The Efficacy of Using Natural Antioxidant Blends to Control Oxidative Rancidity in Headed and Gutted, Filleted, and Minced Rainbow Trout (*Oncorhynchus mykiss*) During Frozen Storage

by

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
IN
FOOD SCIENCE AND TECHNOLOGY

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ABSTRACT

The antioxidant properties of various blends of rosemary extract and tocopherols, either alone or with citric and ascorbic acid, were compared in filleted, headed and gutted, and minced rainbow trout (*Oncorhynchus mykiss*). The filleted and headed and gutted products were stored at -29°C for twelve months, while the minced was stored at the same temperature for 24 weeks. Oxidation was measured by following changes in thiobarbituric reactive substances, conjugated diene hydroperoxides, texture, drip loss, pH, sensory evaluation, and gas chromatographic detection of aldehydes. Natural antioxidants, in particular those that contained citric and ascorbic acids, were effective at retarding the development of conjugated diene hydroperoxides and malonaldehyde (*p*<0.05). Furthermore, sensory evaluation indicated that treated samples were less oxidized. In subsequent studies, however, it was determined that the herbal flavor notes associated with natural antioxidants complicated the ability of the experienced panel to judge extent of oxidation. Also, using filleted samples, further consumer sensory panels indicated that after 12 months frozen storage, the treated and control samples were equally acceptable. For both the filleted and headed and gutted samples, no texture differences were noted over storage time or between control or variable treatments. When using natural antioxidant products, drip loss and pH were found unreliable predicators of oxidation or muscle degradation.
ACKNOWLEDGMENTS

I would first like to thank God for His continued blessings throughout my life and
His support in completing this endeavor.

I would like to express my sincere appreciation to my advisor, Dr. George J. Flick,
Jr., for his support and guidance throughout my graduate studies. I also extend my
appreciation to the members of my committee: Drs. Frank D. Conforti, Susan E. Duncan
and C. Gene Haugh. I thank each of you for bringing your unique expertise to this
project; your advice and recommendations have been invaluable.

I would also like to thank the staff personnel and my fellow graduate students at
Virginia Tech. You were there to provide me with whatever I needed--from test tubes to
a good joke or two!

I extend enormous gratitude to my best friend and fellow graduate student, Lisa L.
Scott. Since we were children she has always been a constant source of support and
encouragement. Lisa has spent many nights in the lab with me, either helping me when
needed or simply keeping me company. I could never begin to thank her enough.

A special thanks is extended to my fiancé, Terry T. Ashby, for his encouragement,
patience and support. I truly appreciate having you in my life.

Finally, I would like to acknowledge Kalsec, Inc. and Thomas Jones for supporting
this research endeavor and supplying the antioxidant treatments.
DEDICATION

This work is dedicated to my parents, General J. and Gertha M. Turner, who inspired my brothers and me to always reach for the stars. I admire them for their work ethic, their insight, and their willingness to always sacrifice for the better good. Mom and Dad---here's to you! Thanks!
TABLE OF CONTENTS

TITLE
ABSTRACT
ACKNOWLEDGMENTS
DEDICATION
TABLE OF CONTENTS
LIST OF TABLES AND FIGURES
INTRODUCTION
SECTION 1: REVIEW OF LITERATURE
The Marketing of Seafood in the United States
The Proximate Composition of Fish
Nutritional Value of the Omega-3 Fatty Acids
Lipid Oxidation
  Autoxidation
    Initiation
    Propagation
    Termination
  Other Mechanisms of Lipid Oxidation
    Photo-oxidation
    Enzymatic Oxidation
Factors Influencing the Rate of Lipid Oxidation in Fish
Exposure to Oxygen 15
Water Glaze 15
Vacuum Packaging 15
Modified Atmospheric Packaging (MAP) 16
Temperature of Storage 16
Dietary Intake 17
Region Within Fish 18
Water Activity 19
Fat Content 19
Degree of Butchery 20
Exposure to Light 20
Presence of Pro-oxidants or Antioxidants 20
Antioxidants 21
Characteristics of an Ideal Antioxidant 22
Limitation of Antioxidants 23
Types of Antioxidants 23
   Synthetic Primary Antioxidants 23
       BHA 23
       BHT 24
       Propyl Gallate 24
       TBHQ 24
   Synthetic Secondary Antioxidants 25
SECTION 2: The Efficacy of Using Natural Antioxidant Blends to Control
Oxidative Rancidity in Filleted and Headed and Gutted Rainbow Trout During
Frozen Storage

Abstract 53
Introduction 54
Materials and Methods 57
Results and Discussion 65
Conclusions 71
References 73
Acknowledgments 75

SECTION 3: The Efficacy of Using Natural Antioxidant Blends to Control
Oxidative Rancidity in Minced Rainbow Trout During Frozen Storage 76

Abstract 76
Introduction 77
Materials and Methods 80
Results and Discussion 85
Conclusions 89
References 91
Acknowledgments 92

Appendix I: DataTables 93
Appendix II: Figures 103
Appendix III: Sensory Evaluation 110
Vitae 116
LIST OF TABLES AND FIGURES

Appendix 1--Tables

Table 1: Changes in percent drip loss of headed and gutted rainbow trout as affected by treatment and storage time 94

Table 2: Changes in percent drip loss of filleted rainbow trout as affected by treatment and storage time 95

Table 3: Changes in percent drip loss of minced rainbow trout as affected by treatment and storage time 96

Table 4: Changes in pH of headed and gutted rainbow trout as affected by treatment and storage time 97

Table 5: Changes in pH of filleted rainbow trout as affected by treatment and storage time 98

Table 6: Changes in pH of minced rainbow trout as affected by treatment and storage time 99

Table 7: Paired comparison of headed and gutted rainbow trout to fresh and stored controls 100

Table 8: Paired comparison of filleted rainbow trout to fresh and stored controls 101

Table 9: Paired comparison of minced rainbow trout to fresh and stored controls 102

Appendix 2--Figures

Figure 1: The effect of storage time and treatment on the development of TBARS in headed and gutted rainbow trout 104

Figure 2: The effect of storage time and antioxidant treatment on the development of TBARS in filleted rainbow trout 105

Figure 3: The effect of storage time and treatment on the development of conjugated diene hydroperoxides in headed and gutted rainbow trout 106

Figure 4: The effect of storage time and treatment on the development of conjugated diene hydroperoxides in headed and gutted rainbow trout 107
Figure 5: The effect of storage time and treatment on the development of conjugated diene hydroperoxides in filleted rainbow trout

Figure 6: The effect of storage time and treatment on the development of conjugated diene hydroperoxides in minced rainbow trout
INTRODUCTION

During the 1980’s a shift in eating habits was observed in the United States, as health conscious Americans became aware of the numerous benefits of consuming a diet low in saturated fats and cholesterol (Six, 1994). Fortunately, this renewed interest in health has in many ways benefited the seafood industry, as consumers believe seafood products are excellent alternative to less healthy food choices. Fish, in particular, has received a great deal of attention because of its high protein content and its high proportion of unsaturated fatty acids (Wada and Fang, 1992; Vareltzis et al., 1997).

While the protein content in fish is advantageous to consumers, it is the lipid portion that is often of most interest to the American public. This is because fish contain omega-3 fatty acids, a group of polyunsaturated fatty acids (PUFA) that are reported to have tremendous health benefits. The intake of omega-3 fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been associated with a lowered risk of many medical problems, including heart disease (Kinsella, 1986), stroke, numerous cancers, tumorigenesis and diabetes, among others (Waagbo et al., 1993; Garcia, 1998).

Throughout the world, this greater interest in omega-3 fatty acids has led to significant increases in fish consumption. Such increases have been reported in salmonoids (No and Storebakken, 1991), and catfish (Erickson, 1992), but this is actually a general trend throughout the fish industry. In fact, Flick et al. (1991) reported that the United States has a per capita annual fish and shellfish consumption of over sixteen pounds, with this value expected to exceed twenty pounds by the year 2000. With growing interest and
greater consumption of fish products, processors have responded by increasing production volumes. While this will likely bring more revenue to the fish industry, it also brings the risks often associated with market instability. If at any time there are excessive supplies of fish products, the market price may shift downward and processors may lose potential profit (Erickson, 1993).

Erickson (1993) reported that the answer to market instability may be to freeze fish products. By freezing products, processors can better control how much fish saturates the market, and thus the commodity will have more price stability (No and Storebakken, 1991; Erickson, 1993). In addition to regulating the market supply, frozen storage may become more necessary as the distribution area for many processors increases (Erickson, 1992). Finally, frozen storage may also be critical in situations such as in the wild salmon industry. Processors would like to capture sufficient fish to supply the market until the next season, nearly a year later (Thomas Jones, Kalsec, Inc., personal communication, 1997). To accomplish such endeavors, frozen storage is a necessity. Unfortunately, long term freezing has been associated with product deterioration due to oxidative reactions. This is obviously a major concern of food processors.

In addition to the frozen shelf life of fish products, seafood processors are also concerned about producing more products from recovery operations. It is estimated that the yield of fish fillets from a whole fish is around 35%, depending on the species. This estimate is based on both hand and machine filleting procedures (Nettleton, 1985). Many seafood processors are currently marketing the fillets, a more valuable product per weight basis, and discarding the remainder of the backbone, frame, belly flap, and other remnants
of the fish which still contain some edible tissue (George Flick, Jr., Virginia Tech, personal communication, 1998). In an effort to increase profitability, many of these items could be further processed using a mechanical bone separator. Brannan and Erickson (1996) reported that 50-200 % more usable meat could be obtained per fish than is currently being utilized. In addition to significantly increasing the usable meat obtained per fish, this technology would also greatly reduce waste costs (Nettleton, 1985; Brannan and Erickson, 1996). Therefore, this recovery operation could increase profit in two ways: through marketing of the minced product from currently discarded food material and by lowering waste processing cost.

While mechanical mincing procedures could prove extremely beneficial to fish processors, the industry must make such products more acceptable to the public. To do this, the seafood industry will have to effectively control oxidative reactions in the product (Erickson, 1992). Nettleton (1985) reported that minced fish prepared from whole headless frames is higher in fat, cholesterol and calcium than minced prepared from fillets. Such mince also has a higher concentration of metals, such as iron and copper. With higher levels of fat and more pro-oxidants, along with greater surface area, minced products are extremely susceptible to oxidation. While freezing minced tissue will slow oxidation processes, low temperatures alone are not enough to significantly retard deterioration (Hamilton et al., 1994; Nawar, 1996).

Due to the high content of polyunsaturated fatty acids, frozen storage of fish products typically results in many undesirable changes in tissue, including development of off odors and flavors and changes in texture, water holding capacity, color and nutritive
properties (Eun et al., 1994; Thed and Erickson, 1994). Fish processors may use several methods to control oxidation such as vacuum packaging, glazing, and modified atmospheric packaging (MAP). Although many choices are available, when evaluated on cost, effectiveness and ease of use, antioxidants are the most widely used method to control lipid oxidation (Harris and Tall, 1994). Antioxidants are substances that retard lipid oxidation by acting as either radical scavengers, oxygen quenchers, or metal chelators. There are two major classes of antioxidants available, synthetic and natural (Rajalakshmi and Narasimhan, 1996).

Dating back to the 1940s, synthetic antioxidants have been used to extend the shelf life of numerous lipid containing products. The most widely used synthetic antioxidants are BHA, BHT, TBHQ and propyl gallate (Giese, 1996; Edlefsen and Brewer, 1998). Although these substances are very effective in controlling oxidation, recently there has been growing concern about the toxicity of these synthetic products, especially BHA. Many countries, including the United States, are re-evaluating the use of BHA and countries such as Japan have actually banned this antioxidant (Inatani et al., 1983; Brookman, 1991). These actions have obviously alarmed the public. As a result, there have been increased efforts to obtain antioxidant compounds from natural sources, such as spices and plants.

Natural antioxidants are often obtained from various spices such as rosemary, oregano, nutmeg, sage, and others whose main antioxidant activity is due to various phenolic compounds (Rajalakshmi and Narasimhan, 1996). With a major consumer trend towards more “natural” products, there has been great interest in such antioxidants
because they are perceived as safer (Dorko, 1994; Six, 1994). While perhaps calming consumer fears, food processors still question if these natural products will be the answer to the on-going question of how food quality is preserved.

The objective of this study was to determine the efficacy of using natural antioxidants to control oxidative rancidity during frozen storage of headed and gutted, filleted and minced rainbow trout (*Oncorhynchus mykiss*). Commercial natural antioxidant blends were used, primarily consisting of varying concentrations of rosemary extract, tocopherols, citric acid and ascorbic acid. Overall, this research will allow the scientific community to better determine the feasibility of using natural antioxidant systems to retard oxidative rancidity during frozen storage of fish. The information gained from this study will be used in the wild salmon industry to provide consumers with consistent, good quality salmon products throughout the year. Such conclusions may be made due to the close genetic relation between rainbow trout and salmon, both being salmonoids. Although this research will be used primarily in the salmon industry, it is relevant to the long-term frozen storage of fish products in general.
SECTION 1: REVIEW OF LITERATURE

THE MARKETING OF SEAFOOD IN THE UNITED STATES

In a 1984 Food Marketing Institute survey, over 63% of supermarket shoppers reported they were “very concerned” about the nutritional content of what they ate. An overwhelming 95% were either “very” or “somewhat” concerned about nutritional aspects of their food. This study demonstrates a strong consumer awareness and interest in nutrition, an interest that has intensified over the years as Americans have become more and more health conscious (Food Marketing Institute, 1984; Peavey et al., 1994).

The 1980’s witnessed a shift in eating habits, as health conscious Americans became aware of the nutritional benefits of consuming a diet low in saturated fats and cholesterol (Six, 1994). Fortunately, this renewed interest in health has in many ways benefited the seafood industry, as consumers believe seafood products are an excellent alternative to less healthy food choices. By the end of the 1980’s, the average American had a greater consumption of seafood and poultry products, while the consumption of beef and pork considerably decreased (Harvey, 1994).

The United States has a per capita annual fish and shellfish consumption of over sixteen pounds, with this value expected to exceed twenty pounds by the year 2000 (Flick et al., 1991). While the increasing interest in seafood products may be attributed to several factors, most researchers agree that heightened consumer awareness of the nutritional value of marine products has been extremely influential (Hwang and
Regenstein, 1988; Redmayne, 1989; Peavey et al., 1994). Fish is a valuable natural resource that has received a great deal of attention because of its high protein content and its high proportion of unsaturated fatty acids (Wada and Fang, 1992; Vareltzis et al., 1997).

**PROXIMATE COMPOSITION OF FISH**

The edible flesh of fish consists of three major components: water, protein and lipid, which together constitute nearly 98% of the total flesh mass (Nettleton, 1985). The actual proportion of these components in the flesh varies with several factors, including species, type of muscle, size, maturity, sex, feed intake, activity level, season of year, geographical location were harvested, and if the animal was farm raised or wild (Nettleton, 1985; Chanmugam et al., 1986; Flick et al., 1992).

In general, the flesh has a water content in the range of 50 to 85%, and a crude protein content of between 11 to 24%, making fish an excellent source of high quality protein (Sikorsky et al., 1990). In addition to their abundance, proteins from seafoods are also highly digestible, making them extremely advantageous to the consumer (Nettleton, 1985). Along with the water and protein contents, fish has a lipid concentration in the range of 0.5 to 25% (Ogata et al., 1996). It is the lipid portion that is often of most interest to researchers and consumers, alike.

Fish oils have at least two distinguishing properties not shared by any other food products. First, they have a relatively large amount of odd carbon chain fatty acids, typically with 15, 17 or 19 carbon atoms (Nettleton, 1985). The second unique property is a significant proportion of highly polyunsaturated fatty acids, consisting of more than
four double bonds (Ackman, 1996; Garcia, 1998). The omega-3 fatty acids are in this group of polyunsaturated fatty acids (PUFA) and they are reported to have the greatest health benefit (Ackman, 1996).

**NUTRITIONAL VALUE OF OMEGA-3 FATTY ACIDS**

There are a total of about seven omega-3 fatty acids which can be isolated from fish oil, however, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the major two. Of the omega-3 fatty acids, approximately 10% are EPA, while 30-33% are DHA (Ogata *et al.*, 1996). EPA consists of a twenty carbon chain with five double bonds (C20:5), while DHA has twenty-two carbons and six double bonds (C22:6). These omega-3 fatty acids remain liquid at cold temperatures (Nettleton, 1985).

Intake of omega-3 fatty acids has been associated with a lowered risk of many medical problems, including heart disease (Kinsella, 1986), stroke, numerous cancers, tumorigenesis and diabetes (Waagbo *et al.*, 1993; Garcia, 1998). In addition, it has been associated with a favorable high-density to low-density lipoprotein ratio, low triacylglycerol and cholesterol levels, and a low concentration of very low-density lipoproteins in the blood serum (Sikorsky *et al.*, 1990).

Since the 1970’s, more and more evidence has been presented to support the health benefits of omega-3 fatty acids, and in particular EPA and DHA. Several countries, including Denmark, Canada, Japan and England, have even established recommended daily allowances for this specific group of fatty acids (Garcia, 1998). All of this positive publicity has led to a greater consumer interest in fish products; however, Americans are mainly increasing their consumption of lean, low-fat fish species, which have a lower
omega-3 content than in fattier species (Hwang and Regenstein, 1988). The explanation for this trend is that fattier fish are much more susceptible to oxidation and thus the production of off-flavors and odors which consumers find unacceptable (Peavey et al., 1994; Ogata et al., 1996).

While the advantageous health implications of EPA and DHA are directly related to the increased annual consumption of fish, seafood processors have also been presented with greater challenges because of the high content of polyunsaturated fatty acids (PUFA). To fully take advantage of consumers’ interest in fish products, the seafood industry must work diligently to better understand and control oxidation reactions.

**LIPID OXIDATION**

Of all the factors that can cause changes in product quality, oxidation of lipids most significantly results in the deterioration and limited shelf life of many fish products (Erickson, 1993). This causes substantial product loss every year and makes the oxidation process a serious economic concern to the seafood industry. The oxidation problem becomes even greater when dealing with the captured fish industry because there is typically a longer period of time between harvesting, processing, packaging, distribution and marketing (Flick et al., 1992).

Lipid oxidation is typically thought of as a free-radical chain reaction that results in drastic changes in sensory properties and nutritional value of the fish product (Nawar, 1996). It is responsible for many changes in texture, color, odor and flavor quality, often resulting in an unacceptable product (Erickson, 1993; Brannan and Erickson, 1996a). In addition to causing deterioration reactions, there is growing concern that lipid oxidation
may play a significant role in coronary heart disease, atherosclerosis, cancer, and aging. This is due to the numerous free radicals in the oxidation process (Chen et al., 1992; Giese, 1996).

**Autoxidation of Lipids**

In general, the mechanism of lipid oxidation is still not yet greatly understood. Unfortunately, the majority of information on lipid oxidation in fish has been determined through various studies on fish oils, a much simpler system. The true nature of oxidative reactions in fish is much more difficult to study because of the heterogeneous nature of the food matrix (Harris and Tall, 1994). While there are other types of oxidation, autoxidation has long been believed to be the most significant oxidation process. Based on what is known, scientists have divided the reaction mechanism into three basic steps: initiation, propagation and termination (Nawar, 1996).

**Initiation.** Autoxidation of a lipid material is first initiated with the formation of a free radical. This radical may be formed by several external energy sources, such as light, heat, high energy radiation or by chemical presence of metal ions or metalloproteins such as heme. Fish contain a plethora of such metals, particularly copper, iron, and heme, all of which may act as catalysts. In the presence of such catalysts and atmospheric oxygen, the lipid molecule, LH, will react to produce a free radical, L’, as in equation 1. In this reaction, a hydrogen radical is taken from an allylic methylene group of an unsaturated fatty acid (Hamilton, 1994; Jadhav et al., 1996; Nawar, 1996).

Lipid hydroperoxides may also play a role in the initiation of oxidation. These hydroperoxides are produced by several pathways, such as the reaction of a singlet oxygen
with unsaturated lipids. These lipid hydroperoxides, which already exists in trace amounts, may react to produce free radicals as well. This occurs when the hydroperoxides undergo homolytic cleavage to produce alkoxy radicals, \( \text{LO}^* \), as in equation 2. The initiation step can also involve lipid hydroperoxides that undergo bimolecular decomposition to produce free radicals, as seen in equation 3. No matter what pathways are taken to produce the free radicals, all of these radicals are extremely reactive and are able to propagate further oxidation reactions (Hamilton, 1994; Jadhav et al., 1996; Nawar, 1996; Fernandez et al., 1997).

\[
\begin{align*}
\text{LH} & \rightarrow \text{L}^* + \text{H}^* \quad \text{Equation 1} \\
\text{LOOH} & \rightarrow \text{LO}^* + \text{HO}^* \quad \text{Equation 2} \\
2\text{LOOH} & \rightarrow \text{LO}^* + \text{LOO}^* + \text{H}_2\text{O} \quad \text{Equation 3}
\end{align*}
\]

**Propagation.** The second step of the mechanism is characterized by a chain reaction of free radicals to produce additional radicals. Propagation often consists of a lipid free radical reacting with atmospheric oxygen, resulting in the production of peroxy radicals, \( \text{LOO}^* \), as seen in equation 4. These peroxy radicals then abstract a hydrogen from \( \alpha \)-methylene groups of other lipid molecules to yield hydroperoxides (LOOH) and additional free radicals, as in equation 5. As this reaction is repeated, an accumulation of hydroperoxides results (Hamilton, 1994; Jadhav et al., 1996; Nawar, 1996).

\[
\begin{align*}
\text{L}^* + \text{O}_2 & \rightarrow \text{LOO}^* \quad \text{Equation 4} \\
\text{LOO}^* + \text{LH} & \rightarrow \text{LOOH} + \text{L}^* \quad \text{Equation 5}
\end{align*}
\]
The primary products of autoxidation, hydroperoxides, have no sensory attributes of taste or odor. However, hydroperoxides are rather unstable and they will undergo decomposition into secondary oxidation products. Initially, hydroperoxides break down to produce alkoxy and hydroxy free radicals. The alkoxy radical further decomposes, by cleavage on either side of the oxygen-bearing carbon, producing numerous products, including aldehydes, ketones, hydroxyacids, and alcohols (Flick, 1992; Nawar, 1996). These secondary oxidation products, particularly the numerous aldehydes, are responsible for the off-flavors and odors often associated with oxidation (Hamilton, 1994).

**Termination.** Possessing an unpaired electron, free radicals will react to restore a complete outer valence shell whenever possible. As the amount of unsaturated lipid materials shrinks, radicals bond to one another to produce stable compounds. With the combination of radicals to produce nonreactive compounds, the propagation step ceases and the termination phase progresses (Hamilton, 1994; Jadhav et al., 1996). The occurrence of termination reactions is determined by the concentration of the radicals and stereochemistry. The radical concentration is responsible for the frequency of interactions between radical compounds, while the stereochemistry governs the orientation of the radical collisions (Nawar, 1996). Equations 6-8 provide examples of termination reactions.

\[
L^\cdot + L^\cdot \rightarrow L-L \quad \text{Equation 6}
\]

\[
L^\cdot + LOO^\cdot \rightarrow LOOL \quad \text{Equation 7}
\]

\[
LOO^\cdot + LOO^\cdot \rightarrow LOOL + O_2 \quad \text{Equation 8}
\]
**Other Mechanisms of Lipid Oxidation**

It was long believed that the process of lipid oxidation in muscle tissue was primarily a free-radical nonenzymatic reaction, such as autoxidation. However, the significance of photo-oxidation and enzymatic oxidation has become increasingly apparent over the years (Ashie et al., 1996). Currently, researchers cannot thoroughly explain the relationship between the various mechanisms; however, it is well understood that several oxidation mechanisms play significant roles.

**Photo-oxidation.** This form of oxidation occurs in the presence of visible or ultraviolet radiation and a photosensitizer molecule, often pigment compounds such as chlorophyll or myoglobin. The significance of this mechanism is the ability to produce singlet oxygen compounds, as in equation 9. These singlet oxygen molecules ($^{1}\text{O}_2*$) are extremely reactive and can react directly with an unsaturated lipid molecule, LH, to produce a hydroperoxide (LOOH), as seen in equation 10 (Nawar, 1996; Hamilton et al., 1997). As previously mentioned, hydroperoxides are the precursors of numerous undesirable oxidation products.

\[ ^3\text{Sens}^* + ^3\text{O}_2 \rightarrow ^1\text{O}_2^* + ^1\text{Sens} \quad \text{Equation 9} \]

\[ ^1\text{O}_2^* + \text{LH} \rightarrow \text{LOOH} \quad \text{Equation 10} \]

**Enzymatic Oxidation.** The importance of enzymatic oxidation in fish has become increasingly apparent. Enzymes such as lipoxygenase, peroxidase and microsomal enzymes from fish tissues can potentially initiate lipid oxidation (Hamilton, 1994).

Currently, two lipoxygenases have been detected in fish tissue. While generally they do not contain a heme structure, most lipoxygenases contain iron at their active sites.
Lipoxygenase enzymes are believed to catalyze the direct addition of oxygen to free fatty acids. The hydroperoxides formed may further react either enzymatically or non-enzymatically to produce hydroxyl products, or they may decompose to produce free radicals. These free radicals can initiate the chain reactions of autoxidation (Hultin, 1992; Eun et al., 1994; Harris and Tall, 1994). Studying fish samples, Wada and Fada (1992) found that lipoxygenase activity was inactivated by heat treatment, however, after treatment, non-enzymatic oxidation was accelerated during subsequent frozen storage.

Microsomal enzymes function somewhat differently than lipoxygenases, however, with these enzymes being involved in reducing iron complexes in a form capable of stimulating lipid oxidation. Microsomal enzyme lipid oxidation requires the presence of the co-factor NADH/NADPH and iron. The functionality of fish microsomes is improved by the co-factor NADH more so than NADPH. The reaction is enhanced by ADP or ATP, which appear to aid in the solubility of Fe$^{3+}$ so that it may be reduced by the NADH enzyme system (Harris and Tall, 1994; Hultin, 1994). Slabyj and Hultin (1983) reported that the dark muscle of fish had more microsomal activity than the light muscle.

All of the oxidation mechanisms, autoxidation, photo-oxidation and enzymatic oxidation, produce hydroperoxides. These compounds are very unstable and will decompose to produce numerous compounds associated with the off-flavor and odor associated with rancidity (Flick et al., 1992; Erickson, 1993).
Factors Influencing Rate of Lipid Oxidation in Fish

A. Exposure to Oxygen

There are several factors that may influence the rate of oxidation in food products. Perhaps the most critical factor is the amount of oxygen present (Hamilton, 1994). In conditions where oxygen supply is abundant, the oxidation rate is independent of the concentration of oxygen. However, under limited oxygen concentrations the reaction rate is proportional to this concentration (Nawar, 1996). Overall, since the presence of oxygen is essential for oxidation to occur, several methods of controlling the process are based on the elimination or retardation of oxygen contact with the fish product. Methods often used by seafood processors include glazing, vacuum packaging and modified atmospheric packaging (Harris and Tall, 1994).

Water glaze. In water glazing, processors create a thin coating of ice on the exterior of the fish by dipping the frozen product into water. A simple water glaze has the disadvantage of being brittle, so many processors use roughly a 3% solution of corn syrup or other commercial products to produce a more flexible coating. This coating acts as a barrier, slowing the rate of oxygen permeation. While water glazing is often used for headed and gutted fish and sometimes fish fillets, it is less often used for minced products. The major disadvantage of glazing is that, while it can retard the permeation of oxygen, the oxygen does eventually come into contact with the fish and thus oxidation can proceed (Harris and Tall, 1994).

Vacuum Packaging. This technique allows processors to greatly reduce the amount of oxygen available to initiate oxidation. Using gas impermeable packaging, fish products
can be vacuum packed under substantially reduced oxygen levels. While vacuum packaging will not completely inhibit oxidation, it is the single most effective method to control oxidation in refrigerated or frozen fish products (Flick et al., 1991). Hwang and Regenstein (1988) reported that vacuum packaging was more effective than numerous antioxidants, including ascorbic acid, tocopherols, rosemary and TBHQ, in retarding oxidative rancidity in menhaden mince. Despite much success, vacuum packaging does, however, have disadvantages. For most seafood processors this method is not very economical.

**Modified Atmosphere Packaging (MAP).** Modified atmosphere packaging (MAP) is commonly used to retard the rate of microbial growth, however, since it greatly reduces the amount of oxygen present, it can be used to retard oxidation as well. Although it has been reported that mixtures of carbon dioxide and nitrogen have been successful in increasing the shelf life of seafood products, Flick et al. (1991) reported that packaging in carbon dioxide is no more beneficial than simple vacuum packaging. Like vacuum packaging, MAP is usually not very economical for most seafood processors.

**B. Temperature of Storage**

To supply consumers with a consistent, acceptable quality of seafood products, oxidation reactions must be controlled. This is often attempted through freezing, which the seafood industry has long recognized as one of the best methods of preserving fish products (Peavey et al., 1994). However, unlike microbial deterioration, oxidation is not as dependent on temperature factors. Through previous research, scientists have found that while oxidative processes may be significantly slower at low temperatures, it is
impossible to reach a temperature low enough to completely stop product deterioration (Hamilton, 1994; Nawar, 1996).

In general, the rate of oxidation increases as the temperature increases. This trend is supported by numerous research studies, including Hwang and Regenstein (1988) who found that the rate of oxidation in menhaden products at -20°C was slower than at -7°C. Also, it has been reported that the rate of reaction of oxygen with lipid material roughly doubles with each 10°C increase in temperature (Nawar, 1996). An interesting exception to this trend is that the oxidation rate is faster at -5°C than at 0°C. This is because at -5°C the formation of ice crystals creates a concentration of oxidation reactants and thus the reaction occurs faster, even at a lower temperature. As temperatures fall below -5°C, however, the lowered temperature dominates the reaction, and thus the rate slows (Harris and Tall, 1994).

C. Dietary Intake

While the diet of wild fish cannot be controlled, the dietary intake of wild and aquacultured fish plays a major role in oxidative stability. Brannan and Erickson (1996a) reported that increased levels of dietary α-tocopherol (vitamin E) have been effective in delaying oxidation in catfish. This has also been reported with supplementation of ascorbic acid in catfish (Thed and Erickson, 1994). The increased protection against oxidation is due to an increase in antioxidant content in the animal’s flesh. This was demonstrated by Waagbo et al. (1993), who reported that the vitamin E content in salmon fillets was elevated by increasing the level of dietary supplementation. Also, Frigg et al.
(1990) reported significant sensory preferences for trout fillets with higher levels of vitamin E.

While often used to increase levels of antioxidants, dietary supplementation may also alter the total fatty acid composition of the fish. Bakir et al. (1993) found that the fatty acid composition of neutral lipids and phospholipids of white amur was dependent on fatty acids found in their diet. Similar results were reported by Waagbo et al. (1993) in working with Atlantic salmon fillets. Altering the diet of the fish can also be used to increase the amount of omega-3 fatty acids, which may be very desirable from a nutritional standpoint. After reporting that wild catfish had higher levels of omega-3, Chanmugam et al. (1986) suggested supplementing the feed of aquacultured catfish with greater levels of omega-3. Obviously, dietary supplementation may be used to decrease the levels of omega-3, as well, thus making the fish less susceptible to oxidative reactions.

D. Region Within Fish

Because factors such as the lipid composition and enzyme activity may vary in different regions in the fish, the rate of oxidation is dependent on location within the fish product (Freeman and Hearnsberger, 1994). Flick et al. (1992) reported that the rate of lipid oxidation in fresh or frozen round fishes decreases from a maximum in the skin, to intermediate rates in dark muscle and slowest in ordinary muscle tissue. These rates may be explained by the large concentration of pro-oxidants in the skin and a higher concentration of fat in the dark muscle compared to the light. Also, Hultin et al. (1992) found that the dark muscle of mackerel oxidized much faster than the light muscle during
frozen storage at -20°C. A higher lipid content and a higher concentration of pro-oxidants in the dark muscle explained this difference observed between regions.

E. Water Activity

Flick et al. (1992) reported that at low levels, water has antioxidative properties. This occurs because water reduces the ability of metals to act as catalysts. It can also assist in joining free radicals and promote nonenzymatic browning, which produces active antioxidants. Also, as water interacts with hydroperoxides, it reduces the rate of free radical formation. At higher water activities, however, the rate of oxidation is increased because of the greater mobility of oxidation components.

F. Fat Content

The fat content between fish may vary with numerous factors, including the season, species, sex, maturity, type of muscle, size, feed intake, activity level, geographical location were harvested, and if the animal was farm raised or wild (Nettleton, 1985; Flick et al., 1992). Differences in the amount of lipid present, as well as the type of lipid material, influence the rate of oxidation.

While the fat content may vary with numerous factors, the total level of lipid greatly affects the rate of oxidation. Fattier fish tend to have higher contents of polyunsaturated fatty acids (PUFA), including EPA and DHA. They experience greater susceptibility to oxidation due to their degree of unsaturation (Hwang and Regenstein, 1988; Ogata et al., 1996). While even saturated fatty acids undergo oxidative attack, the rate of such reactions is extremely slow and is often negligible (Harris and Tall, 1994; Nawar, 1996).
G. Degree of Butchery

Oxidation occurs much faster as the degree of butchery increases because tissue integrity is destroyed and cell contents, especially pro-oxidants, are better able to migrate. Another explanation for this increase is the larger surface area of further processed seafoods. This increased surface area, such as with minced fish, results in more exposure to oxygen and thus, oxidation progresses (Harris and Tall, 1994). Brannan and Erickson (1996) supported this explanation in their investigation of the oxidative stability of frozen minced and filleted catfish.

H. Exposure to Light

It has been well documented that exposure to visible, ultraviolet and gamma-radiation significantly increases the rate of oxidation. Such forms of light aid in the initiation reactions of oxidation. The influence of light can thus induce undesirable changes in food products, such as off-flavors, loss of vitamin activity, photosensitized oxidation of lipids and oxidation of pigments. Bjerkeng and Johnsen (1995) found that illumination significantly decreased the oxidative stability of frozen rainbow trout at -18°C. To prevent such oxidation and degradation of pigments, Bjerkeng and Johnsen recommended using packaging materials with UV-light absorbers or light-impermeable materials.

I. Presence of antioxidants/Presence of pro-oxidants

Antioxidants and pro-oxidants can have great influence on the rate of oxidative reactions. Pro-oxidants are typically thought of as transition metals such as copper, iron or nickel. Obviously, such metals are naturally occurring components of all muscle
tissues, including fish products. If present even at very low concentrations, these metals can act as catalysts for the initiation of oxidation. Functioning in this manner, pro-oxidants act to decrease the induction period and thus increase the rate of oxidation. Flick et al. (1992) reported that at 40°C, metal ions and hemin affected oxidation of fish in the decreasing order: Fe$^{2+}$, hemin, Cu$^{2+}$, and Fe$^{3+}$.

Antioxidants are also very influential in the oxidation process, functioning to lengthen the induction period. There are many forms of antioxidants, with many different functionalities. As this topic is extremely involved, it will be investigated more extensively in the following sections.

**ANTIOXIDANTS**

It is estimated that over 10,000 shelf stable products are available to the American consumer. In addition to these numerous products, scientists have also been able to increase the time frame that even a perishable product remains in a high quality and edible form. Interestingly, the use of antioxidants has played a major role in these advances (Brookman, 1991; Dorko, 1994).

The term antioxidant is often used to describe “a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell and Gutteridge, 1989). Dating back to the 1940s, antioxidants have been used in one form or another to extend the shelf life of numerous lipid containing products. While antioxidants cannot reverse the process, they can function by a variety of different mechanisms to retard oxidative reactions.

Antioxidants are obviously not the only method available to control oxidation;
however, it is oftentimes the most applicable and economical (Hwang and Regenstein, 1988). This is because only a little oxygen is needed to initiate and maintain oxidation, and to fully remove all oxygen is extremely difficult in terms of equipment needed and expense (Coppen, 1994). Antioxidants are frequently used in the seafood industry because they are generally effective, easily applied and inexpensive (Harris and Tall, 1994).

**Characteristics of an Ideal Antioxidant**

Ideally, there are several major attributes that an antioxidant should possess. First, the antioxidant should be safe in use. This is obviously the first and foremost concern to consumers, food scientists and government agencies. Second, the antioxidant should contribute no odor, flavor or color to the product it is intended to protect. The goal of using antioxidants is to retard oxidative reactions and preserve the original sensory properties of the food substance. Third, antioxidants should be effective at low concentrations. Government agencies, such as the FDA and USDA, provide strict guidelines as to the permitted concentration of various antioxidants in food products. Fourth, antioxidants should be easy to incorporate into the specified food product. Some antioxidants are more compatible with lipid base foods, while others are more water soluble. Such affinities should be considered when determining a proper antioxidant for a food system. Fifth, an ideal antioxidant should have good carry-through properties, meaning that it can survive cooking processes without thermally decomposing. This is important to impart oxidative stability to the final product after a cooking process. Finally, antioxidants should be relatively inexpensive (Coppen, 1994). Price is essential
because processors do not want to add excessive costs to a product by incorporating antioxidant materials.

**Limitation of Antioxidants**

While antioxidants can retard oxidative reactions, there are many misconceptions about the ability of these products. First of all, antioxidants cannot improve the flavor of poor quality lipid systems. Likewise, they cannot function to improve a system in which oxidative rancidity has already occurred. For these reasons, it is extremely important to add the antioxidant to the food product as early as possible (Brookman, 1991).

Other limitations of antioxidants include the prevention of hydrolytic rancidity. This type of rancidity results from the chemical hydrolysis of fat to produce free fatty acids. Since antioxidants will not prevent the formation of these free fatty acids, they cannot inhibit this type of rancidity. Also, in general, antioxidants cannot prevent microbial decay, although many phenolic antioxidants have been shown to have limited antimicrobial activity against select microorganisms (Coppen, 1994).

**Types of Antioxidants and Their Uses**

**Synthetic Primary Antioxidants**

**BHA.** Butylated hydroxyanisole (BHA) is one of the most commonly used commercial antioxidants. This hindered phenolic was the first antioxidant of its kind to be used in foods, beginning around the late 1940s. BHA is sold as white, waxy tablets consisting of a mixture of approximately 10% of 2-tert-butyl-4-hydroxyanisole (2-BHA) and 90% of 3-tert-butyl-4-hydroxyanisole (3-BHA). This antioxidant is very soluble in fats and oil products, however, it has poor solubility in water-based applications (Giese, 1996). With
a molecular weight of 180g/mole, BHA has a relatively low melting point, between 48-65°C. Also, BHA is steam volatile, so during frying applications much is lost. The residual BHA, however, has demonstrated impressive carry-through properties in baked and fried foods (Dorko, 1994). Interestingly, BHA may sometimes develop an undesirable pinkish color due to interactions with alkaline metals in the lipid system (Coppen, 1994).

**BHT.** Butylated hydroxytoluene (BHT) is commercially available as a white, crystalline solid that is very similar to BHA (Giese, 1996). However, BHT is not as effective as BHA, primarily due to steric hindrance created by two tert-butyl groups. Like BHA, BHT is also very soluble in fats and oil products and lacks solubility in aqueous phases. BHT is more steam volatile than BHA and thus it lacks favorable carry-through characteristics. In addition, in the presence of iron ions BHT produces an undesirable yellow pigment (Dorko, 1994; Madhavi et al., 1996).

**Propyl Gallate.** Propyl gallate is commercially sold as a white, crystalline powder that decomposes above 148°C. In a family of gallates including octyl and dodecyl, propyl gallate is one of the most widely used antioxidants. While less soluble in fats and oils than either BHA or BHT, propyl gallate has considerable solubility in water (Giese, 1996). Like BHT, propyl gallate lacks significant carry-through properties. Also similar to BHT, this antioxidant produces a purplish pigment in the presence of iron ions (Dorko, 1994; Madhavi et al., 1996).

**TBHQ.** First approved as a food antioxidant in 1972, tert-Butyl hydroquinone (TBHQ) is often used to stabilize fats and oils, in particular polyunsaturated crude vegetable oils. Its structure contains two para-hydroxyl groups that give the compound its antioxidant
activity (Madhavi et al., 1996). This white crystalline powder is very stable at high
temperatures and is less steam volatile than BHA and BHT, often making it the
antioxidant of choice for commercial fryer operations. TBHQ has good solubility in
various fat and oil systems and is slightly soluble in water. Unlike many other synthetic
antioxidants, TBHQ does not form a colored complex with copper or iron ions (Dorko,
1994; Giese, 1996).

**Synthetic Secondary Antioxidants**

The primary synthetic antioxidants presented function by ending the propagation of
the oxidation reaction. Scientists believe that these antioxidants can react with various
radicals to convert them to more stable products, thus quenching the chain of reaction.
Secondary antioxidants, however, function by different mechanisms. These antioxidants
may act as electron or hydrogen donors to the primary antioxidant radicals, therefore
regenerating the primary antioxidant. They may also function as metal chelators, oxygen
scavengers, or to decompose hydroperoxide to a non-radical product. Examples of
synthetic secondary antioxidants include various sulfites acting as oxygen scavengers and
ethylenediaminetetraacetic acid (EDTA) which functions as a metal chelator (Dorko,

**Synergistic Use of Synthetic Antioxidants**

When investigating the control of oxidation in the skin lipids of mackerel, Ke et al.
(1977) found that the order of effectiveness of synthetic antioxidants at 0.02%
concentration was TBHQ > BHA > BHT. With an exception of TBHQ, no single

A synthetic antioxidant is highly effective in retarding oxidation in fish products. This has led researchers to investigate other methods of using these synthetic products.

Scientists have observed that a combination of two or more antioxidants may sometimes better retard oxidation than the equivalent quantity of any one antioxidant. When this occurs, it is termed synergism (Hamilton, 1994). Many of the synthetic antioxidants are used synergistically because there are many free radicals produced during oxidation and different antioxidants have different efficiencies in dealing with these radicals (Coppen, 1994).

The two most widely synergistically used primary antioxidants are BHA and BHT. However, most often, at least one primary antioxidant is combined with at least one secondary antioxidant to achieve greatest stability. This is the case with BHA, BHT and propyl gallate, which are nearly always used with a metal chelator, such as EDTA to bind metals and prevent the formation of off-colors (Coppen, 1994; Giese, 1996). When used synergistically, synthetic antioxidants are quite effective at controlling oxidation in fish. Flick et al. (1992) reported that the shelf life of fresh salmonoids was significantly increased by treatments of BHA or TBHQ and EDTA.

**The Future of Synthetic Antioxidants**

Since the 1940s, synthetic antioxidants have been the primary means by which researchers have extended the shelf life of food products. In numerous studies, such as those mentioned, synthetic antioxidants have been used to significantly retard lipid oxidation in fish. However, despite many successes, the synthetic antioxidant market has in recent years come under attack because of suspected toxicity. In particular, scientists in
Japan have reported that BHA is responsible for pre-cancer lesions in test animals (Inatani et al., 1983; Brookman, 1991). In other international studies, BHA and BHT were identified as promoters of carcinogenesis (Chen et al., 1992). These studies have resulted in the banning of BHA and BHT in several international arenas. While no banning has occurred in the United States, there is growing concern from the Food and Drug Administration, especially with regards to BHA (Boyd et al., 1993; Brookman, 1991).

After all the publicity, the American public has also grown very concerned about the use of synthetic antioxidants (Brookman, 1991; Vareltzis et al., 1997). In fact, with the new interest in a health-oriented lifestyle, many Americans have begun to negatively view food additives in general. In the United States, various polls have shown that the top concern about the food supply is the use of food additives. As a result, there has been a major consumer trend towards more “natural” products, which are seen as safer (Dorko, 1994; Six, 1994).

Natural Antioxidants

The consumer trend towards more natural food products has had a major effect on the food industry. This trend is evident as the U.S. food industry witnessed a 175% increase in “all-natural” products between 1989 and 1990. During this same period, there was a 99% increase in the number of products claiming to be “additive or preservative

Henkel Corporation, 1991). Food processors obviously want to respond to consumer demands, thus there has been greater interest and research into natural food antioxidants. Although many questions remain unanswered, such research has resulted in the food industry having many more antioxidant choices.
Sources of Naturally Occurring Antioxidants

Researchers have found that many spices are potential sources of natural antioxidants. In a study by Chipault et al. (1956), scientists investigated the antioxidant activity of 78 samples representing 36 spices in lard. The samples were prepared by simple grinding or in some cases, more elaborate extraction procedures. Overall, it was determined that, among others, allspice, rosemary, cloves, thyme, sage, and oregano all demonstrated antioxidant activity. In other studies, Bracco et al. (1981) identified additional spices, including paprika, red chili, cinnamon leaf, turmeric, black pepper, dry ginger, nutmeg and cocoa shells as sources of natural antioxidants. Through several studies, Chipault et al. (1956) demonstrated that of all antioxidants from spices, rosemary (Rosmarinus officinalis L.) and sage (Salvia officinalis L.), both of the family Labiatae, have the greatest promise of commercial use. While this work was conducted many years ago, recent studies have also suggested such findings (Six, 1994).

In addition to various spices, other natural antioxidants have been isolated from oils, oilseeds, fruits, vegetables and protein sources, to name a few (Madhavi et al., 1996). Along with the previously mentioned rosemary and sage, tocopherols, ascorbic acid and citric acid have also shown great promise in retarding oxidation (Six, 1994). The antioxidant potency and mode of action of these naturally occurring products has been heavily debated and researched. Similar to the synthetic antioxidants, these natural products may function by different mechanisms, from that of primary to secondary antioxidants.
**Tocopherols**

Tocopherols, often considered to be the most effective natural antioxidants, function by scavenging lipid free radicals, hydroxyl radicals, hydroperoxy radicals and singlet oxygen (Erickson, 1991). While they may have superior antioxidant ability, Hwang and Regenstein (1988) reported that tocopherols may act as pro-oxidants if used in too high concentrations.

Tocopherols, all of which exhibit vitamin E activity, are widely found in vegetable oil and fats, with the prime commercial industry being soybeans. Other rich sources of tocopherols are wheat germ, corn, sunflower seed, rapeseed, soybean oil, alfalfa and lettuce. These antioxidants are extracted from tocopherol-rich deodorized distillates, by-products of steam stripping of vegetable oils during final processing. This produces tocopherols that are pale yellow, clear, viscous, oily substances that are insoluble in water and fat/oil soluble (Six, 1994, Madhavi et al., 1996).

The antioxidant activity of the $\alpha$, $\beta$, $\gamma$, and $\delta$-tocopherols significantly change with temperature. Madhavi et al. (1996) reported that around 37°C, the antioxidant activity of tocopherols are in the order: $\alpha > \beta > \gamma > \delta$. In fish applications, the most widely used form is $\alpha$-tocopherol (Fang and Wada, 1993; Hopia et al., 1996). However, at higher temperatures, between 50-100°C, the potency of the various tocopherols is reversed, with $\delta$ having the strongest inhibitory effect (Madhavi et al., 1996).

Tocopherols may be incorporated into seafood products by many methods. One possible method is through dietary supplementation. Waagbo et al. (1993) reported that
dietary tocopherols improved the oxidative stability and sensory perception of salmon fillets. While dietary supplementation may be feasible for aquacultured products, wild fish may be treated with tocopherols through sprays, injections, or dips. Ogata et al. (1996) found that α-tocopherol inhibited the formation of malonaldehyde, a toxic oxidation by-product, from DHA by 28%, but it did not show any antioxidative activity against EPA oxidation. In this study, it appears that α-tocopherol inhibited oxidation more effectively from lipids with less unsaturation. In another study, Erickson (1993) reported the effectiveness of tocopherols in retarding lipid oxidation in channel catfish. This investigation noted that a decrease in the levels of tocopherols was correlated with an increase in oxidation rate.

While tocopherols have shown great promise for use in the seafood industry, they are typically not as effective as popular synthetic antioxidants. Working with various antioxidants, including α-tocopherol, Ogata et al. (1996) demonstrated that BHT was the most effective at inhibiting oxidation in omega-3 fatty acids. To improve the effectiveness of tocopherols, they are often used synergistically.

**Rosemary**

Commercial processes have been developed to produce purified antioxidants from spices such as rosemary. There are two published methods to produce rosemary antioxidant extract; however, many private companies have developed their own patented procedures. In a published procedure developed by Chang et al. (1977), crude rosemary antioxidant is extracted using ethyl ether under refluxing conditions. Following the
removal of the solvent, the crude material is repeatedly washed and then dissolved in methanol and bleached with activated carbon by stirring for 15 minutes at 60°C. This yields a light-colored, fine purified powder with minimal odor of the original spice. Using vacuum distillation, researchers reported an odorless and tasteless product can be manufactured. Interestingly, in more recent studies, Chen et al. (1992) noted that the antioxidant activity of the extract was very dependent on the solvent system. Other possible solvent systems include acetone and hexane among others.

In another process developed by Bracco et al. (1981), rosemary leaves were milled to a particle size near 2 mm and whirl-sieved, collecting particles smaller than 600 µ. Suspending the particles in peanut oil, a moisture content of 2-4% is maintained. The principal step in this process is when the lipid suspension is microionized in a ball mill. This step serves to rupture the cell wall, free the antioxidant from the protoplasm, and permit contact of the lipid phase and the microionized protoplasm. A lipid suspension is thus produced for the molecular distillation step. Since the dispersed antioxidant compounds have a lower molecular weight than the triglycerides of the oil used, they can be physically separated using molecular distillation (Bracco et al., 1981).

The first research of the antioxidant potential of rosemary was reported by Ostric-Matijasevic in 1955 (Houlihan et al., 1984). Through the years, many compounds possessing antioxidant character have been isolated from rosemary. Those isolated have included many phenolic acids, diterpenes and flavonoids, all of which are phenolic compounds possessing significant antioxidant character. While the antioxidative behavior
and synergistic action of most of these compounds remain unknown, scientists have made notable advances (Cuvelier et al., 1996).

Brieskorn et al. (1964) isolated and determined the structure of the first major antioxidant from rosemary, a phenolic diterpene named carnosol (Houlihan et al., 1984). Since then, Inatani et al. (1983) have isolated and characterized several additional antioxidants, including epirosmanol, isorosmanol and rosmanol. These three compounds were determined to be more potent antioxidants than carnosol and in lard samples it was reported that they demonstrated an activity more than four times higher than BHA and BHT. Following the isolation of these antioxidants, several others have been reported, including rosmaridiphenol and rosmariquinone. Houlihan et al. (1985) isolated these compounds and determined their structures and antioxidant potency using 0.02% concentrations in lard. This study found the antioxidant activity of rosmariquinone and rosmaridiphenol to be superior to the same concentration of BHA, but slightly less than BHT.

In more recent studies, scientists have attempted to clarify the potency of the numerous phenolic compounds from rosemary. Cuvelier et al. (1996) reported that the most effective antioxidative compounds in rosemary are carnosol, rosmarinic acid, and carnosic acid, followed by caffeic acid, rosmanol, rosmadial, genkwanin and cirsimaritin. Unfortunately, there is still a great deal of conflicting results. Aruoma et al. (1992) reported that 90% of the antioxidant character in rosemary extract is attributed to the phenolic diterpenes carnosol and carnosic acid. Yet Cuvelier et al. (1996) have reported
the principal components to be equally rosmarinic acid, carnosol, and carnosic acid. However, there are explanations for these discrepancies. Although extensive research has been undertaken on the antioxidant character of rosemary, it has oftentimes been difficult to realistically assess the feasibility of such usage. This is primarily due to the variability in test results based on the type of test system used, for example bulk oil versus oil-in-water emulsions, and method of rosemary extraction. The type of test system can greatly influence results as a particular antioxidant will have varying effectiveness in these different systems. The method of rosemary extraction can also cause sizable variability. Frankel and Huang (1996) and in another study, Chen et al. (1992) determined that the potency of various antioxidants in rosemary are dependent on, among other factors, the solvent used to extract the compounds. Obviously, more research is needed to better understand the components present and their functionalities.

Rosemary, with its numerous antioxidative compounds, can provide significant stability to the lipids in fish products. Vareltzis et al. (1997) investigated the effectiveness of rosemary extract on the oxidative stability of filleted and minced fish during four months of frozen storage at -18°C. Using both a low and high fat fish, the results indicated that rosemary extract significantly retarded the oxidation in filleted and minced samples for both species. While this study may indicate the effectiveness of using rosemary extract in the fish industry, the duration of the test period was not satisfactory. It is generally accepted that filleted fish will have a four month shelf life at -18°C. Therefore, more research is needed to better assess the efficacy of rosemary extracts over longer storage periods.
Ascorbic Acid

Widely found in fruits and vegetables, L-ascorbic acid, commonly known as vitamin C, is readily available. Ascorbic acid is a reducing agent that aids in preventing oxidation by acting as a free radical terminator or hydrogen donor, oxygen scavenger, and metal chelator. Acting as a hydrogen donor, ascorbic acid is able to regenerate phenolic antioxidants such as rosemary or BHA (Hwang and Regenstein, 1988).

In meat products, ascorbic acid is used not only to retard oxidation, but also to prevent discoloration by delaying the formation of metmyoglobin. In fish products, ascorbic acid is effective when used alone, or even more so when used synergistically. In addition to retarding oxidation, ascorbic acid also delays rusting, a yellowing of fresh or marine fish during frozen storage (Harris and Tall, 1994). Madhavi et al. (1996) reported that during frozen storage, ascorbic acid increased the shelf life of freshwater lake herring from three months to at least seven months. In -7°C frozen storage of menhaden mince, Hwang and Regenstein (1988) reported that ascorbic acid was more effective than tocopherols, rosemary extracts or TBHQ in retarding oxidation. Interestingly, Thed and Erickson (1992) have reported that ascorbic acid acts as a pro-oxidant at low levels and as an antioxidant at higher concentrations.

Citric Acid

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is a white, odorless solid that is naturally found in plants and animals (Madhavi et al., 1996). It is widely used, often along with primary antioxidants or oxygen scavengers, as an acidulant and a metal chelator in food products. Madhavi et al. (1996) reported that citric acid with BHA or
Propyl gallate was effective in controlling oxidation in frozen minced Atlantic whiting for up to eight weeks. Researchers also reported mixtures of ascorbic and citric acids significantly retard oxidation in dried herring. Furthermore, a dip may also be prepared from citric and absorbic acids to prevent rusting in the surface tissue of oily fish (Madhavi et al., 1996).

**Synergistic Use of Natural Antioxidants**

In most research, natural antioxidants used alone were not as effective as synthetic antioxidants such as BHA or BHT. To increase their ability to inhibit or retard oxidation, most natural antioxidants are used synergistically with other natural or synthetic antioxidants. With such a large number of free radicals produced during oxidation, synergism is beneficial because it takes advantage of the efficiencies of various antioxidants in dealing with these radicals (Coppen, 1994).

Many researchers have investigated the use of natural antioxidants in combination with the synthetic types, such as BHA, BHT or TBHQ. Boyd et al. (1993) reported that rosemary extract used synergistically with TBHQ was less effective than TBHQ with ascorbic acid in controlling rancidity in frozen cooked fish flakes. The rosemary and TBHQ mixture was more effective, however, than when rosemary was used alone.

With increasing interest in "all-natural" products, more researchers are investigating the inhibition of oxidation using solely natural antioxidants. Using a mixture of rosemary and α-tocopherol, Fang and Wada (1993) reported that used synergistically, these antioxidants were more effective in inhibiting Fe⁺² catalyzed fish lipid oxidation than when used alone. Researchers also reported that when used synergistically, rosemary may
act as an electron reservoir to regenerate $\alpha$-tocopherol from $\alpha$-tocopheroxy radicals.

Several commercial antioxidant blends consist of combinations of citric acid, ascorbic acid, tocopherols, and rosemary extracts to retard oxidation reactions in a variety of foods, including numerous fish products (Thomas Jones, Kalsec, Inc., personal communication, 1997). By using such blends, oxidation may be retarded in several ways, including the chelating of metals, scavenging for oxygen and quenching radical ion reactions.

**Advantages of Using Natural Antioxidants**

Antioxidants from natural sources have two major advantages. First, the most obvious advantage of using natural antioxidants is that consumers readily accept these products as safe (Rajalakshmi and Narasimhan, 1996). When the American consumer reads a label, most are more comfortable with names such as rosemary or sage, for example, than *tert*-butyl hydroquinone or butylated hydroxyanisole. Also, interesting enough, the majority of Americans will simply think of natural antioxidant ingredients as spices or flavorings, and will not think of these ingredients as actual “preservatives” or

The other major advantage of using natural antioxidants is from a government regulation standpoint. In many countries, legislation does not require safety tests if the antioxidant is from a generally recognized as safe food product (Rajalakshmi and Narasimhan, 1996). While many synthetic antioxidants are undergoing speculations of causing adverse health problems, few have voiced concern about natural products.
Disadvantages of Using Natural Antioxidants

While using natural antioxidants is advantageous in many regards, like anything else, there are drawbacks as well. The first concern is expense. These natural antioxidant products are usually more expensive if they have undergone standardization and purification processes. If the antioxidant has not undergone such processes, it will be much less expensive, however, it is typically less efficient as well. In addition to price concerns, natural variations in geographic location, season, maturity, and a host of other factors contribute to different plants having more or less antioxidant potency (Brookman, 1991; Rajalakshmi and Narasimhan, 1996). These variations make it more difficult to work with these natural products compared to the synthetic types, which are very consistent in quality.

Another consideration when using natural antioxidants is that the safety of these products has not been adequately studied. While the antioxidants may be derived from GRAS substances, toxicity studies may still need to be performed. Once it has been extracted, the concentration of the antioxidants being consumed is much greater than normal. A substance may be natural and commonly found in a food, however there is no guarantee that it is safe at such high concentrations (Six, 1994).

The last major concern when using natural antioxidants is that many of these products may impart a color, aftertaste, or off-flavor to the product in which it is incorporated (Brookman, 1991). Food processors typically want to have the protection of antioxidants without any indication of their presence. Often, this is not possible when using natural products due to the extraction and purification methods. Therefore, natural
antioxidants are often used in foods when they are compatible with the texture, color and flavor of the end product. To make these antioxidants more feasible for a wider variety of foods, scientists must continue to conduct extensive research into methods of obtaining more desirable natural antioxidant products (Rajalakshmi and Narasimhan, 1996).

The Future of Natural Antioxidants

With several European and Asian countries banning the use of some synthetic antioxidants and re-evaluating the use of others, the interest in natural antioxidants will surely increase. Even in America, the public has become much more health conscious and concerned about the food they consume. This has resulted in a rejection of many “synthetic” products and a tremendous interest in “natural” foods, including natural antioxidants (Dorko, 1994; Six, 1994). To increase profitability, the food industry must respond to such consumer concerns and trends.

Currently, there is a significant increase in the use of natural antioxidants in nearly every segment of the food industry, and seafood is no exception. In future years, the American public will most likely see an even greater increase in the number of food products containing natural antioxidants. There is a critical need, however, for more research into the use of various natural antioxidants in food systems. Through continued research, food processors will be able to determine the most effective treatments for specific food systems. The future also holds promise for genetic manipulation of natural sources by breeding, genetic mutation, or selection to increase the antioxidant concentration (Six, 1994). The true success of natural antioxidants, however, may depend on action from the federal government.
Federal Regulation of Antioxidants

Synthetic. The Food and Drug Administration and the United States Department of Agriculture have the responsibility of regulating the use of antioxidants. Specifically, antioxidants are regulated under the Food Additives Amendment of 1958. When this amendment was enacted, BHA, BHT, propyl gallate and TBHQ were given the status of generally recognized as safe. GRAS regulations, however, limited the concentration of these antioxidants to 0.02% (200 ppm) of the lipid material in the food product. If these antioxidants are used synergistically, the total antioxidant content cannot exceed 0.02%. Interestingly, federal law prohibits the synergistic use of TBHQ and propyl gallate because of possible interactions (Dorko, 1994).

After international concern, in 1978 the FDA re-evaluated the health risks of BHA. At the time, it was determined to be safe at normal levels of human consumption. However, since this time, new issues have risen concerning the toxicity of BHA. While this antioxidant is still permitted in American products, it has been banned in several European and Asian countries (Inatani et al., 1983; Brookman, 1991). Unlike BHA, there have been few safety concerns with BHT, TBHQ or propyl gallate. FDA regulations require that antioxidants must be declared on the ingredient labels of products, followed by an explanation of their purpose (Giese, 1996)

Natural. Federal law states that tocopherols are permitted in food products according to good manufacturing practice (21 CFR 182.3890). These antioxidants can be used in products containing animal fats or combinations of such fat with vegetable fats at 0.03%, or 300 ppm (9 CFR 318.7). Ascorbic acid is also permitted in foods and has a generally
recognized as safe (GRAS) status for use as a chemical preservative. There are currently no restrictions on its usage levels (21 CFR 182.3013). Also, citric acid is approved and has a federal usage levels of 0.1 to 0.3%.

Currently, there is no known FDA filing for any isolated antioxidants from plant sources to be used as antioxidants. Such natural antioxidants would be considered an additive and thus extensive toxicological testing would be required. This would make such a filing an extremely costly process (Six, 1994). Despite plant extracted antioxidants not being officially approved as antioxidants in the United States, these products are still commercially produced and often used in industry. It has been reported that some companies use the term “oxidation inhibitors” instead of “antioxidant”. This allows for the use of plant extracted antioxidants without coming under the FDA guidelines for “antioxidants.” For labeling purposes, such oxidation inhibitors are typically labeled as “natural flavoring” or “rosemary extract”, for example (anonymous, personal communication, 1997). In the future, it is reasonable to expect that the United States will develop and enforce stricter guidelines for natural plant antioxidants.

THE MARKETING OF FROZEN FISH

American fish processors are relying increasingly on frozen storage of seafood products. While long-term storage may have several disadvantages, there are many reasons for freezing fish products. No and Storebakken (1991) reported a sizable increase in production of aquacultured salmonoids over the last fifteen years, with production levels expected to rise. Erickson (1992) also noted such a trend in the catfish industry. In fact, as reported by Flick et al. (1991), there is a general increase in consumption of all fish
throughout the industry. With growing interest and greater consumption of fish products, processors have responded by increasing production volumes. While this will likely bring more revenue to the fish industry, it also brings the risks often associated with market instability. If at any time there are excessive supplies of fish products, the market price may shift downward and processors may lose potential profit (Erickson, 1993).

Erickson (1993) reported that the answer to market instability may be to freeze fish products. By freezing products, processors can better control how much fish saturates the market, and thus the commodity will have more price stability (No and Storebakken, 1991; Erickson, 1993). In addition to regulating the market supply, frozen storage of fish products may become necessary as the distribution area increases for many processors (Erickson, 1992). Finally, frozen storage may also be critical in situations such as in the wild salmon industry. Processors would like to capture sufficient fish to supply the market until the next season, nearly a year later (Thomas Jones, Kalsec, Inc., personal communication, 1997). To accomplish such endeavors, frozen storage is a necessity. Unfortunately, long term freezing has long been associated with product deterioration due to oxidative reactions. This is obviously a major concern of food processors.

**Oxidation in Frozen Fish Products**

While frozen storage of fish products may have many advantages, there are obvious disadvantages. Long term freezing has long been associated with product deterioration due to oxidative reactions. The shelf life of frozen fish products depend on several factors, including the temperature of storage, species of fish and degree of butchery (Harris and Tall, 1994). In general, however, frozen storage of fish products
results in many undesirable changes in tissue, including development of off odors and flavors, changes in texture, water holding capacity, color and nutritive properties (Erickson, 1992).

**Protein Damage**

Due to the high concentration of protein in muscle tissue and its close proximity to numerous free radical initiators, such as iron and copper, muscle proteins are very susceptible to interactions with hydroxyl radicals. These hydroxyl radicals are extremely unstable and will react with nearly any tissue component with which it comes in contact. Srinivasan and Hultin (1995) reported that the side chains of proteins are attacked by hydroxyl radicals to produce protein free radicals. Molecular oxygen can then react with these protein free radicals to produce peroxyl radicals. These radicals may then abstract a hydrogen from another molecule to form a protein hydroperoxide. Similar to lipid hydroperoxides, protein hydroperoxides are unstable and can decompose to produce additional products.

In general, when attacked by free radicals, proteins are chemically crosslinked and their electric charge is altered (Eun et al., 1994). This results in less stable proteins with a greater susceptibility to denaturation. The oxidized proteins have reduced solubility and water-holding capacity compared to non-oxidized proteins (Srinivasan and Hultin, 1995). This oxidation and denaturation of protein results in a toughened texture in fish products and changes in nutritional value (Flick et al., 1992; Jadhav et al., 1996). Obviously, such undesirable changes may result in consumer rejection of frozen stored fish products.
**Flesh Discoloration**

Carotenoids, such as astaxanthin and canthaxanthin, are responsible for the characteristic pigment of many seafood products, including salmon, red snapper, redfish and tuna (No and Storebakken, 1991). Like other animals, fish are not capable of synthesizing these pigments *de novo*, so they must be obtained from the diet. In aquacultured products, carotenoids are often used as dietary supplements to increase consumer acceptability of the product (Ingemansson *et al.*, 1993). Once consumed, the pigments are deposited into the fish flesh in unesterified form, where it binds with actomyosin (Bjerkeng and Johnsen, 1995). Unfortunately, during frozen storage of many fish products, the flesh becomes discolored because of a loss of pigments (No and Storebakken, 1991).

In addition to lipid oxidation, Ingemansson *et al.* (1993) reported that a major problem in the frozen storage of fish species is the oxidation of pigments. Carotenoids are actually considered as antioxidants, since they often protect unsaturated lipids by themselves being oxidized. Unfortunately, while they may protect lipids, carotenoid oxidation leads to discoloration of the food product. Overall, it has been reported that carotenoids can be oxidized by both enzymatic (lipoxygenases or peroxidases) or non-enzymatic (light, heat, oxygen) oxidation reactions (Ingemansson *et al.*, 1993). Terao (1989) and Miki (1991) demonstrated the effectiveness of carotenoids as singlet oxygen quenchers and radical scavengers. Also, Bjerkeng and Johnsen (1995) found that astaxanthin was effective in extending the shelf life of frozen rainbow trout. These
researchers also reported that carotenoids protect lipids from photo-oxidation by acting as preferred substrates.

Discoloration during frozen storage of fish is obviously a major concern to those in the seafood industry. These carotenoids are very important in the marketing of numerous seafood products because their intensity is critical to the desirability and overall consumer acceptance of the product (No and Storebakken, 1991). While carotenoids may be useful in extending the shelf life of frozen fish products, their discoloration is sometimes more detrimental than the increase in lipid oxidation products.

**Sensory Quality**

Oxidation reactions produce numerous compounds from the decomposition of hydroperoxide, which itself has no sensory attributes. These compounds are often associated with off odors and flavors that can prove detrimental to the marketability of fish products, especially those that have been frozen. Yasuhara and Shibamoto (1995) reported that volatile aldehydes such as formaldehyde, acetaldehyde and propanal were isolated from fish flesh after heat treatment. In addition to these aldehydes, Kawai (1996) identified such compounds as ethanal, acetone, ammonia, formic acid, acetic acid, hydrogen sulfide, and dimethyl disulfide, among others. While there are numerous possible oxidation products, all of these compounds are associated with a reduced acceptance rate as their threshold concentrations are reached.

These off flavors and odors are extremely important because the sensory attributes of food substances are perhaps the most influential factor in consumption. From information learned in focus group studies, Peavey *et al.* (1994) reported that consumers
felt freshness was the paramount concern when purchasing seafood items. Consumers assessed the freshness of products in terms of appearance and smell, with numerous panelists perceiving fish that smelled “fishy” as not being fresh.

Although frozen fish products allow greater flexibility to seafood processors, to effectively market these products, the industry must be able to convince consumers that frozen products are not nutritionally or otherwise inferior to fresher fish. Through proper use of antioxidants and temperature control, fish processors can reduce the extent of oxidation, and thus many of the detrimental sensory attributes in frozen fish products.

**Minced Fish from Recovery Operations**

In addition to the frozen shelf life of fish products, seafood processors are also concerned with producing more products from recovery operations. It is estimated that the yield of fish fillets from a whole fish is around 35%, depending on the species. This estimate uses both hand and machine filleting procedures (Nettleton, 1985). Many seafood processors are currently marketing the fillets, a more valuable product per weight basis, and discarding the remainder of the backbone, frame, belly flap, and other remnants of the fish (Flick, 1998). In an effort to increase profitability, many of these items currently being discarded could be further processed using a mechanical bone separator. Brannan and Erickson (1996b) reported that 50-200% more usable meat could be obtained per fish than is currently being utilized. In addition to significantly increasing the usable meat obtained per fish, this technology would also greatly reduce waste costs (Nettleton, 1985; Brannan and Erickson, 1996b). This recovery operation could therefore
increase profit in two ways: through marketing of the minced product from currently discarded food material and by lowering waste processing costs.

While mechanical mincing procedures could prove extremely beneficial to fish processors, the industry must make such products more acceptable to the public. To achieve this objective, the seafood industry will have to effectively control oxidative reactions in the product (Erickson, 1993). Nettleton (1985) reported that minced fish prepared from whole headless frames is higher in fat, cholesterol and calcium than minced prepared from fillets. Such mince also has a higher concentration of metals, such as iron and copper. With higher levels of fat and more pro-oxidants, along with greater surface area, minced products are extremely susceptible to oxidative reactions. Freezing minced tissue will slow oxidation, however, low temperatures alone are not enough to significantly retard this reaction (Hamilton et al., 1994; Nawar, 1996). However, if the minced product is treated with antioxidants and frozen, the rate of oxidation can be better controlled, the shelf life may be significantly extended, and the acceptance of such products may increase.
REFERENCES


SECTION 2

The Efficacy of Using Natural Antioxidant Blends to Control Oxidative Rancidity in Filleted and Headed and Gutted Rainbow Trout (Oncorhynchus mykiss) During Frozen Storage

by

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ABSTRACT

The antioxidant properties of various blends of rosemary extract and tocopherols, either alone or with citric and ascorbic acid, were compared to each other in filleted and headed and gutted rainbow trout. Oxidation was measured by following changes in thiobarbituric reactive substances, conjugated diene hydroperoxides, detection of aldehydes using solid phase microextraction and mass spectroscopy, texture, drip loss, pH and sensory evaluation. While researchers determined that rainbow trout at -29°C does not undergo extensive oxidation, natural antioxidant blends were effective at retarding the development of conjugated diene hydroperoxides and malonaldehyde. Sensory evaluation indicated that treated samples were less oxidized; however, in subsequent studies, it was determined that the herbal flavor notes associated with natural antioxidants complicated the ability of the experienced panel to judge extent of oxidation. Also, further consumer sensory panels indicated that after 12 months frozen storage, the treated and control samples both remained acceptable. No texture differences were noted over storage time or between control or variable treatments.
INTRODUCTION

The intake of omega-3 fatty acids, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been associated with a lowered risk of many medical problems, including heart disease (Kinsella, 1986), stroke, numerous cancers, tumorigenesis and diabetes, among others (Waagbo et al., 1993; Garcia, 1998). These reported health benefits have resulted in several countries, including Denmark, Canada, Japan and England, establishing recommended daily allowances for this specific group of fatty acids (Garcia, 1998).

Such reported health benefits have been extremely advantageous to the fish industry, since fish contain the most concentrated natural form of omega-3 fatty acids. Throughout the world, greater interest in omega-3 fatty acids has led to significant increases in fish consumption. Such increases have been reported in salmonoids (No and Storebakken, 1991), and catfish (Erickson, 1992), but this is actually a general trend throughout the fish industry. In fact, Flick et al. (1991) reported that the United States has a per capita annual fish and shellfish consumption of over 16 pounds, with this value expected to exceed twenty pounds by the year 2000. With growing interest and greater consumption of fish products, processors have responded by increasing production volumes. While this will likely bring increased revenue to the fish industry, it also brings the risks often associated with market instability. If at any time there are excessive
supplies of fish products, the market price may shift downward and processors may lose potential profit (Erickson, 1993).

The answer to market instability may be to freeze fish products (Erickson, 1993). By freezing products, processors can better control how much fish saturates the market, and thus the commodity will have more price stability (No and Storebakken, 1991; Erickson, 1993). In addition to regulating the market supply, frozen storage may become necessary as the distribution area for many processors increases (Erickson, 1992). Finally, frozen storage may also be critical in situations such as in the wild salmon industry. Processors would like to capture sufficient fish to supply the market until the next season, nearly a year later (Thomas Jones, Kalsec, Inc., personal communication, 1997). To accomplish such endeavors, frozen storage is a necessity. Unfortunately, long term frozen storage has been associated with product deterioration due to oxidative reactions. This is obviously a major economic and quality concern of seafood processors.

Due to the high content of polyunsaturated fatty acids, frozen storage of fish products typically results in many undesirable changes in tissue, including development of off odors and flavors and changes in texture, water holding capacity, color and nutritive properties (Eun et al., 1994; Thed and Erickson, 1994). Fish processors may use several methods to control oxidation such as vacuum packaging, glazing, and modified atmospheric packaging (MAP). However, although many choices are available, when evaluated on cost, effectiveness and ease of use, antioxidants are the most widely used method to control oxidation (Harris and Tall, 1994). Antioxidants retard lipid oxidation by acting as radical scavengers, oxygen quenchers, or metal chelators.
There are two major classes of antioxidants available, synthetic or natural (Rajalakshmi and Narasimhan, 1996). While synthetic antioxidants, such as BHA, BHT and TBHQ, have traditionally been used to control oxidation there has been growing concern about the toxicity of these synthetic products, especially BHA. In fact, after reports of BHA causing tumor growth in the forestomach of rats, Japan banned this antioxidant and several other countries are re-evaluating the use of synthetic antioxidants (Inatani et al., 1983; Brookman, 1991). These actions have obviously alarmed the public and have resulted in increased efforts to obtain antioxidant compounds from natural sources, such as spices and plants (Giese, 1996).

Natural antioxidants have been obtained from various spices such as rosemary, oregano, nutmeg, sage, and others whose main antioxidant activity is due to various phenolic compounds (Rajalakshmi and Narasimhan, 1996). Other natural antioxidants include tocopherols, citric acid and ascorbic acid. With a major consumer trend towards more “natural” products, there is tremendous interest in natural antioxidants because they are perceived as safer (Dorko, 1994; Six, 1994). While perhaps calming consumer fears, food processors still question if these natural products will be the answer to the on-going question of how food quality is preserved.

The objective of this study was to determine the efficacy of using various natural antioxidant blends, consisting of rosemary extract, tocopherols, citric acid and ascorbic acid, to control oxidative rancidity during frozen storage of filleted and headed and gutted rainbow trout (Oncorhynchus mykiss). The information gained from this study will be used in the commercial salmon industry to provide consumers with consistent, good
quality salmon products throughout the year. Such conclusions may be made due to the close genetic relation between rainbow trout and salmon, both being salmonoids.

Although this research will be used primarily in the salmon industry, it is relevant to the long-term frozen storage of fish products in general.

MATERIALS AND METHODS

Fish Supply

Fresh mature rainbow trout were obtained from an aquaculture facility in western Virginia. To simulate commercial salmon fishing practices, the fish were held on ice for three days prior to further processing. Whole rainbow trout fish were randomly assigned to one of two groups: headed and gutted (n=120); or filleted (n=405). The fillet samples were cut to obtain two skinless fillets per fish. Fillets were packed in plastic freezer bags, with ten fillets per bag. The fish temperature did not exceed C during processing. All samples were iced packed in insulated containers and transported to laboratory facilities (2.5-3 h).

Antioxidant Preparation

Five natural antioxidant blends were obtained from Kalsec Inc., Kalamazoo, MI. The antioxidants used were: 1) Duralox Blends AMNC-202, 2) AMNC-203, 3) ANC-204, and 4) MANC-213, and 5) Herbalox Seasoning KS-15. The Herbalox Seasoning is a mixture of rosemary extract and tocopherols, while the Duralox blends contain rosemary extract, tocopherols, and citric and ascorbic acids. The ingredient proportions of these blends varied. Using pre-chilled water (3-7°C), solutions of antioxidants were
prepared at the manufacturer's recommended usage rates. These rates were: 10% for ANC-204 and MANC-213; 13.3% for KS-15, and 15% for AMNC-202 and AMNC-203.

**Fish and Fillet Treatment and Processing**

**Headed and Gutted**

To prepare samples for glazing, headed and gutted fish were placed in a -29°C industrial freezer until the exterior of the samples were lightly frozen. Samples were removed from freezer and randomly assigned to one of three treatment groups: control, AMNC-202 or MANC-213. Control fish were glazed using a traditional commercial dextrose glazing solution (Roquette America, Inc., Keokuk, IA). The antioxidant treated fish were briefly dipped in their respective solutions, either AMNC-203 or MANC-213, to produce an antioxidant glaze. Several representative fish from each treatment group were weighed both before and after glazing to determine the increase in fish body mass, henceforth termed percent pick-up. After glazing, all fish were shatterpacked in boxes (Frontier Packaging Inc., Seattle, WA) and returned to the -29°C freezer.

**Filleted**

Samples were removed from gallon size freezer bags and randomly assigned to one of six groups: control, Duralox Blends AMNC-202, AMNC-203, ANC-204, MANC-213 or Herbalox Seasoning KS-15. The control fish were shatterpacked in boxes and placed in an industrial -29°C freezer. The treated samples were randomly assigned to batches of 10-20 fish, placed in a large colander, weighed and dipped into their respective antioxidant treatment for one minute with agitation. Following dipping, the samples were allowed to
drain of excess solution for 0.5 minutes and re-weighed. The samples were then shatterpacked in boxes and returned to the -29°C freezer.

**Percent Pick-Up**

Weighing the samples both before and after treatment allowed for the determination of percent pick-up. This value is the percent of mass gained by the sample following treatment with glazing solution or antioxidant dip. It is calculated as:

\[ \% \text{ Pick-Up} = \left( \frac{\text{Final Mass} - \text{Initial Mass}}{\text{Final Mass}} \right) \times 100 \]

**Evaluation Periods**

Sufficient samples were removed from frozen storage at two-month intervals for the fillets and six-month intervals for headed and gutted samples, over a twelve month frozen storage period. All headed and gutted evaluations, with an exception of drip loss, used fillets obtained from the samples after they were thawed. All measurements were made in triplicate for two duplicated experiments.

**Percent Drip Loss**

Samples were removed from the -29°C freezer, placed in pre-weighed freezer bags and the net frozen mass determined. The samples were allowed to thaw overnight in an industrial refrigerator unit (-1°C). Thawed samples were removed from the freezer bags and placed on a wire mesh screen for 10 min to equilibrate. The samples were then re-weighed and drip loss was calculated as:

\[ \% \text{ Drip Loss} = \left( \frac{\text{Loss in Mass}}{\text{Initial Mass}} \right) \times 100 \]
**pH**

Using a composite sample of three fillets, the muscle pH was determined using the procedure described by Rhee *et al.* (1984). Minced rainbow trout (10 g) was homogenized with 100 mL distilled, deionized water in two 25-second periods at high speed using a Waring commercial blender. The pH of the slurry was measured using a pH meter (Accumet, Model 10) with a glass electrode.

**Conjugated Diene Hydroperoxides (CDHP)**

Using a composite sample of three fillets, a solution of chloroform/methanol (2:1 v/v) (Fisher Scientific, New Jersey) was used to extract lipid from 1.00 ± 0.02 g of muscle tissue as described by Erickson (1992). A 5 mL aliquot of this extract was dispensed into a clean test tube and washed with 1 mL of 0.88% KCl (Fisher Scientific, New Jersey). Following aspiration of the upper layer, 2 mL of the lower layer was evaporated to dryness under nitrogen gas, and reconstituted in 5 mL of cyclohexane (Fisher Scientific, New Jersey). Using quartz cuvettes, the absorbance was read at 232 nm using cyclohexane as a reference (Shimadzu, UV-2101PC, Maryland).

**2-Thiobarbituric Acid Reactive Substances**

Using a composite of three fillets, the concentration of 2-thiobarbituric acid reactive substances (TBARS) was determined by the distillation method developed by Tarladgis *et al.* (1960). Where applicable, procedures were performed under nitrogen, and n-propyl gallate and ethylenediaminetetraacetic acid (Sigma Chemical Co., Missouri) were used to inhibit further oxidation during testing. A standard curve was prepared with 1,3,3,3,-tetraethoxypropane (Sigma Chemical Co., Missouri). Absorbance of samples was read at
532 nm (Spectronic Genesys, Model 20). TBARS values were reported as μmole malonaldehyde per kg sample.

**Texture**

Breaking strength was measured on fillets using an Instron model 1011 (Instron Ltd., Buckinghamshire, England). Testing was performed on samples that had been cooked as whole fillets in a covered baking dish at 177°C for 20 min in a convection oven (Blodgett, Mark V Model). After cooling to room temperature, a uniform 6 cm² area (2 cm x 3 cm) was cut from the head, middle and tail region of each fillet sample. Each sample was weighed and placed in the Kramer shear press cell with skin-side down and muscle fibers running perpendicular to the blades. Using a 500 kg load transducer, samples were stretched at 10 cm min⁻¹ and the breaking strength was measured. An air comparison pycnometer was used to determine the density of the various regions for each applicable treatment. There were no significant differences in density with respect to the region sampled or the treatment. Using the sample mass, breaking strength and density of the fish, the modulus of toughness was calculated as the energy per unit volume (Nm⁻²).

**Solid Phase Microextraction (SPME)/Mass Spectroscopy**

The SPME device (Supelco, USA) used for this analysis was a poly(dimethysiloxane) coated fused silica fiber. Capillary separation was performed using a Hewlett Packard model 6890/5973 GC/MS and a HP-5 (crosslinked 5% Ph Me Silicone) 25 m x 0.53 mm ID, 5.0 μm film column. The oven temperature was set at 30°C for 5
min, then ramped at 8 degrees per min to 220°C, and held for 3 min. The carrier gas, helium, was set at 1 ml per min. The injector temperature was 250°C and the detector was set at 280°C.

For the mass spectroscopy, capillary separation was also performed using a HP model 6890/5973 GC/MS and a HP-5MS column, 30 m x 0.25 mm and 0.25 μm film thickness. The oven temperature was set at 30°C for 5 min, then ramped at 10 degrees per min to 220°C and held for 3 min. Helium, the carrier gas, was set at 1 ml per min. The injector temperature was 250°C and the transfer line was set at 300°C. Researchers used a 70eV electron impact ionization technique, and scanned from 50 to 400 mass units.

**Sensory Evaluation**

The extent of oxidation among samples was assessed at each testing period by experienced panelists. This panel, which consisted of 15 staff personnel and graduate students from Virginia Tech, was heterogeneous in nature, having nearly equal numbers of both sexes and individuals ranging from 20 to 50 years of age. Prior to the study, panelists were familiarized with rancid odor and flavor notes and their relative intensities. The training sessions also served to acquaint panelists with fish products and the tastes associated with natural spices, since the antioxidant blends imparted an herbal flavor character. Paired comparison tests were used to assure that panelists could differentiate between higher levels of oxidation, both with and without herbal background notes. In the sensory evaluation, panelists were presented with pairs of an antioxidant treated sample and a control sample of the same age. They were also presented with pairs of an
antioxidant treated sample and a fresh control sample that had been recently purchased from a local supermarket. One pair was presented at a time, with panelists completing the evaluation of the first before moving on to the next. This methodology allowed researchers to answer two essential questions: 1) Are the stored antioxidant treated samples more oxidized than the freshly purchased samples; and 2) Are the stored control samples more oxidized than the stored antioxidant treated samples.

The panelists demonstrated difficulty in assessing the extent of oxidation between samples in which one had an herbal flavor and the other did not. Therefore, researchers decided that an herbal background note would be necessary in all products to allow panelists to assess oxidation properly. At the appropriate test interval, samples were removed from the -29°C freezer and place in a -1°C ± 2°C industrial refrigerator to thaw. After thawing, both the stored and fresh control fish were randomly assigned an antioxidant treatment group, and dipped in their respective treatment as described previously. Samples were cooked in covered baking dishes in a convection oven at 177°C for 20 min (Blodgett, Mark V Model). Afterwards, the fish were flaked to achieve a more homogeneous sample and about 10 g of sample were placed in randomly coded aluminum foil and kept warm until consumption. In the filleted study, panelist were presented with a total of 12 pairs of samples, the stored control versus each treated sample and the fresh control, and also the fresh control compared to each treated sample and the stored control. In a forced-choice test, panelists were asked which sample in the pair was more oxidized. To prevent overload, panelists were given a break between each group of six pair. In the headed and gutted study, panelists were presented with six pair of samples and no break
was necessary. Data were analyzed using statistical table T8 (Meilgaard, 1991a) for one-sided paired comparison test for difference.

To gain additional information, researchers performed two additional sensory tests. The first, a paired comparison test, used a balanced incomplete block design to determine if a difference existed in the filleted samples herbal intensity levels associated with antioxidant dipping before and after one month of storage. Thirty-five untrained panelists participated in the sensory evaluation, and each panelist received two pairs of samples. Each pair was from the same treatment group, either dipped before freezing, or after one month of frozen storage. Pairs were replicated a total of 14 times. The dipping and cooking procedures were followed as described earlier. Data were analyzed using statistical table T9 (Meilgaard, 1991b) for two-sided paired comparison test for difference.

In the second study, researchers also wanted to investigate the overall acceptability of control and treated filleted samples after twelve months of frozen storage. Using a balanced complete block design, 35 untrained panelists were each asked to judge the acceptability of six samples, the control and five treated groups, using a nine point hedonic scale. The scale values corresponded to, 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much, and 9=like extremely. The handling and cooking procedures were followed as described previously. To determine any statistical significance, paired t-tests were conducted for each treated group vs. control.
Proximate Composition

The moisture contents of skinless rainbow trout fillets were determined using the standard convection air oven method (AOAC, 1984). About 10 g (9.5 to 10.5 g) of the sample were dried in an aluminum dish with lid. The moisture was determined in a standard forced air convection oven (Hotpack model 14512, Hotpack Corp. Philadelphia, PA) with the temperature set at 100-102°C for 16-18 h. Protein content was determined using the Kjeldahl method (AOAC, 1984b), and the lipid content used the Soxtec 1043 method with ether solvent extraction.

Statistical Analyses

Data analyses were conducted by analysis of variance (ANOVA) using SAS (SAS, version 6.09, Cary, NC). The overall design model was a two way factorial. Mean separation (p<0.05) was determined using Tukey analysis.

RESULTS AND DISCUSSION

Proximate Composition

The average percent moisture among skinless fillets from both the filleted and headed and gutted samples was determined to be 79.08 ± 0.31, with no significant difference between replications of the experiment. Fish samples were also found to contain 82.46 ± 0.00 percent protein and 11.27 ± 0.27 percent lipid on a dry weight basis. There were no significant differences between replications of the experiment.
**Percent Pick-Up**

The percent pick-up of the filleted samples was calculated to be 3.59 ± 0.64 for AMNC-202, 4.09 ± 1.04 for AMNC-203, 3.45 ± 0.86 for ANC-204, 2.05 ± 1.04 for KS-15, and 3.09 ± 1.00 for MANC-213. For the headed and gutted samples, the percent pick-up was 7.42 ± 0.55 for the control glaze, 7.46 ± 1.17 for AMNC-202, and 7.66 ± 1.22 for MANC-213. Percent pick-up values exhibited considerable variability and there were no statistical differences among treatments for either level of butchery.

**Percent Drip Loss**

The capacity of fish muscle to bind water, often reflected as percent drip loss, has been traditionally used as a method to assess freshness in fish. Previous research has found that a decrease in fluid-holding capacity was associated with ATP-depletion. It has also been reported that lowered pH values contribute to increases in drip loss through modification of protein charges, which result in less moisture being held in the fish product (Ashie *et al.*, 1996). Therefore, drip loss is in effect, a reflection of protein denaturation. The headed and gutted fish had no significant differences (p<0.05) in percent drip loss (Appendix I, Table 1). In the filleted samples, significant differences were noted in months 2, 4 and 6 (Appendix I, Table 2). However, within specific storage times, increases in drip loss did not correspond with higher levels of oxidation, as reflected by TBARS or CDHP values (Appendix II, Fig. 2 and 5). In fact, even when the antioxidant treated samples exhibited significantly less oxidation, many of these samples displayed higher percent drip losses than the control samples. This is likely due to the nature of the antioxidant blends.
These blends contain varying concentrations of emulsifying agents such as polyglycerol fatty acid esters and distilled monoglycerides. Emulsifying agents would obviously affect the water holding ability, and thus in this application, it is difficult to use drip loss as an assessment of lipid oxidation.

**pH**

Although reportedly pH values tend to generally increase over frozen storage, the pH values of both the headed and gutted and filleted samples generally decreased over the twelve month storage period (Appendix I, Tables 4 and 5). The metabolic activity in fish tissue still exists at low temperatures, and thus it is believed that the decline in pH is due to the degradation of muscular glycogen to produce lactic acid. Environmental and stress factors may also play a role in such trends.

As detailed in Tables 4 and 5, there was a general trend of decreasing pH values as storage time progressed. Significant differences were noticed between treatments at specific time frames, however, this is likely a result of the natural antioxidant blends. The Duralox blends contain the chelating agents citric acid and ascorbic acid, and thus such ingredients would obviously function to reduce the pH values. The differences in concentrations of these chelating agents between Duralox blends may also affect apparent differences in pH values. In general, Herbalox Seasoning KS-15 and control samples exhibited higher pH values when compared to Duralox blends. Both in the headed and gutted and filleted samples, as pH values neared 6 slight gaping was noticed.
Chemical Analyses

As one of the primary products of oxidation, the concentration of conjugated diene hydroperoxides (CDHP) increased during the first two months of filleted frozen storage (Appendix II, Fig. 5). Between months two and six, little increase in conjugated dienes was noted, however, elevated levels were detected in month eight. While CDHP values did not greatly increase during the first six months, the development of thiobarbituric reactive substances (TBARS) substantially increased throughout storage of filleted trout (Appendix II, Fig. 2). Such elevation in TBARS coupled with little increase in CDHP indicates that the hydroperoxides are being quickly decomposed to secondary oxidation products.

In both the CDHP and TBARS analyses, the control fillets demonstrated the greatest susceptibility to oxidation (Appendix II, Fig. 2 and 5). While significantly less (p<0.05) oxidized than the control, the KS-15 treated samples still exhibited significant oxidation (p<0.05) when compared to the other antioxidant treatments. These data support the importance of chelating agents, such as citric acid or ascorbic acid, in retarding oxidation reactions. Antioxidant blends that contained such chelators exhibited greatly retarded oxidation rates. These chelators are extremely beneficial in products such as fish, which contain a plethora of metals, including iron, copper and heme. Chelators act to bind such metals, making them unavailable to initiate oxidative reactions and thus slowing the oxidation rate. Such trends in CDHP and TBARS products can also be seen in the headed and gutted data (Fig. 1 and 4). Oxidation increases with the level of
butchery, thus the development of CDHP and TBARS products is not as extensive in the headed and gutted trout when compared to filleted samples of the same age.

**Solid Phase Microextraction**

Rainbow trout sampled over storage times 0, 6, and 12 months were analyzed for pentane, hexane, heptane, propanal, heptanal, and hexanal. None of these alkanes or aldehydes were detected at any storage time. However using mass spectroscopy, BHT, a synthetic antioxidant, was repeatedly detected in all fish samples. Upon further investigation, researchers isolated the source of the BHT to be the aquaculture fish feed. The presence of BHT may have acted to further retard oxidative reactions by imparting antioxidant character to the flesh prior to processing and during frozen storage.

**Sensory Evaluation**

With the methodology selected, panelists were initially able to judge the extent of oxidation in the headed and gutted and filleted samples. In both groups of fish, no differences were noted between the fresh and stored controls and the treated samples at time zero (Appendix I, Tables 7 and 8). However, in the filleted samples, at two months storage panelists reported that the stored control samples were more oxidized. This judgment may in fact be true, however, researchers also believe that the herbal flavors associated with the natural antioxidant blends were difficult for the panelists to overlook. Even though, prior to cooking, the control samples were dipped in their respective antioxidant treatment, further research indicated that there was a difference between dipping fresh fillets then freezing and freezing fresh fillets and then dipping before cooking. Such statistical differences (p<0.05) were found for blends MANC-213,
AMNC-202 and AMNC-203 after one month of storage. Using 35 untrained panelists, this study concluded that fish that was first frozen for a month, and subsequently dipped in antioxidant had a more intense herbal flavor than fish of the same age that was dipped before frozen storage. It is likely that this higher herbal intensity played a large role in panelists’ decisions concerning oxidation, as many may have associated the intense flavor notes with oxidation. Also, researchers observed no reasonable correlation between TBARS and sensory performance of fish samples. Thus, TBARS concentrations should not be used as an indicator of human perception.

At the conclusion of the study, 35 untrained panelists were asked to judge the acceptability of the twelve-month treated and control filleted samples, without dipping the controls. Panelists responded that both the treated and control samples were equally acceptable. The mean values were: control, 5.8; AMNC-202, 5.6; AMNC-203, 5.5; ANC-204, 5.8; KS-15, 5.5; and MANC-213, 5.8. There were no statistical differences among treatments or control, due to large deviations in data. This further supports the opinion that the fish were not extensively oxidized at any point in this study, and the flavor attributes were still acceptable.

It is interesting to note that in this consumer panel there appeared to be two groups of people, those who found bland fish (the control) more acceptable, and those who found the herbal flavor notes of the antioxidant treatments more acceptable. Panelists who assigned higher (more acceptable) scores to the control fish also assigned lower scores to the treated fish. The opposite was true as well, with those who found the herbal flavors more acceptable ranking the control samples lower. These data
demonstrate how the acceptability of natural antioxidants may widely differ based on the consumer.

Texture

To determine the modulus of toughness, the density of the rainbow trout had to be determined. Using a pycnometer, researchers measured the density of the head, middle and tail regions for the various treatments. As stated previously, there were no significant differences in density with respect to the region sampled or the treatment, and the general density was determined to be $1.1 \pm 0.1 \text{ g cm}^{-3}$.

Using this density and the breaking force data collected from the Instron, the modulus of toughness was calculated for the various treatments, storage times and regions within the fish. There were no statistical differences with respect to storage time or treatment, with the average modulus of toughness being $2.62 \times 10^5$ Pascal for the filleted samples and $2.17 \times 10^5$ Pascal for the headed and gutted. The slightly tougher texture in the filleted samples is most likely due to the increased initial processing, which likely caused some crosslinking of protein and lipid structures. In general, increases in length of frozen storage and extent of oxidation have been correlated with increases in toughness due to the oxidation of proteins and the crosslinking of oxidized lipids and protein structures. However, in this study, increases in storage time had no effect on the quality of flesh texture.

CONCLUSIONS

In this study, rainbow trout were not extensively oxidized in either the headed and gutted or filleted samples. This is likely due, in part, to low storage temperatures (-29°C)
and the presence of BHT in the samples. While researchers can not be certain, it is very likely that BHT imparted a protection against oxidation, serving to further slow reactions. Although even the control samples did not undergo extensive oxidation, the antioxidant treated samples were significantly less oxidized than the controls. Thus, this study demonstrates the ability of natural antioxidants to control oxidative rancidity in filleted and headed and gutted rainbow trout. Used synergistically, rosemary and tocopherols significantly reduced oxidative reactions. By acting as metal chelators, the addition of citric or ascorbic acid improves the effectiveness of natural blends. Natural antioxidants may also improve the sensory attributes of frozen stored fish, as these blends have desirable natural flavors that many may prefer. These natural flavors may also function to mask the off-flavors and odors associated with oxidation, thus improving sensory perception. Overall, natural antioxidants are effective tools in the management of oxidative reactions. This effectiveness would likely be even more pronounced in fattier fish.
REFERENCES


ACKNOWLEDGMENT

The authors would like to thank Thomas Jones for supplying the antioxidant blends, and David Johnston for providing the trout samples. The authors also thank Joe W. Boling for performing the statistical analysis of the data, and Diane Bourne for assisting in the G-C/Mass Spec. studies. Finally, the assistance of Lisa L. Scott in the sensory evaluation procedures was greatly appreciated.
ABSTRACT

The percent recovery yields of rainbow trout belly flap was 74.8%, while the yield from the back bone of a butterfly filleted rainbow trout was 64.8%. Such recoveries from currently discarded fish by-products could be a major economic advantage to the seafood industry. Using minced rainbow trout from fillets as a model system, the antioxidant properties of various blends of rosemary extract and tocopherols, either alone or with citric and ascorbic acids, were compared to each other and untreated mince. Oxidation was measured by following changes in thiobarbituric reactive substances, conjugated diene hydroperoxides, drip loss, pH and sensory evaluation. Minced rainbow trout at -29°C undergoes significant oxidation, with control minced exceeding 80 μmole malonaldehyde/kg in 24 weeks. Natural antioxidants, in particular those that contained citric and ascorbic acids, were effective at retarding the development of conjugated diene hydroperoxides and malonaldehyde. Sensory evaluation indicated that treated samples were less oxidized. Natural antioxidants can effectively control oxidative reactions, and thereby maintain the quality of minced fish.
INTRODUCTION

It is estimated that the yield of fish fillets from a whole fish is around 35%, depending on the species. This estimate uses both hand and machine filleting procedures (Nettleton, 1985). Many seafood processors are currently marketing the fillets, a more valuable product per weight basis, and discarding the remainder of the backbone, frame, belly flap, and other remnants of the fish which still contain some edible tissue (Flick, 1998). In an effort to increase profitability, many of these items could be further processed using a mechanical bone separator. Brannan and Erickson (1996) reported that 50-200% more usable meat could be obtained per fish than is currently being utilized. In addition to significantly increasing the usable meat obtained per fish, this technology would also greatly reduce waste costs (Nettleton, 1985; Brannan and Erickson, 1996). Therefore, this recovery operation could increase profit in two ways: through marketing of the minced product from currently discarded food material; and by reducing waste processing costs.

While mechanical mincing procedures could prove extremely beneficial to fish processors, the industry must make such products more acceptable to the public. To do this, the seafood industry will have to effectively control oxidative reactions in the product (Erickson, 1992). Nettleton (1985) reported that minced fish prepared from whole headless frames is higher in fat, cholesterol and calcium than minced prepared from fillets. Such mince also has a higher concentration of metals, such as iron and copper. With
higher levels of fat and more pro-oxidants, along with greater surface area, minced products are extremely susceptible to oxidation. Frozen storage of minced fish would benefit processors by extending the shelf life to allow more time for further processing and distribution. However, while freezing minced tissue will slow oxidation processes, low temperatures alone are not enough to significantly retard deterioration (Hamilton et al., 1994; Nawar, 1996).

Due to the high content of polyunsaturated fatty acids, frozen storage of fish products typically results in many undesirable changes in tissue, including development of off odors and flavors and changes in texture, water holding capacity, color and nutritive properties (Eun et al., 1994; Thed and Erickson, 1994). Fish processors may use several methods to control oxidation such as vacuum packaging, glazing, and modified atmospheric packaging (MAP). Although many choices are available, when evaluated on cost, effectiveness and ease of use, antioxidants are the most widely used method to control lipid oxidation (Harris and Tall, 1994). Antioxidants retard lipid oxidation by acting as either radical scavengers, oxygen quenchers, or metal chelators.

There are two major classes of antioxidants available, synthetic or natural (Rajalakshmi and Narasimhan, 1996). While synthetic antioxidants, such as BHA, BHT and TBHQ, have traditionally been used to control oxidation there has been growing concern about the toxicity of these synthetic products, especially BHA. In fact, after reports of BHA causing tumor growth in the forestomach of rats, Japan banned this antioxidant and several other countries are re-evaluating the use of synthetic antioxidants (Inatani et al., 1983; Brookman, 1991). These actions have obviously alarmed the public
and have resulted in increased efforts to obtain antioxidant compounds from natural
sources, such as spices and plants (Giese, 1996).

Natural antioxidants have been obtained from various spices such as rosemary,
oregano, nutmeg, sage, and others whose main antioxidant activity is due to various
phenolic compounds (Rajalakshmi and Narasimhan, 1996). Other natural antioxidants
include tocopherols, citric acid and ascorbic acid. With a major consumer trend towards
more “natural” products, there is tremendous interest in natural antioxidants because they
are perceived as safer (Dorko, 1994; Six, 1994). While perhaps calming consumer fears,
food processors still question if these natural products will be the answer to the on-going
question of how food quality is preserved.

The objective of this study was to determine the efficacy of using natural
antioxidant blends, consisting of rosemary extract, tocopherols, citric acid and ascorbic
acid, to control oxidative rancidity during frozen storage of minced rainbow trout
(Oncorhynchus mykiss). The information gained from this study will be used primarily in
the commercial salmon industry to provide consumers with consistent, good quality
salmon products throughout the year. Such conclusions may be made due to the close
genetic relation between rainbow trout and salmon, both being salmonoids. Although this
research will be used primarily in the salmon industry, it is relevant to the long-term frozen
storage of minced fish products in general.
MATERIALS AND METHODS

Fish Supply

Fresh rainbow trout were obtained from an aquaculture facility in western Virginia. To simulate commercial salmon fishing practices, the fish had been held on ice for three days prior to further processing. Whole rainbow trout fish were processed to obtain two skinless fillets per fish and packed in plastic freezer bags, with ten fillets per bag. The fish temperature did not exceed 7°C during processing. These bags were iced packed in insulated containers and transported to laboratory facilities (2.5-3 h).

Antioxidant Preparation

Five natural antioxidant blends were obtained from Kalsec Inc., Kalamazoo, MI. The antioxidants used were: Duralox Blends AMNC-202, AMNC-203, ANC-204, and MANC-213, and Herbalox Seasoning KS-15. The Herbalox Seasoning is a blend of rosemary extract and tocopherols, while the Duralox blends contain these ingredients with citric and ascorbic acid as chelating agents. Using pre-chilled water (3-7°C), solutions of antioxidants were prepared at the manufacturer's recommended usage rates. These rates were: 10% for ANC-204 and MANC-213; 13.3% for KS-15; and 15% for AMNC-202 and AMNC-203.

Fish Treatment and Processing

Samples were removed from gallon size freezer bags and randomly assigned to one of six groups: control, Duralox Blends AMNC-202, AMNC-203, ANC-204, MANC-213 or Herbalox Seasoning KS-15. The control fish were minced using a mechanical meat bone separator with a separator chamber screen size of 0.060/0.040/0.031 inches and a
revolving valve setting of 6D (BeeHive, Inc., Utah). The treated samples were randomly assigned to batches of 10-20 fish, placed in a large colander, weighed and dipped into their respective antioxidant treatment for one minute with agitation. Following dipping, the samples were allowed to drain of excess solution for 0.5 min and re-weighed. The samples were then minced as the control samples, and all minced product was packaged separately in approximately 454 g (1 lb) lots using gallon size plastic freezer bags. The samples were stored in a -29°C freezer.

**Percent Pick-Up**

Weighing the samples both before and after treatment allowed for the determination of percent pick-up. This value is the percent of mass gained by the sample following treatment with glazing solution or antioxidant dip. It is calculated as:

\[
\% \text{ Pick-Up} = \left( \frac{\text{Final Mass} - \text{Initial Mass}}{\text{Final Mass}} \right) \times 100
\]

**Recovery Operations**

To better assess the feasibility of using rainbow trout fillets to obtain a significant quantity of edible minced tissue, researchers saved the leftover belly flap and back bone from the filleting procedures. Using mincing procedures as described earlier, these products were minced separately and the percent yield was calculated.

**Evaluation Periods**

Sufficient samples were removed from frozen storage at six week intervals, for a total of 24 weeks. All measurements were made in triplicate for two replicate experiments.
**Percent Drip Loss**

Samples were removed from the -29°C freezer, placed in preweighed freezer bags and the net frozen mass determined. The samples were placed in an industrial refrigerator unit (-1°C ± 2°C) and the samples allowed to thaw overnight. Thawed samples were removed from the freezer bags and placed on a wire mesh screen for 10 min to equilibrate. The samples were then reweighed and drip loss was calculated as:

\[
\% \text{ Drip Loss} = \left( \frac{\text{Loss in Mass}}{\text{Initial Mass}} \right) \times 100
\]

**pH**

Muscle pH was determined using the procedure described by Rhee *et al.* (1984). Minced rainbow trout (10g) was homogenized with 100mL distilled, deionized water in two 25-second periods at high speed using a Waring commercial blender. The pH of the slurry was measured using a pH meter (Accumet, Model 10) with a glass electrode.

**Conjugated Diene Hydroperoxides (CDHP)**

A solution of chloroform/methanol (2:1 v/v) (Fisher Scientific, New Jersey) was used to extract lipid from 1.00 ± 0.02 g of muscle tissue as described by Erickson (1992). A 5 mL aliquot of this extract was dispensed into a clean test tube and washed with 1 mL of 0.88% KCl (Fisher Scientific, New Jersey). Following aspiration of the upper layer, 2 mL of the lower layer was evaporated to dryness under nitrogen gas, and reconstituted in 5 mL of cyclohexane (Fisher Scientific, New Jersey). Using quartz cuvettes, the absorbance was read at 232 nm using cyclohexane as a reference (Shimadzu, UV-2101PC, Maryland).
2-Thiobarbituric Acid Reactive Substances

The concentration of 2-thiobarbituric acid reactive substances (TBARS) in the control and treated samples was determined by the distillation method developed by Tarladgis et al. (1960). Where applicable, procedures were performed under nitrogen, and n-propyl gallate and ethylenediaminetetraacetic acid (Sigma Chemical Co., Missouri) were used to inhibit further oxidation during testing. TBARS values were reported as µmole malonaldehyde per kg sample. A standard curve was prepared with 1,3,3,3,-tetraethoxypropane (Sigma Chemical Co., Missouri). Absorbance of samples was read at 532 nm (Spectronic Genesys, Model 20)

Sensory Evaluation

The extent of oxidation between samples was assessed at each testing period by semi-trained panelists. This panel, which consisted of 15 staff personnel and graduate students from Virginia Tech, was heterogeneous in nature, having nearly equal numbers of both sexes and individual ranging from 20 to 50 years of age. Prior to the study, panelists were familiarized with rancid odor and flavor notes and their relative intensities. The training sessions also served to acquaint all panelists with fish products and the tastes associated with natural spices, since the antioxidants used imparted a slight herbal flavor character. Paired comparison tests were used to assure that panelists could differentiate between higher levels of oxidation, both with and without herbal background notes.

In the sensory evaluation, panelists were presented with pairs of an antioxidant treated sample and a control sample of the same age. They were also presented with pairs of an antioxidant treated sample and a fresh control sample that had been recently
purchased from a local supermarket. One pair was presented at a time, with panelists completing the evaluation of one pair before moving on to the next. This methodology allowed researchers to answer two essential questions: 1) Are the stored samples more oxidized than the freshly purchased samples; and 2) Are the stored control samples more oxidized than the stored antioxidant treated samples.

The panelists demonstrated difficulty in assessing the extent of oxidation between samples in which one had an herbal flavor and the other did not. Therefore, researchers decided that an herbal background note would be necessary in all products to allow panelists to assess oxidation properly. At the appropriate test interval, samples were removed from the -29°C freezer and placed in a -1°C ± 2°C industrial refrigerator to thaw. After thawing, both the stored and fresh control fish were randomly assigned an antioxidant treatment group, and dipped in their respective treatment as described previously. Samples were cooked in covered baking dishes in a convection oven at 177°C for 20 min. Afterwards, the fish was flaked to achieve a more homogeneous sample and about 10 g of sample were placed in randomly coded aluminum foil and kept warm until consumption. Panelists were presented with a total of 12 pairs of samples, the stored control versus each treated sample and the fresh control, and also the fresh control compared to each treated sample and the stored control. In a forced-choice test, panelists were asked which sample was more oxidized. To prevent overload, panelists were given a break between each group of six pair. Data were analyzed using statistical table T8 (Meilgaard, 1991a) for one-sided paired comparison test for difference.
**Proximate Composition**

The moisture contents of skinless rainbow trout fillets were determined using the standard convection air oven method (AOAC, 1984). About 10 g (9.5 to 10.5 g) of the sample was dried in an aluminum dish with lid. The moisture was determined in a standard forced air convection oven (Hotpack model 14512, Hotpack Corp. Philadelphia, PA) with the temperature set at 100-102°C for 16-18h. Protein content was determined using the Kjeldahl method (AOAC, 1984b), and the lipid content used the Soxtec 1043 method with ether solvent extraction.

**Statistical Analyses**

Data analysis were conducted by analysis of variance (ANOVA) using SAS (SAS, version 6.09, Cary, NC). The overall design model was a two way factorial. Mean separation (p<0.05) was determined using Tukey analysis.

**RESULTS AND DISCUSSION**

**Recovery Yields**

Using belly flap and backbone material from the filleting procedures, researchers recovered a mean percent yield of 74.8% and 64.8%, respectively. While, researchers operated at only one machine setting, different yields may be obtained by varying the settings of the mechanical separator. Such recovery yields demonstrate the potential of using mechanical processes to obtain more edible fish and thus increase revenues. Currently, most processors discard of such fish by-products, resulting in significant losses in potential revenue and increased waste management costs.
Proximate Composition

The average percent moisture among skinless fillets was determined to be $79.46 \pm 0.31$, with no significant difference between replications of the experiment. Fish samples were also found to contain $79.25 \pm 0.37$ percent protein and $13.01 \pm 0.10$ percent lipid on a dry weight basis. There were no significant differences between replications of the experiment.

Percent Pick-Up

The mean percent pick-up of the treated samples was calculated to be 1.8% for AMNC-202; 2.8% for AMNC-203; 2.7% for ANC-204; 1.9% for KS-15; and 1.6% for MANC-213. Variations in percent pick-up values are most likely a factor of differences in antioxidant treatments.

Percent Drip Loss

The capacity of fish muscle to bind water, often reflected as percent drip loss, has been traditionally used as a method to assess freshness in fish. Previous research has found that a decrease in fluid-holding capacity was associated with ATP-depletion. It has also been reported that lowered pH values, which can modify protein charges, contribute to increases in drip loss. Therefore, drip loss is in effect, a reflection of protein denaturation. Significant differences in drip loss between treatments were noted throughout the various storage times (Appendix I, Table 3). However, increases in drip loss did not correlate with higher levels of oxidation, as reflected by TBARS or CDHP values (Appendix II, Figs. 3 and 6). In fact, even when the antioxidant treated samples exhibited significantly less oxidation, many of these samples displayed higher percent drip
losses than the control samples. This is likely due to the nature of the antioxidant blends. These blends contain varying concentrations of emulsifying agents such as polyglycerol fatty acid esters and distilled monoglycerides. Emulsifying agents would obviously affect the water holding ability, and thus in this application, it is difficult to use drip loss as an assessment of lipid oxidation. It is interesting to note, however, that within treatments, all groups exhibited an increase in drip loss over the duration of the study. Thus, in this case, percent drip loss appears to be a function of both extent of oxidation and antioxidant treatment.

**Chemical Analyses**

As one of the primary products of oxidation, the concentration of conjugated diene hydroperoxides increased during the first six weeks of minced frozen storage (Appendix II, Fig.6). Between weeks 6 and 12, little increase in conjugated dienes was noted, however, elevated levels were detected in weeks 18 and 24. While CDHP values did not greatly increase between weeks 6 and 12, the development of thiobarbituric reactive substances in the control mince significantly increased throughout the storage of minced trout (Appendix II, Fig. 3). After week 12, TBARS also significantly increased in the treated samples as well. This most likely indicates a reduction in the concentrations of antioxidants, and thus the product is left more susceptible to oxidative attack. Minced products are extremely susceptible to oxidation due to the high level of butchery and excessive exposure of tissue to oxygen during mincing procedures.

In both the CDHP and TBARS analyses, the control mince demonstrated the greatest susceptibility to oxidation (Appendix II, Figs. 3 and 6). While significantly less
(p<0.05) oxidized than the control, the KS-15 treated samples still exhibited significant oxidation (p<0.05) when compared to the other antioxidant treatments. These data support the importance of chelating agents, such as citric acid or ascorbic acid, in retarding oxidation reactions. Antioxidant blends that contained such chelators exhibited greatly inhibited oxidation rates. These chelators are extremely beneficial in products such as fish, which contains a plethora of metals, including iron, copper and heme. Chelators act to bind such metals, making them unavailable to initiate oxidative reactions and thus slowing the oxidation rate.

**pH**

The pH values of minced rainbow trout generally increased over storage time (Appendix I, Table 6). Such trends have also been reported by Vareltzis et al. (1997). It is reported that enzymatic activity in fish tissue can cause such increases in pH, as more basic groups are released from cellular structures. In addition to differences being noticed as storage time progressed, differences were also noticed between treatments at specific time frames. This is a result of the natural antioxidants used. The Duralox blends contain the chelating agents citric acid and ascorbic acid, and thus such ingredients would obviously function to reduce the pH values. The differences in concentrations of these chelating agents between the various Duralox blends may also affect pH reading. In general, Herbalox Seasoning KS-15 and control samples exhibited higher pH values when compared to Duralox blends. While pH has been used to assess the extent of muscular degradation in fishery products, the introduction of such chelating agents eliminates the reliability of such analysis.
Sensory Evaluation

With the methodology selected, panelists were initially able to judge the extent of oxidation in samples. No differences were noted between the fresh and stored controls and the treated samples at time zero (Appendix I, Table 9). However, as time progressed panelists reported that the stored control samples were more oxidized. This judgment may in fact be true, however, researchers also believe that the herbal flavors associated with the natural antioxidant blends were difficult for the panelists to overlook. Even though, prior to cooking, the control samples were dipped in their respective antioxidant treatment, further research by Turner et al. (1998) indicated that there was a difference between dipping fresh fillets then freezing and freezing fresh fillets and then dipping before cooking. Such statistical differences (p<0.05) were found for blends MANC-213, AMNC-202 and AMNC-203 after one month of storage using rainbow trout fillets. This study concluded that fish that was first frozen for a month, and subsequently dipped in antioxidant had a more intense herbal flavor note than fish of the same age that was dipped before frozen storage. It is likely that this higher herbal intensity played a large role in panelists' decisions concerning oxidation. Overall, there was no correlation between sensory evaluation results and the concentrations of thiobarbituric reactive substances (TBARS). Thus, TBARS concentrations should not be used as an indicator of human perception.

CONCLUSIONS

Minced rainbow trout at –29°C was found to be susceptible to oxidative reactions, despite Turner et al. finding BHT in rainbow trout from the same aquaculture facility. In
this previous study, BHT was isolated from rainbow trout using mass spectroscopy. While researchers cannot be certain, it is very likely that BHT imparted a protection against oxidation, serving to somewhat slow reactions.

This study demonstrates the ability of natural antioxidants to control oxidative rancidity in minced rainbow trout. Used synergistically, rosemary and tocopherols significantly reduced oxidative reactions. By acting as metal chelators, the addition of citric or ascorbic acid improves the effectiveness of natural blends. Natural antioxidants may also improve the sensory attributes of frozen stored fish, as these blends have desirable natural flavors that many may prefer. These natural flavors may also function to mask the off-flavors and odors associated with oxidation, thus improving sensory perception. Overall, natural antioxidants are effective tools in the management of oxidative reactions.

Considering the recovery yields of samples in this study, fish processors could increase profit by using mechanical separators to recover minced tissue. While minced fish is very susceptible to oxidation, with proper storage temperatures and antioxidant treatments, the shelf life can be greatly prolonged.
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91


**ACKNOWLEDGMENT**

The authors would like to thank Thomas Jones for supplying the antioxidant blends, David Johnston for providing the trout samples and Thomas Goldston for the use of the Bee-Hive separator. The authors also thank Joe W. Boling for performing the statistical analysis of the data. Finally, the assistance of Lisa L. Scott in the sensory evaluation procedures was greatly appreciated.