaluation carried out similar results with the peroxide value determination. The treatment containing 1000 ppm TEX inhibited on formation of primary oxidation products effectively. AP+CA+RSEX

decreased delay oxidation of MO more effectively than TEX alone.

Anisidine Value

Anisidine value can use an indication of secondary oxidation products that provide undesirable odors after decomposition of the hydroperoxides (O'Sullivan, Mayr, Shaw, Murphy, & Kerry, 2005). The AV values of control and 200 ppm AP + 100 ppm CA were steadily increased and had the highest anisidine values among the samples at 40 ℃ storage after eight days. However, the mixture of 200 ppm AP+ 100 ppm CA + 2000 ppm RSEX + 1000 ppm TEX or 500 ppm TEX has much lower AV than control and 200 ppm AP + 100 ppm CA during eight days storage at 40℃. Combinations of 200 ppm AP, 100 ppm CA, TEX (500 and 1000 ppm) and RSEX (1000 ppm and 2000 ppm) inhibited on oxidation and improved stability of MO containing mixed tocopherols at 40℃ storage for eight days (Table 8.3).

Drusch (2008) reported mixtures of mixed tocopherols (γ-, or δ tocopherol and low concentration of α-tocopherol), AP, RSEX and CA efficiently improve stability of bulk fish oil rich in PUFAs at ambient temperature (Drusch, Groß, & Schwarz, 2008). Because AP helped regenerating tocopherols in the fish oil formulation during the storage test, the stability of fish oil was improved by AP addition (Han, Yi, & Shin, 1991; Kul & Ackman, 2001). Especially, AP protected against α -tocopherol degradation during storage (Yi, Andersen, & Skibsted, 2011). However, ascorbic acid or AP could be changed to a prooxidant if transition metals were present in fish oil (Indrasena & Barrow, 2010). Addition of CA as metal chelating agent helped increase stability for the fish oil formulation containing tocopherols, RSEX and AP by reduction of primary oxidation

products, and sunflower oil blending with RSEX, α-tocopherol and AP delayed oxidative deterioration as well (Drusch, Groß, & Schwarz, 2008; Yi, Andersen, & Skibsted, 2011). Although AP and CA possibly helped delay oxidative degradation of MO mixture formulations, the stability of the MO formulations was not improved by addition of AP and CA in our study.

CONCLUSION

In twenty seven natural antioxidants treatments, 200 ppm, 500 ppm and 1000 ppm Teng Cha (TEX) effectively delayed oxidation of MO compared to control at 40℃ storage for eight days. The combination of TEX and RSEX more effectively improved stability of MO containing mixed tocopherols at 40℃ storage for eight days, and RSEX treatments increased effectiveness of TEX antioxidant effectiveness. The mixture of 500 ppm TEX and 2000 ppm RSEX was the most effective combination examined on lipid oxidation of the MO during the storage at 40℃. RSEX had an additive antioxidant effect in the mixed formulations as measured by peroxide value, headspace oxygen and anisidine value, since the combination of TEX and RSEX inhibited on oxidation more than TEX itself. Meanwhile, 1000 ppm RSEX alone in the MO did not improve stability of MO against oxidation.

From this study, TEX can be used as a potential natural antioxidant in food industry, especially in combination with RSEX and tocopherols, extending shelf life of menhaden oil.

Table 1. Fatty acid composition of and area percent of menhaden fish oil fatty acids methyl ester higher than 1% using internal standard tricosanoic acid (C 23:0)

Fatty Acids	Area % mean ± sd
Myristic,methyl ester C14:0	9.40 ± 0.05
Palmitic, methyl ester C16:0	22.4 ± 0.26
Palmitoleic,methyl ester C16:1 n-7	13.2 ± 0.14
Stearic, methyl ester C18:0	3.95 ± 0.32
Oleic, methyl ester C18:1 n-9	6.76 ± 0.23
Linoleic, methyl ester C18:2 n-6	1.46 ± 0.04
Alpha linolenic, methyl ester C18:3 n-3	1.12 ± 0.03
Arachidonic, methyl ester C20:4 n-6	2.52 ± 0.13
Eicosaoentaenoic, methyl ester C20:5 n-3	12.7 ± 0.38
Docasapentaenoic methyl ester C22:5 n-3	2.27 ± 0.21
Docosahexanoic, methyl ester C22:6 n-3	11.9 ± 0.30

Area % was reported as mean, standard deviation (sd), and n=2 for the fatty acid composition of menhaden fish oil methyl ester

Table 2. Quantification of mg of EPA and DHA per g of menhaden oil sample using formula Ce1b-89 (AOCS 1999)

Weight of EPA and DHA (mg/g)	
EPA	73.5± 2.63
DHA	66.8± 0.62

Weights of EPA and DHA was indicated as mean, standard deviation (sd) and n=2

Table 3. Total phenolic content of DHM, GREX, GTEX and TEX (gallic acid equivalents mg/g) and means of treatments by different letters indicates significantly different by using Tukey-analysis ($p<0.05$)

Treatments	GAE (mg/g) mean ± sd
GREX	380 ± 0.0024^{a}
DHM	370 ± 0.0027^{b}
TEX	310 ± 0.0036^{c}
GTEX	180 ± 0.0044^{d}

Total phenolic contents of GREX, DHM, TEX and GTEX (GAE mg/g)
were indicated as mean, ± standard deviation(sd) and n=3.

Table 4. Alpha, gamma and delta tocopherol contents of menhaden oil was determined by external standard procedure using HPLC analysis (n=2)

Alpha Tocopherol (mg/g)	Gamma Tocopherol (mg/g)	Delta Tocopherol (mg/g)
0.18± 0.0014	0.37± 0.0028	0.14±0.0008

The amounts of alpha, gamma and delta tocopherol of menhaden fish oil were reported as mean, ± standard deviation (sd) and n=2

Table 5.1 Head space oxygen content (%) of control, 200 ppm AP, 500 ppm AP and 1000 ppm AP were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.26^{a}	17.53^{b}	13.85^{a}	7.91^{d}	4.78^{d}
200 AP	20.46^{a}	18.34^{a}	13.64^{b}	8.64^{a}	6.34^{a}
500 AP	20.26^{a}	18.36^{a}	12.86^{c}	8.51^{b}	6.37^{b}
1000 AP	20.53^{a}	17.49^{b}	12.03^{d}	8.39^{c}	6.29^{c}

$Means^{abcd}$ (n=3) of control, 200 ppm AP, 500 ppm AP and 1000 ppm AP by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.2 Head space oxygen content (%) of control, 50 ppm CA, 100 ppm CA and 150 ppm CA were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.26^{a}	17.5^{b}	13.8^{a}	7.9^{c}	4.78^{d}
50 CA	20.41^{a}	18.3^{a}	13.5^{b}	8.9^{b}	5.3^{c}
100 CA	20.27^{a}	18.6^{a}	13.8^{a}	9^{a}	5.6^{b}
150 CA	20.2^{a}	18.4^{a}	13.7^{a}	9^{a}	5.8^{a}

$Means^{abcd}$ (n=3) of control, 50 ppm CA, 100 ppm CA and 150 ppm CA by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.3 Head space oxygen content (%) of control, 200 ppm TEX, 500 ppm TEX and 1000 ppm TEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.2^{b}	17.5^{b}	13.8^{d}	7.9^{c}	4.8^{d}
200 TEX	20.4^{ab}	18.8^{a}	17.7^{a}	17.4^{a}	11.8^{c}
500 TEX	20.3^{ab}	18.8^{a}	16.5^{c}	16.7^{b}	13.2^{b}
1000 TEX	20.6^{a}	18.1^{a}	16.9^{b}	16.8^{b}	14.4^{a}

$Means^{abcd}$ (n=3) of control, 200 ppm TEX, 500 ppm TEX and 1000 ppm TEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.4 Head space oxygen content (%) of control, 200 ppm GSEX, 500 ppm GSEX and 1000 ppm GSEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.3^{a}	17.5^{c}	13.8^{d}	7.9^{c}	4.8^{c}
200 GSEX	20.4^{a}	19.3^{ab}	17.7^{a}	9.2^{a}	6.3^{a}
500 GSEX	20.2^{a}	18.9^{b}	16.6^{c}	8.9^{ab}	6.1^{b}
1000 GSEX	20.4^{a}	19.4^{a}	17.3^{b}	8.9^{b}	6.1^{b}

$Means^{abcd}$ (n=3) of control, 200 ppm GSEX, 500 ppm GSEX and 1000 ppm GSEX by different letter are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.5 Head space oxygen content (%) of control, 500 ppm GTEX, 1000 ppm GTEX and 2000 ppm GTEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.3^{a}	17.5^{c}	13.8^{d}	7.9^{d}	4.8^{d}
500 GTEX	20^{b}	18.7^{b}	16.3^{c}	8.9^{c}	5.8^{c}
1000 GTEX	20.1^{ab}	18.8^{b}	17.3^{b}	10.7^{b}	7.3^{b}
2000 GTEX	20.2^{ab}	19.4^{a}	18.1^{a}	11.8^{a}	7.8^{a}

Means^{abcd} (n=3) of control, 500 ppm GTEX, 1000 ppm GTEX and 2000 ppm GTEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.6 Head space oxygen content (%) of control, 200 ppm MT, 500 ppm MT and 1000ppm MT were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.3^{a}	17.5^{b}	13.8^{c}	7.9^{d}	4.8^{d}
200 MT	20.3^{a}	18.5^{a}	15.8^{a}	9.3^{c}	6.4^{c}
500 MT	20.4^{a}	18.5^{a}	15.3^{b}	9.5^{b}	6.5^{b}
1000 MT	20.4^{a}	18.4^{a}	15.7^{a}	10^{a}	6.8^{a}

Means^{abcd} (n=3) of control, 200 ppm MT, 500 ppm MT and 1000 ppm MT by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.7 Head space oxygen content (%) of control, 200 ppm QT, 500 ppm QT and 1000 ppm QT were measured after storage at 40°C at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.3^{b}	17.5^{b}	13.8^{a}	7.9^{c}	4.8^{b}
200 QT	20.5^{a}	18.3^{a}	13^{c}	7.9^{c}	5.5^{c}
500 QT	20.4^{ab}	18.5^{a}	13.4^{b}	8.5^{b}	5.9^{a}
1000 QT	20.4^{ab}	16.8^{c}	13.4^{b}	8.6^{a}	5.9^{a}

Means^{abcd} (n=3) of control, 200 QT ppm, 500 QT ppm and 1000 QT ppm by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 5.8 Head space oxygen content (%) of control, 500 ppm RSEX, 1000 ppm RSEX and 2000 ppm RSEX were measured after storage at 40°C at 0, 2,4,6,8 days.

Treatment	headspace oxygen%				
	0 day	2 day	4 day	6 day	8 day
Control	20.3^{a}	17.5^{c}	13.8^{c}	7.9^{d}	4.8^{d}
500 RSEX	20.2^{a}	18.7^{ab}	13.4^{d}	9.7^{c}	5.7^{c}
1000 RSEX	20.2^{a}	18.8^{a}	15.5^{b}	12.7^{b}	7.7^{b}
2000 RSEX	20.3^{a}	18.4^{b}	18.1^{a}	15.9^{a}	10.4^{a}

Means^{abcd} (n=3) of control, 500 ppm RSEX, 1000 ppm RSEX and 2000 ppm RSEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.1 Anisidine values of control, 200 ppm AP, 500 ppm AP and 1000 ppm AP were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.23^{c}	8.58^{c}	14.84^{a}	16.02^{a}	20.7^{b}
200 AP	9.70^{ab}	9.64^{b}	11.53^{a}	18.44^{b}	24^{a}
500 AP	10.01^{a}	9.59^{b}	11.9^{a}	20.97^{c}	23.95^{a}
1000 AP	9.37^{b}	10.34^{a}	13.22^{a}	23.09^{d}	26.44^{a}

Means^{abcd} (n=3) of control, 200 ppm AP, 500ppm AP and 1000 ppm AP by different letter are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.2 Anisidine values of control, 50 ppm CA, 100 ppm CA and 150 ppm CA were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.29^{a}	8.6^{b}	14.8^{a}	16^{a}	20.7^{a}
50 CA	8.62^{a}	8.9^{a}	9.4^{c}	12.7^{b}	16.6^{ab}
100 CA	8.82^{a}	11^{a}	11.2^{b}	12.5^{b}	16.1^{b}
150 CA	8.44^{a}	10.7^{a}	12^{b}	12.9^{b}	15.7^{b}

Means^{abcd} (n=3) of control, 50 ppm CA, 100 ppm CA and 150 ppm CA by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.3 Anisidine values of control, 200 ppm TEX, 500ppm TEX and 1000ppm TEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.3^{a}	8.6^{a}	14.8^{a}	16.1^{a}	20.7^{a}
200 TEX	4.4^{b}	6.4^{b}	6.7^{b}	8.7^{b}	9.9^{b}
500 TEX	4.4^{b}	6.9^{b}	7.4^{b}	8.3^{bc}	9^{b}
1000 TEX	3.8^{b}	6.1^{b}	7.1^{b}	7.9^{bc}	9.3^{b}

Means^{abcd} (n=3) of control, 200 ppm TEX, 500 ppm TEX and 1000 ppm TEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.4 Anisidine values of control, 200 ppm GSEX, 500 ppm GSEX and 1000ppm GSEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.3^{ab}	8.6^{a}	14.8^{a}	16^{a}	20.7^{a}
200 GSEX	7.3^{b}	7.6^{a}	8.2^{c}	9^{b}	10.5^{b}
500 GSEX	8.8^{a}	7.2^{a}	9.4^{b}	10.8^{b}	10.9^{b}
1000 GSEX	8.5^{ab}	8^{a}	9.1^{bc}	10.1^{b}	11.2^{b}

Means^{abcd} (n=3) of control, 200 ppm GSEX, 500 ppm GSEX and 1000 ppm GSEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.5 Anisidine values of control, 500ppm GTEX, 1000ppm GTEX and 2000ppm GTEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.3^{a}	8.6^{a}	14.8^{a}	16.1^{a}	20.7^{a}
500 GTEX	5.6^{ab}	8.5^{a}	5.6^{b}	8.8^{b}	9^{b}
1000 GTEX	5^{b}	6.8^{b}	5^{b}	9.9^{b}	9.7^{b}
2000 GTEX	6.1^{ab}	7.1^{b}	6.1^{b}	8.6^{b}	8.8^{b}

Means^{abcd} (n=3) of control, 500 ppm GTEX, 1000 ppm GTEX and 2000 ppm GTEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.6 Anisidine values of control, 200 ppm MT, 500 ppm MT and 1000 ppm MT were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.3^{a}	8.6^{a}	14.8^{a}	16.1^{a}	20.7^{a}
200 MT	5.2^{c}	5.8^{bc}	6.3^{c}	10.4^{b}	9.6^{b}
500 MT	4.5^{c}	4.6^{c}	6.5^{c}	11.2^{b}	10.2^{b}
1000 MT	6.7^{b}	6.6^{b}	7.8^{b}	9.8^{b}	9.6^{b}

Means^{abcd} (n=3) of control, 200 ppm MT, 500 ppm MT and 1000 ppm MT by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.7 Anisidine values of control, 200 ppm QT, 500 ppm QT and 1000 ppm QT were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.3^{a}	8.6^{b}	14.8^{c}	16.1^{c}	20.7^{b}
200 QT	4.5^{c}	11.1^{b}	16.9^{b}	18.5^{bc}	22.3^{b}
500 QT	5.6^{bc}	17.2^{a}	19.1^{a}	20.5^{ab}	22.5^{b}
1000 QT	6.1^{b}	17^{a}	19.3^{a}	22.1^{a}	26^{a}

Means^{abcd} (n=3) of control, 200 ppm QT, 500ppm QT and 1000ppm QT by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table 6.8 Anisidine values of control, 500 ppm RSEX, 1000 ppm RSEX and 2000 ppm RSEX were measured after storage at 40℃ at 0, 2,4,6,8 days.

Treatment	Anisidine value				
	0 day	2 day	4 day	6 day	8 day
Control	8.3^{a}	8.6^{a}	14.8^{a}	16.1^{a}	20.7^{a}
500 RSEX	7.8^{a}	9.5^{a}	11.5^{b}	15.1^{a}	19.4^{a}
1000 RSEX	7.3^{a}	9.4^{a}	9.5^{c}	11.9^{b}	19.2^{a}
2000 RSEX	7.9^{a}	9.1^{a}	9.7^{c}	11.7^{b}	17.3^{b}

Means^{abcd} (n=3) of control, 500 ppm RSEX, 1000 ppm RSEX and 2000 ppm RSEX by different letters are significantly different using Tukey's analysis. ($p<0.05$)

Table7.1. Peroxide values (meq/kg) of mixtures of TEX and RSEX were evaluated after 40℃ storage at 0, 2,4,6,8 days.

Treatment (ppm)	Peroxide Value (meq/kg)				
	0 day	2 days	4 days	6 days	8 days
Control	$1±0.2^{a}$	$4.2±0.8^{a}$	$16.6±1.6^{a}$	$29.4±0.18^{a}$	$37.4±1.3^{a}$
200TEX	$0.9±0.1^{a}$	$2.9±0.3^{ab}$	$6.9±1.5^{bc}$	$15.3±1.4^{c}$	$27.5±0.7^{b}$
500TEX	$1.7±1^{a}$	$3.0±1.2^{ab}$	$9.5±1.3^{b}$	$13.3±0.8^{cd}$	$24.8±2.2^{bc}$
1000RSEX	$1.1±0.21^{a}$	$2.2±0.8^{b}$	$9.3±1.4^{b}$	$24.6± 1.9^{b}$	$35.7± 1.3^{a}$
1000RSEX + 200TEX	$1.1±0.5^{a}$	$2.2±0.2^{ab}$	$6.1±1.1^{c}$	$9.7±0.5^{de}$	$21.9±3.5^{cd}$
1000RSEX + 500TEX	$1.1±0.1^{a}$	$2.7±0.4^{ab}$	$6.2±0.5^{c}$	$11.1±0.6^{d}$	$16.3±0.2^{de}$
2000RSEX	$1.3±0.4^{a}$	$2.2±0.2^{b}$	$5.6±0.1^{c}$	$10.7± 1.9^{d}$	$19.9±3.2^{cde}$
2000RSEX + 200TEX	$1.1±0.3^{a}$	$2.1±0.1^{b}$	$6.3±1.7^{c}$	$10.0±0.6^{d}$	$14.8±1.5^{ef}$
2000RSEX + 500TEX	$1.3±0.1^{a}$	$1.9±0.4^{b}$	$4.3±1.0^{c}$	$6.2±1.2^{e}$	$10.7±1.3^{f}$

Values are reported as means ± standard deviation of peroxide value (meq/kg) on each test day and different super script letters are indicated significantly different in Tukey's analysis($p<0.05$)

Table7.2. Head space oxygen content (%) of mixtures of TEX and RSEX were evaluated after 40℃ storage at 0, 2,4,6,8 days.

Treatment (ppm)	Oxygen content %				
	0 day	2 days	4 days	6 days	8 days
Control	20.2 ± 0.1^{ab}	18.4 ± 0.2^{c}	11.9 ± 0.01^{f}	7.4 ± 0.1^{g}	5.1 ± 0^{i}
200TEX	20.2 ± 0.2^{a}	18.5 ± 0.2^{c}	17.6 ± 0.1^{e}	13.5 ± 0^{f}	10.7 ± 0^{f}
500TEX	20.2 ± 0.1^{ab}	18.6 ± 0.2^{c}	17.8 ± 0.1^{de}	14.8 ± 0^{d}	11.5 ± 0^{e}
1000RSEX	20.2 ± 0.1^{a}	19.5 ± 0.1^{ab}	18 ± 0.1^{cd}	14.7 ± 0.1^{d}	8.4 ± 0.1^{h}
1000RSEX + 200TEX	20.3 ± 0.5^{ab}	19.3 ± 0.1^{b}	17.9 ± 0.1^{cde}	15.2 ± 0.1^{d}	11.9 ± 0.1^{d}
1000RSEX + 500TEX	20.1 ± 0.1^{ab}	19.5 ± 0.1^{ab}	18 ± 0.1^{bcd}	17.8 ± 0.1^{a}	14.2 ± 0.1^{b}
2000RSEX	19.6 ± 0.08^{b}	19.3 ± 0.1^{b}	18.3 ± 0.1^{abc}	14.4 ± 0.1^{e}	10.1 ± 0.01^{g}
2000RSEX + 200TEX	20.1 ± 0.3^{ab}	19.4 ± 0.1^{b}	18.4 ± 0.1^{ab}	$17.3\pm0.1^{.b}$	13.9 ± 0.02^{c}
2000RSEX + 500TEX	20.1^{ab}	19.8 ± 0.04^{a}	18.6 ± 0.1^{a}	17.9 ± 0.06^{a}	14.5 ± 0.03^{a}

Values are reported as means ± standard deviation of headspace oxygen content (%)on each test day and different super script letters are indicated significantly different in Tukey's analysis($p<0.05$)

Table7.3. Anisidine values of mixtures of TEX and RSEX were evaluated after 40℃ storage after at 0, 2,4,6,8 days.

Treatment (ppm)	Anisidine value				
	0 day	2 days	4 days	6 days	8 days
Control	$7.7±0.2^{a}$	$9.7±0.7^{a}$	$13±1.1^{a}$	$18.1±0.4^{a}$	$21.2±0.2^{b}$
200TEX	$7.5±0.2^{a}$	$8.3±0.1^{b}$	$10.6±0.2^{ab}$	$13.4±0.4^{c}$	$15.2±0.4^{c}$
500TEX	$7.7±0.4^{a}$	$9.5±0.2^{ab}$	$11±1.4^{ab}$	$14.3±0.5^{c}$	$15.5± 0.3^{c}$
1000RSEX	$7.5±0.2^{a}$	$9.1±0.2^{ab}$	$10.3±0.2^{ab}$	$16.5± 0.8^{b}$	$24.6± 0.2^{a}$
1000RSEX + 200TEX	$7.4±0.4^{a}$	$9.5±0.4^{ab}$	$9.5±0.3^{b}$	$11.4±0.4^{de}$	$15.6±0.7^{c}$
1000RSEX + 500TEX	$6.4±0.6^{b}$	$8.8±0.2^{ab}$	$11.1±2.9^{ab}$	$11.8±1.3^{d}$	$13.5±1.4^{d}$
2000RSEX	$8.3±0.2^{a}$	$9.5±0.2^{ab}$	$9.8±1.12^{ab}$	$10.5± 1.98^{e}$	$14.9±3.2^{c}$
2000RSEX + 200TEX	$7.6±0.4^{a}$	$9.2±0.2^{ab}$	$10.6±0.7^{ab}$	$11±0..4^{de}$	$13.2±0.5^{d}$
2000RSEX + 500TEX	$7.9±0.1^{a}$	$9.1±0.1^{ab}$	$9.3±2.2^{ab}$	$10.4±0.2^{e}$	$11.2±1.31^{e}$

Values are reported as means ± standard deviation of anisidine value on each test day and different super script letters are indicated significantly different in Tukey's analysis ($p<0.05$)

Table 8.1. Peroxide value (meq/kg) of mixtures of AP, CA, TEX and RSEX were determined after 40℃ storageat 0, 2,4,6,8, days.

Treatment (ppm)	Peroxide value (meq/kg) (p<0.05)				
	0 day	2 days	4 days	6 days	8 days
Control	1.7±0.4 ^{ab}	3.1±0.8^{a}	18.1±1.3 ^{a}	26.9± 0.6^{a}	35± 0.6^{a}
200AP +100CA	1.3±0.5 ^{ab}	3.1±0.1^{a}	13.9±1.1 ^{b}	25±0.5 ^{b}	35.3±2.2 ^{a}
200AP +100CA 100+ 500TEX	1.2± 1.0^{ab}	3.5±0.2^{a}	5.1±1^{c}	7.8± 0.4^{de}	11.8± 0.3^{d}
200AP +100CA + 1000TEX	1.5±0.6^{ab}	3±1.7^{a}	5.88±0.9 ^{c}	7.3±0.4 ^{de}	9.8± 0.4^{de}
200AP +100CA + 1000RSEX	1.3±0.2^{ab}	2.9±0.2^{a}	5.8± 0.2^{c}	11± 0.5^{c}	22.7±1.1 ^{b}
200AP +100CA +1000RSEX+500TEX	1.8±0.2 ^{ab}	3.8±0.3^{a}	6± 0.7 ^{c}	8.1± 0.9^{d}	10±0.9 ^{de}
200AP +100CA + 1000RSEX+1000TEX	1.4±0.2 ^{ab}	2.7±0.2^{a}	5.4± 0.6^{c}	6.3± 0.7^{ef}	7.3± 0.1^{ef}
200AP +100CA + 2000RSEX	1.3±0.2 ^{ab}	2.7±0.5^{a}	5.2± 0.5^{c}	11.3± 1.0^{c}	16.6±1.2 ^{c}
200AP +100CA+ 2000RSEX+500TEX	0.9±0.1 ^{ab}	3.4±0.2^{a}	4.8± 1.1^{c}	6.2±0.1 ^{ef}	7.6±0.2 ^{ef}
200AP +100CA + 200RSEX + 1000TEX	0.4± 0.7^{ab}	3±0.8^{a}	3.8± 0.1^{c}	5.3± 0.2^{f}	6.7± 0.2^{f}

Values are reported as means ± standard deviation of peroxide value (meq/kg) on each test day and different super script letters are indicated significantly different in tukey analysis(p<0.05)

Table 8.2. Head space oxygen content (%) of mixtures of AP, CA, TEX and RSEX were determined after 40℃ storageat 0, 2,4,6,8, days.

Treatment (ppm)	Oxygen % (p<0.05)				
	0 day	2 days	4 days	6 days	8 days
Control	19.8±0.2^{ab}	18.9±0.1 ^{a}	14.3±0.02 ^{f}	9.1± 0.01^{a}	6± 0.01^{g}
200AP +100CA	19.6±0.1b	18±0.1 ^{d}	13.2± 0.2^{g}	8.9±0.01^{a}	5.9±0 ^{g}
200AP +100CA 100+ 500TEX	19.9±0.1 ^{ab}	18.6±0.2^{abc}	17.7± 0.1^{bc}	16.3±0.02^{c}	13.9± 0.01^{d}
200AP +100CA + 1000TEX	19.9±0.1 ^{ab}	18.3± 0.1^{cd}	17.6±0.03^{bc}	17.6±0.04^{bc}	15.3± 0.01^{f}
200AP +100CA + 1000RSEX	20± 0.7^{ab}	18.3± 0.1^{cd}	15± 0.01^{e}	11.3±0.01^{g}	7.9±0.01^{f}
200AP +100CA +1000RSEX+500TEX	20.5±0.1 ^{a}	18.3±0.2^{bcd}	17.4± 0.01^{c}	16.6±0.02^{d}	14.7±0.3 ^{c}
200AP +100CA + 1000RSEX+1000TEX	20.3±0.03^{ab}	18.6±0.1^{abc}	18.5±0.2 ^{a}	17.7±0.1^{b}	15.5±0.02 ^{b}
200AP +100CA + 2000RSEX	20.1± 0.3^{ab}	18.3±0.1^{bcd}	16.6±0.03^{d}	14.4±0.1^{f}	9.80±0^{e}
200AP +100CA+ 2000RSEX+500TEX	20.4± 0.1^{ab}	18.4±0.3^{cd}	18±0.02^{b}	17.4±0.03^{c}	15.3±0.02^{b}
200AP +100CA + 200RSEX + 1000TEX	20.3±0.1^{ab}	18.3±0.03^{bcd}	18.5±0.2^{a}	18.1±0.2^{a}	16.4±0.04^{a}

Values are reported as means ± standard deviation of head space oxygen content (%) on each test day and different super script letters are indicated significantly different in Tukey analysis(p<0.05)

Table 8.3. Anisidine values of mixtures of AP, CA, TEX and RSEX were determined after 40℃ storage at 0, 2,4,6,8, days.

Treatment (ppm)	Anisidine (p<0.05)				
	0 day	2 days	4 days	6 days	8 days
Control	7.6±0.1^{abc}	8.7±0.6^{b}	12.5±0.3^{a}	16.9±0.3^{a}	23.5±1.3^{a}
200AP +100CA	7.2±0.2^{bc}	10.7±0.2^{a}	11.5±0.3^{a}	16.3±0.5^{a}	24.7±1.7^{a}
200AP +100CA 100+ 500TEX	7±0.3^{c}	8.6±0.6^{b}	9.2±0.2^{b}	10.6±0.2^{c}	11.5±1^{cde}
200AP +100CA + 1000TEX	7±0.1^{c}	8.4±0.2^{bc}	9.1±0.4^{b}	10.7±0.3^{b}	12.1±0.5^{bcd}
200AP +100CA + 1000RSEX	7.8±0.1^{ab}	8.8±0.5^{b}	8.7±0.2^{b}	11.8±0.2^{cd}	13.9±0.2^{b}
200AP +100CA +1000RSEX+500TEX	8±0.1^{a}	9.1±0.2^{b}	9.7±1^{b}	10.4±0.3^{cd}	10.8±0.4^{cde}
200AP +100CA + 1000RSEX+1000TEX	7.5±0.4^{abc}	8.2±0.2^{cbd}	9.2±0.2^{b}	9.6±0.2^{de}	9.8±0.2^{de}
200AP +100CA + 2000RSEX	7.4±0.2^{abc}	7.2±0.4^{d}	9.4±0.9^{b}	10.9±0.4^{c}	12.7±0.2^{bc}
200AP +100CA+ 2000RSEX+500TEX	7.5±0.1^{abc}	7.5±0.2^{cd}	9.3±2.3^{b}	9.30±0.5^{e}	10±0.4^{de}
200AP +100CA + 200RSEX + 1000TEX	7±0.1^{c}	7.5±0.2^{cd}	9.5±3.3^{b}	9.10±0.5^{e}	9.4±0.2^{e}

Values are reported as means ± standard deviation of anisidine on each test day and different super script letters are indicated significantly different in Tukey analysis(p<0.05)

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