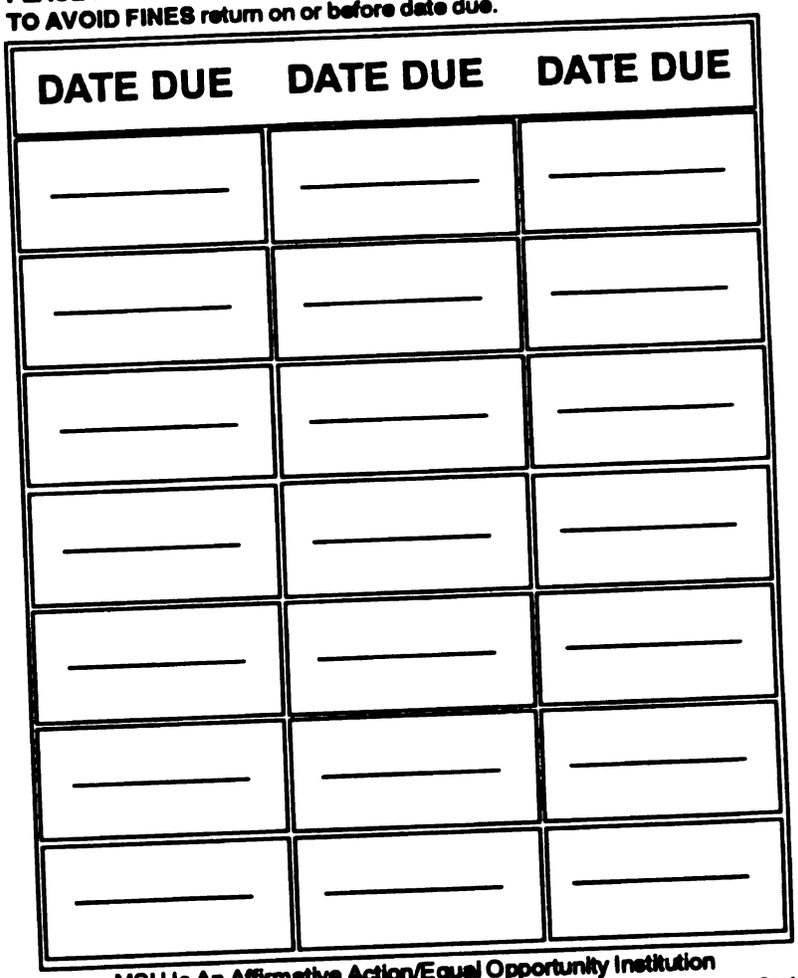

# QUALITY OF RAINBOW TROUT MUSCLE AS AFFECTED BY DIETARY AND PROCESSING TREATMENTS

BY

PERVAIZ AKHTAR

A DISSERTATION

submitted to
Michigan State University
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## ABSTRACT

QUALITY OF RAINBOW TROUT MUSCLE AS AFFECTED BY DIETARY AND PROCESSING TREATMENTS

BY

PERVAIZ AKHTAR

This study was designed to investigate the effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on the stability of color, cholesterol and lipids of rainbow trout (*Oncorhynchus mykiss*) muscle during storage (4°C and -20°C) and processing. The protective effect of these antioxidants on iron/ascorbate-induced lipid oxidation of muscle tissue and microsomes was evaluated. Their effect on lipoxygenase-catalyzed oxidation of arachidonic acid and fish lipids in a model system was also studied.

A significant reduction in α-tocopherol and carotenoid concentrations in muscle tissue as a result of frozen storage was observed. A protective effect of a supranutritional level of α-tocopherol on cholesterol oxide formation in cooked fish muscle and on lipid oxidation in raw muscle during refrigerated and frozen storage was observed. Dietary α-tocopherol supplementation stabilized flesh color of rainbow trout during refrigerated storage. The higher concentration of α-tocopherol (500mg/kg feed) had little effect on color stability during frozen storage. However, oleoresin rosemary was very effective in stabilizing color and lipids including cholesterol. Carotenoid degradation was less in samples with surface

application of oleoresin rosemary.

A beneficial effect of dietary α-tocopherol supplementation and oleoresin rosemary on iron/ascorbate-stimulated oxidation in fish muscle and microsomes was also observed. However, the combination of α-tocopherol and oleoresin rosemary provided greater protection than did α-tocopherol alone.

Cholesterol oxides were observed in all cooked samples. The major cholesterol oxides were 7β-hydroxycholesterol, α- and β-epoxides, and 7-ketocholesterol. Formation of cholesterol oxidation products (COPS) was reduced by dietary α-tocopherol supplementation. COPS formation was further reduced with surface application of oleoresin rosemary.

Specific volatile compounds generated from the lipoxygenase-catalyzed oxidation of arachidonic acid in a model system were 2-octenal, 1-octen-3-ol and 2-nonenal. The oxidation of rainbow trout muscle lipids generated 1-octen-3-ol and 2-nonenal. Dietary α-tocopherol, capsanthin and oleoresin rosemary partially inhibited the generation of these compounds.

Results of the present study support the hypothesis that stabilization of lipids through the supplementation of the diet with supranutritional levels of α-tocopherol and surface application of oleoresin rosemary also protects lipid-soluble pigments against oxidative degradation during storage.

**THIS DISSERTATION IS DEDICATED TO MY**

**LOVING PARENTS ESPECIALLY TO MY MOTHER**

**ACKNOWLEDGEMENTS**

I am highly indebted to almighty GOD who gave me courage, strength and patience during this study and also enabled me to complete this manuscript.

I wish to express my heart felt gratitude to my academic advisor, Dr. James Ian Gray, for his consistent encouragement, inspiring guidance, financial support through the Crop and Food Bioprocessing Center and critical evaluation of the whole script. No words can truly explain my feelings for his kind help and understanding both within and outside of my scientific career.

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**I owe the greatest debt to my sweet mother who always prayed for my success and kept waiting for me to return home till she left for heavens. I certainly will never be able to excuse myself for not being with her at the time she needed me most.**

**Finally and for many reasons, I owe the highest debt to my great and highly respectable father, my loving wife, sweet sons, in-laws, sisters-in-law, sister and brothers for all the sufferings and for providing me love and support.**

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# INTRODUCTION

Fish muscle is susceptible to lipid oxidation because of the presence of large amounts of highly polyunsaturated fatty acids found in organisms from the marine environment (Ackman, 1980). Lipid oxidation in fish and seafood products can occur during frozen and refrigerated storage (Flick et al., 1992). Oxidation of lipids causes adverse changes in flavor, color, texture, nutritive value, and even the safety of fish and seafood products (Khayat and Schwall, 1983; Pearson et al., 1983). It is now generally accepted that phospholipids present in the subcellular membranes (microsomes and mitochondria) are primarily responsible for the initial development of oxidized flavors in both raw and cooked meat products during storage (Buckley and Morrissey, 1992).

Lipid oxidation is brought about by a complex set of reactions which are triggered as a consequence of the formation of free radicals in cells and tissues (Comporti, 1993). As polyunsaturated fatty acids are sensitive to oxidative attack (Gray and Pearson, 1987), it is obvious that the activated methylene bridge represents a critical target site. The production of lipid hydroperoxides leads to their subsequent breakdown to secondary products. However,

termination reactions occur only in the presence of sufficient quantities of radicals (Bors et al., 1988). The initiation and propagation steps could be carried out by several enzymic and nonenzymic reactions (Hultin, 1980). The proposed mechanisms involved in the enzymic and nonenzymic initiation of lipid oxidation have extensively been reviewed (Kanner et al., 1987; Asghar et al., 1988; Hsieh and Kinsella, 1989b; Hultin, 1994). However, the actual species that initiate oxidation still remains the subject of much research and general uncertainty.

The processing of meat and meat products may enhance the likelihood of co-oxidation of cholesterol. The oxidation of cholesterol and the presence of cholesterol oxidation products (COPS) in different foods has been extensively reviewed (Smith, 1981; Finocchiaro and Richardson, 1983; Maerker, 1987; Naresh and Singhal, 1991; Boesinger et al., 1993; Paniangvait et al., 1995). The possible involvement of COPS in sterol metabolism interruption, atherogenicity, cytotoxicity, mutagenicity and even carcinogenicity (Maerker, 1987; Hurrard et al., 1989) has intensified interest in studying the mechanism of cholesterol oxidation in various foods.

Carotenoids are responsible for the pink to red flesh color of salmonids which is of great economic importance because of consumer demand (Torrissen et al., 1989). Salmonids absorb carotenoids from the diet because of their inability to synthesize carotenoids *de novo* (Storebakken and

No, 1992). They are deposited in the flesh in the unesterified form and bind to actomyosin (Henmi et al.,1987, 1989). Astaxanthin is the predominant carotenoid in wild salmonids and is obtained from ingested crustaceans (Torrissen, 1989). Astaxanthin and canthaxanthin are usually supplemented in the feed of farmed salmonids (Pozo et al., 1988; Torrissen et al., 1989). Torrissen et al. (1989) has extensively reviewed pigmentation of salmonids with special emphasis on pigments from naturally occurring sources.

The deterioration of carotenoids in frozen stored rainbow trout muscle is well established (Chen et al., 1984; Pozo et al., 1988; Anderson et al., 1990). Carotenoid deterioration can be attributed to both enzymic and nonenzymic reactions (Krinsky, 1989). Lipoxygenase, through its aerobic and anaerobic pathways (Tsukuda and Amanok, 1968; Eskin et al., 1977), may cause discoloration of certain fish (Tsukuda, 1970), conceivably by bleaching the carotenoids following free radical quenching (Krinsky and Deneki, 1982; Halevy and Sklan, 1987; Kanner et al., 1987). Similarly, Stone and Kinsella (1989) reported bleaching of β-carotene in conjunction with the peroxidation of different polyunsaturated fatty acids by 12-lipoxygenase from trout gill.

This research is based on the hypothesis that besides lipid oxidation, co-oxidation of carotenoids and cholesterol in fish will also occur because of their presence in a highly oxidizable environment. Lipid oxidation in fish muscle during storage is initiated, in part, by the activity

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of lipoxygenases and can adversely affect the safety of the product via cholesterol oxidation, and the color through degradation of carotenoid pigments. Therefore, dietary supplementation of vitamin E and surface application of oleoresin rosemary should stabilize lipids as well as lipid-soluble pigments against oxidative degradation during processing and storage.

Specific objectives of this study are to:

1. Study the effect of dietary supplementation on the deposition of carotenoids and α-tocopherol, and flesh pigmentation in rainbow trout muscle.
2. Study the effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on color and flesh stability during refrigerated and frozen storage of rainbow trout fillets.
3. Investigate the effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in rainbow trout muscle and muscle microsomes.
4. Study the effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on lipid and cholesterol oxidation in cooked rainbow trout muscle.
5. Evaluate, using a model system, the effect of selective antioxidants on the generation of volatile compounds by lipoxygenase-catalyzed oxidation of arachidonic acid and rainbow trout muscle lipids.

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## REVIEW OF LITERATURE

### Lipid Oxidation in Biological Systems

Lipid oxidation is a normal biological process by which energy is generated from fat. However, lipid oxidation or products derived from this reaction, may have serious health and nutrition implications. Lipid oxidation is regarded as one of the major problems, both technically and economically, which limits the stability and acceptability of food products. Lipid oxidation in foods and *in vivo* is generally referred to as autoxidation and peroxidation, respectively.

Lipid oxidation (peroxidation) is a major nonmicrobial cause of quality deterioration in muscle foods and can directly affect many quality characteristics including color, flavor, texture, nutritive value, and safety (Khayat and Schwall, 1983; Pearson et al., 1983). Figure 1 summarizes the key reactions leading to quality changes in foods and feeds and the various factors which enhance and inhibit them (Eriksson, 1982).

#### 1.Lipid Oxidation in Fish

The demand and consumption of sea food products is increasing because of their nutritional benefits, i.e., high

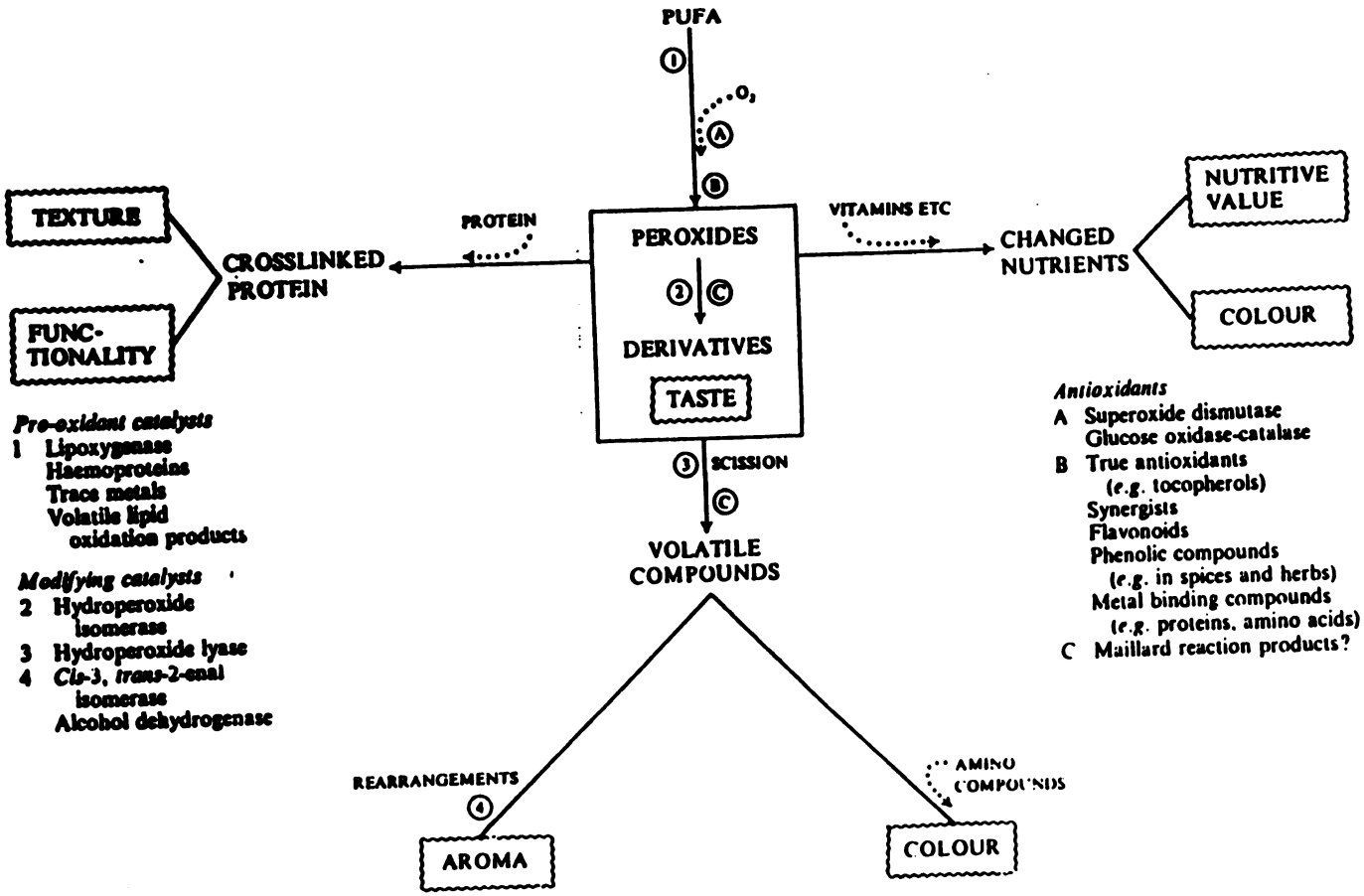


Figure 1. Reactions of polyunsaturated fatty acids (PUFA) leading to quality and nutritional changes in food (Eriksson, 1982).

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content of ω-3 polyunsaturated fatty acids, low content of cholesterol, and high content of good quality protein (Stansby, 1982; Kinsella, 1987, 1988). However, the abundance of polyunsaturated ω-3 fatty acids in fish lipids make them susceptible to oxidation and quality deterioration, especially if the fish are not handled properly. The polyunsaturated ω-3 fatty acids have been shown to function as precursors of prostaglandin and monohydroxy fatty acid biosynthesis (Budowski, 1981). Variation in the composition and content of polyunsaturated fatty acids in fish lipids is large. Several factors including geographic location, catch, season, sex, maturity and feeding habits, affect the composition of fish lipids and thus their susceptibility to oxidation (Stansby, 1982; Kinsella, 1987).

Microsomal enzymes have been suggested as initiators of lipid oxidation in fish tissue (German and Kinsella, 1986a; Kanner et al., 1987; Hsieh and Kinsella, 1989b). The oxidation of unsaturated lipids proceeds through a free radical chain mechanism involving initiation, propagation and termination stages unless mediated by other oxidants or enzyme systems (Hsieh and Kinsella, 1989b).

It is generally accepted that lipid oxidation in meat is initiated at the membrane level, and that the phospholipids in the membranes are the primary centers for initiation (Gray and Pearson, 1987). The localization of relatively large amounts of polyunsaturated fatty acids in the membranes of mitochondria, microsomes and lipoproteins

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makes them highly vulnerable to peroxidative changes (Buege and Aust, 1978). Prooxidants such as oxygen, microsomal enzymes such as peroxidase and lipoxygenase (Vladimirov et al., 1980; Kanner et al., 1987), heme and nonheme iron (Igene and Pearson, 1979), hydrogen peroxide, and superoxide radicals (Kanner and Harel, 1985) are present in muscle cells. These endogenous compounds may play an important role in the formation of the primary pool of biological catalysts in muscle tissues.

## 2. Classical Mechanism of Lipid Oxidation

Free radical lipid oxidation proceeds through different stages include initiation, propagation and termination (Farmer and Sutton, 1943; Uri, 1961; Lundberg, 1962):

Initiation:

$$LH + Initiator \longrightarrow L^{\bullet}$$

Propagation:

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet}$$

$$LOOH \longrightarrow LO^{\bullet} + OH^{\bullet}$$

Termination:

$$LOO^{\bullet} + LOO^{\bullet} \longrightarrow LOOL + O_2$$

$$LOO^{\bullet} + L^{\bullet} \longrightarrow LOOL$$

$$L^{\bullet} + L^{\bullet} \longrightarrow LL$$

$$L^{\bullet}(LOO^{\bullet}, LO^{\bullet}, OH^{\bullet}) + AH \longrightarrow LH(LOOH, LOH, H_2O) + A^{\bullet}$$

Where LH = unsaturated fatty acid, $L^{\bullet}$ = allyl radical, $LO^{\bullet}$ = alkoxyl radical, $LOO^{\bullet}$ = peroxy radical,

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LOOH = hydroperoxide, OH$^{\bullet}$ = hydroxyl radical, AH = phenolic antioxidant and A$^{\bullet}$ = antioxidant radical.

The source of the primary catalysts that initiate oxidation still remains the subject of much research and general uncertainty.

## 3. Initiators of Lipid Oxidation

Free radicals are produced during the metabolism of food where food is oxidized to generate energy. On one hand, free radicals play a beneficial role in the body by providing protection against bacteria and parasites. On the other hand, radical species may also attack the polyunsaturated fatty acids of cell membranes, cell structures and deoxyribonucleic acid (DNA) if natural protective antioxidants are not adequate to quench them. The reaction between oxygen and lipids does not takes place spontaneously because of thermodynamic constraints (Hsieh and Kinsella, 1989b). It needs to be initiated either by the formation of free radicals from the lipids or by the formation of active oxygen species that are able to react directly with the lipid molecule. Thus, numerous species have been suggested as initiators of peroxidation.

The spin restriction of molecular oxygen species can be overcome by any of the following four initiation mechanisms:

(i) Singlet oxygen;

(ii) Partially reduced or activated oxygen species such as hydrogen peroxide, superoxide anion, or hydroxyl

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(iii) Active oxygen-iron complexes (ferryl iron); and

(iv) Iron-mediated homolytic cleavage of the hydroperoxides which generate organic free radicals (Kanner et al., 1987).

In biological systems, active oxygen species such as singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$), superoxide anion ($O_2^{\cdot -}$), and hydrogen peroxide ($H_2O_2$) can be generated via non-enzymatic and enzymatic mechanisms (Fridovich, 1976). Natural pigments such as hematoporphyrin and flavins may serve as sensitizers to yield singlet oxygen in the presence of oxygen and visible light. Enzymes such as microsomal oxidases, lipoxygenase, and prostaglandin synthase may directly or indirectly generate singlet oxygen (Korycka-Dahl and Richardson, 1980).

The sites of free radical generation encompass all cellular constituents including mitochondria, lysosomes and plasma membranes. In biological systems, superoxide anions, which are not particularly reactive, are readily removed by the action of superoxide dismutase, This latter enzyme is part of the natural antioxidant defense system (Tyler, 1975).

$$2O_2^{\cdot -} + 2H \xrightarrow[\text{dismutase}]{\text{Superoxide}} H_2O_2 + O_2$$

The accumulation of hydrogen peroxide is prevented by two enzymes, catalase and glutathione peroxidase, and metabolized to harmless end-products, i.e., water and oxygen

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(Oshino et al., 1973; Fridovich, 1986).

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2O + O_2$$

$$H_2O_2 + GSH \xrightarrow[\text{peroxidase}]{\text{Glutathione}} 2H_2O + GSSG$$

Where GSH = reduced glutathione and GSSG = oxidized glutathione.

The activities of these enzymes may be impaired by a deficiency of selenium or manganese, copper or zinc, resulting in the accumulation of $O_2^{\cdot -}$ and $H_2O_2$. Neither $O_2^{\cdot -}$ nor $H_2O_2$ at the steady state concentrations observed in cells, may be of major concern for reaction with most biochemical entities including polyunsaturated fatty acids (Minotti and Aust, 1987). However, these compounds may be transformed into highly reactive species in the presence of iron (Czapski, 1971; Czapski and Ilan, 1978; McCord and Day, 1978; Butler and Halliwell, 1982):

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe^{2+}} OH^{\bullet} + O_2 + OH^-$$

Minotti and Aust (1987) concluded that the susceptibility of $Fe^{2+}$ to oxidation by either $O_2^{\cdot -}$ and $H_2O_2$ depends on the availability and nature of chelators. They reported that ADP-$Fe^{2+}$ is oxidized preferentially by $H_2O_2$, whereas citrate-$Fe^{2+}$ is oxidized preferentially by $O_2^{\cdot -}$. The hydroxyl radicals are extremely reactive towards organic substrates within their immediate vicinity (Burton, 1994) and attacks the polyunsaturated fatty acids present in the biological membranes. It is one of the primary initiators of lipid

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oxidation in biological tissues (Halliwell and Gutteridge, 1986).

4. Enzymic Catalysis of lipid oxidation in Fish

Hultin and his associates (McDonald et al., 1979; Slabyj and Hultin, 1982) demonstrated the existence of enzymic systems capable of catalyzing the oxidation of microsomal lipid fractions of fish skeletal muscles in the presence of certain co-factors. The enzymic lipid peroxidation in skeletal muscle was found to be more dependent on reduced nicotinamide-adenine dinucleotide (NADH) than on reduced nicotinamide-adenine dinucleotide phosphate (NADPH). It also requires adenosine diphosphate (ADP) and ferrous or ferric ions for maximum reaction rate (McDonald et al., 1979; Slabyj and Hultin, 1982). These investigators also reported that the reaction rate at ultimate (postmortem) muscle pH values was moderate or high compared to the rate at optimum pH values.

In addition to microsomal systems, mitochondrial enzymic systems may play a role in lipid oxidation in raw muscles. An enzymic lipid peroxidation system in fish (trout) muscle mitochondria was demonstrated by Luo (1987) to be similar to that in fish muscle microsomes in terms of co-factor requirements and optimal pH.

German and Kinsella (1985) studied the mechanisms underlying the initiation of lipid oxidation in fish using a crude aqueous extract from trout skin tissue as the source

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of enzyme and exogenous radioactive arachidonic and eicosahexaenoic fatty acids as substrates. They concluded, on the basis of the type of monohydroxy compounds produced from the oxidation reaction and responses to lipoxygenase inhibitors, that the trout skin extract contained lipoxygenase. They also concluded that the skin lipoxygenase released postmortem may be a significant source of free radicals which lead to subsequent lipid oxidation in fish.

## Lipoxygenase-Catalyzed Oxidation of Fish Lipids

The high degree of unsaturation of fish lipids makes them susceptible to oxidation. Quality deterioration of both refrigerated and frozen fish occurs rapidly, especially if the fish are not handled properly. When fish are killed, certain enzymes such as gill and skin lipoxygenase (German and Kinsella, 1985, 1986a,b), blood peroxidase (Kanner and Kinsella, 1983), and muscle microsomal NADH peroxidase (Slabyi and Hultin, 1984) may become uncontrolled and initiate lipid peroxidation to produce hydroperoxides. These hydroperoxides may then be decomposed by homolytic cleavage and β-scission to produce various fragmentation products such as aldehydes, alcohols, ketones and hydrocarbons (Frankel, 1984). These compounds influence the flavor quality and palatability of fish products. Lipoxygenases are highly substrate-and position-specific in nature (KÜhn et al., 1986). Another important property of lipoxygenases is

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the specificity of volatiles produced due to the positional specificity of oxygen addition and double bond rearrangement. This is in contrast to non-enzymic autoxidation which yields a relatively non-specific spectrum of volatiles.

Lipoxygenase is present in gill and skin tissue of several species of fish. German and Kinsella (1985, 1986b,c) identified 12-lipoxygenase in the gill tissue of rainbow trout which was active toward arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid. German and Creveling (1990) discovered an arachidonic-15-lipoxygenase activity in the gill tissue of teleost fishes during purification of the previously recognized and more dominant 12-lipoxygenase enzyme. It was proposed that the postmortem release of lipoxygenase could generate significant quantities of reactive lipid hydroperoxides (Hsieh et al., 1988) which, in conjunction with metal catalysts (such as ferric and ferrous ions) could serve as a source of potent free-radical species (Kanner et al., 1987).

## 1. Mechanism of Lipoxygenase-Catalyzed Lipid Oxidation

All substrates for lipoxygenase-catalyzed oxidation must contain the basic *cis,cis*-non-conjugated pentadiene system. Lipoxygenase readily catalyses the insertion of oxygen into polyunsaturated fatty acids containing a 1,4-pentadiene sequence to form positionally specific acyl hydroperoxides (Yamamoto, 1983). There is a requirement that the central methylene group of the 1,4-pentadiene group

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occupies the ω-8 position on the fatty acid chain and the hydrogen to be removed from the central methylene group must be in the L-position (deMan, 1990).

The initial products from lipoxygenase action are *cis, trans*-conjugated hydroperoxy acids. These products probably do not contribute directly to flavor changes in fish because they are flavorless (Applewhite, 1985). However, their decomposition results in a complex mixture of compounds which contribute to off-flavor development (Frankel, 1984).

Tappel et al. (1953) established that the mechanism of lipoxygenase action does not involve a free radical chain process as observed in autoxidation or in hematin-catalyzed oxidation. The possibility of a chain process was ruled out on kinetic grounds. In animal tissues, the enzymatic reduction of hydroperoxides leads to the production of the corresponding hydroxy analogs. The acyl hydroperoxides, hydroxy fatty acids, and their subsequent metabolites, may be important regulators and mediators of physiological and pathological functions in mammalian cells (Vliegnthart and Veldink, 1980). They are also potential precursors of short-chain carbonyl compounds (Frankel, 1984).

The efforts to characterize the mechanism of action of mammalian lipoxygenases are limited by a lack of information on the isolated enzymes (Kanner et al., 1987). Additionally, the precise nature of the state of the iron environment in the various lipoxygenases from animal origin has not been fully elucidated. Greenwald et al. (1980) have indicated

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that nonheme ferric iron is a required co-factor and that iron chelators remove activity of the enzyme. However, lipoxygenase enzyme from soybeans containing non-heme iron has been well characterized and its resemblance to the animal enzymes has been successfully predictive.

The mechanism proposed by Gaillard (1975) describes the lipoxygenase reaction as a free radical process involving the interaction between the enzyme, substrate and oxygen. Chan (1973) found that one atom of iron was bound per molecule of soybean lipoxygenase enzyme and that the metal was essential for its activity. Hsieh and Kinsella (1989b) reviewed the lipoxygenase reaction and indicated that the activation of this enzyme can be initiated by either the ferric or ferrous form in the presence of oxygen. Moreover, the activation of lipoxygenase also requires the presence of a hydroperoxide fatty acid.

A simplified scheme of the catalytic cycle of lipoxygenase is depicted in Figure 2 (Schewe et al., 1986). According to this scheme, the presence of trace amounts of hydroperoxides are required to convert the ferrous state of lipoxygenase to its active ferric form. Subsequently, this activated ferric form of lipoxygenase abstracts hydrogen from the substrate fatty acid. The resulting fatty acid radical remains bound to the enzyme and the enzyme is again reduced to its ferrous state. At this stage, oxygen is stereospecifically introduced into the substrate fatty acid and a hydroperoxy radical is formed. The hydroperoxy radical

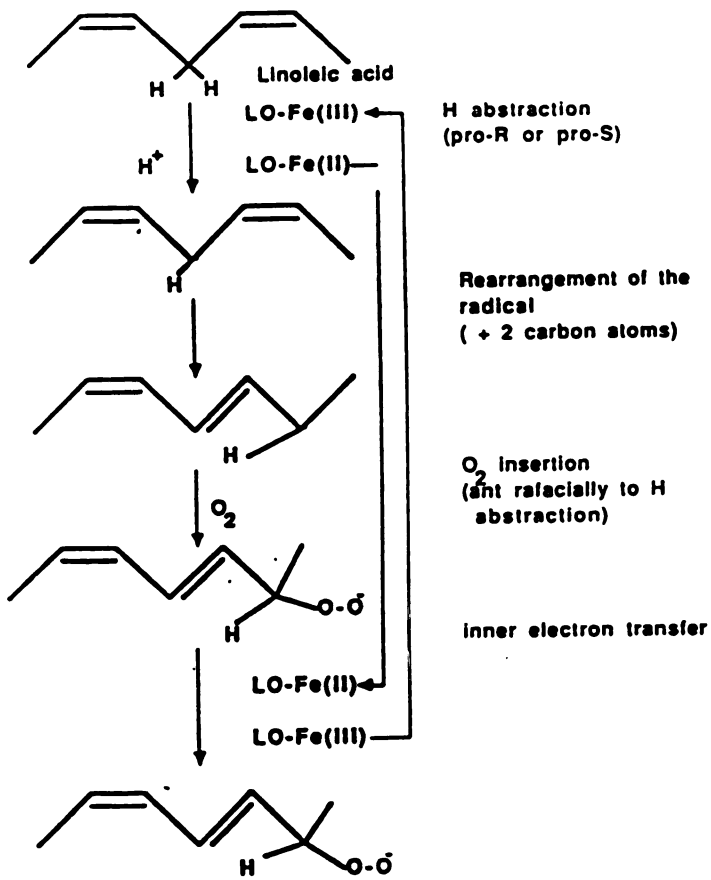


**Figure 2. The simplified reaction scheme of lipoxygenase catalyzed lipid oxidation (Schewe et al., 1986).**

**where LO = Lipoxygenase.**

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then removes an electron from ferrous iron and yields the anion of the hydroperoxy fatty acid. This inner electron transfer regenerates the ferric form of lipoxygenase. This scheme shows that the lipoxygenase reaction is initiated by hydrogen abstraction from the fatty acid and not by the activation of oxygen. This is supported by the fact that fatty acid dimers could arise from free fatty acids generated via the anaerobic lipohydroperoxidase reaction (Hsieh and Kinsella, 1989b). The hydroperoxides are further oxidized by an iron-catalyzed mechanism to generate hydroxy epoxides which are then hydrolyzed to trihydroxy fatty acid derivatives (Pace-Asciak, 1984; German and Kinsella, 1986c). The breakdown of these unstable hydroperoxides and secondary oxidation products may promote further oxidation in fish tissues.

## 2. Factors Influencing Lipoxygenase Activity

Trout gill lipoxygenase displays a broad pH optimum around pH 7 (Hsieh et al., 1988). Lipoxygenase is less sensitive to pH change in the alkaline range compared to the acidic range. A decrease in pH from 7.5 to 6.0 results in the loss of 90% of the lipoxygenase activity. Raising the pH from 7.5 to 9.0 reduced the enzyme activity by only 40%. Hsieh et al. (1988) also observed that gill lipoxygenase retained over 90% of its activity following heating between 10°C and 30°C. Heating the enzyme at 40°C resulted in the retention of 80% of activity. However, an abrupt decline in

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activity occurred at 50°C and only minimal activity was observed at temperatures above 60°C. They suggested that the high residual activity of fish lipoxygenase at temperatures near freezing may be important in initiating lipid oxidation in fish during refrigerated storage.

Hsieh et al. (1988) also observed that lipoxygenase showed equal reactivity towards arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid. However, the enzyme was much less reactive (<20%) towards linoleic acid. The Km of rainbow trout 12-lipoxygenase for arachidonic acid was reported as 20 $\mu$M (Hsieh et al., 1988).

The lipoxygenase enzyme has been shown to be insensitive to the cyclooxygenase inhibitors, indomethacin and aspirin, but is strongly inhibited by stannous chloride (5mM), esculetin (10$\mu$M), and eicosatetraenoic acid (100$\mu$M) (German and Kinsella, 1986a,c; Hsieh and Kinsella, 1989a). Butylated hydroxyanisole (BHA) or esculetin inhibited the formation of specific hydroperoxides and also the resultant volatile products (Hsieh and Kinsella, 1989a). These results indicated that lipoxygenase activity is not only important as an initiator of lipid oxidation, but is also involved in the subsequent generation of oxidative volatile compounds.

German and Kinsella (1985) reported that gill lipoxygenase oxidized arachidonic and eicosapentaenoic acids to produce their respective 12-hydroperoxides (German and Kinsella, 1985). Lipoxygenase can decompose hydroperoxides into specific products. Thus, 12-hydroperoxyarachidonic acid

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decomposes to produce 3-nonenal, 1-octene, or 2-octene radicals (Hsieh and Kinsella, 1989a). The isomerization of 3-nonenal produces 2-nonenal. Acceptance of hydrogen or hydroxyl radical by the 1-octene radical forms 1-octene or 1-octen-3-ol. Similarly, the 2-octene radical may accept a hydrogen or hydroxy radical to produce 2-octene or 2-octen-1-ol (Figure 3a). Hsieh and Kinsella (1989a) also reported that the 12-hydroperoxyeicosapentaenoic acid can be decomposed by similar mechanisms resulting in the formation of 2,6-nonadienal, 1,5-octadien-3-ol, and 2,5-octadiene-1-ol (Figure 3b).

## The Role and Mechanism of Vitamin E as an Antioxidant in Fish

The generation of free radicals *in vivo* is initially minimized by preventive antioxidants such as certain enzymes, carotenoids and chelators of transition metal ion catalysts. Preventive antioxidants reduce the formation of radicals and reactive oxygen species by decomposing fatty acid hydroperoxides and hydrogen peroxide, by sequestering metal ions, and by quenching and dismutating active oxygen. Although small amounts of free radicals may be formed *in vivo*, free radicals may also be taken into the body exogenously (Niki, 1993). The second line of defense include antioxidants which can scavenge the active radicals to suppress initiation reactions and/or break the chain propagation

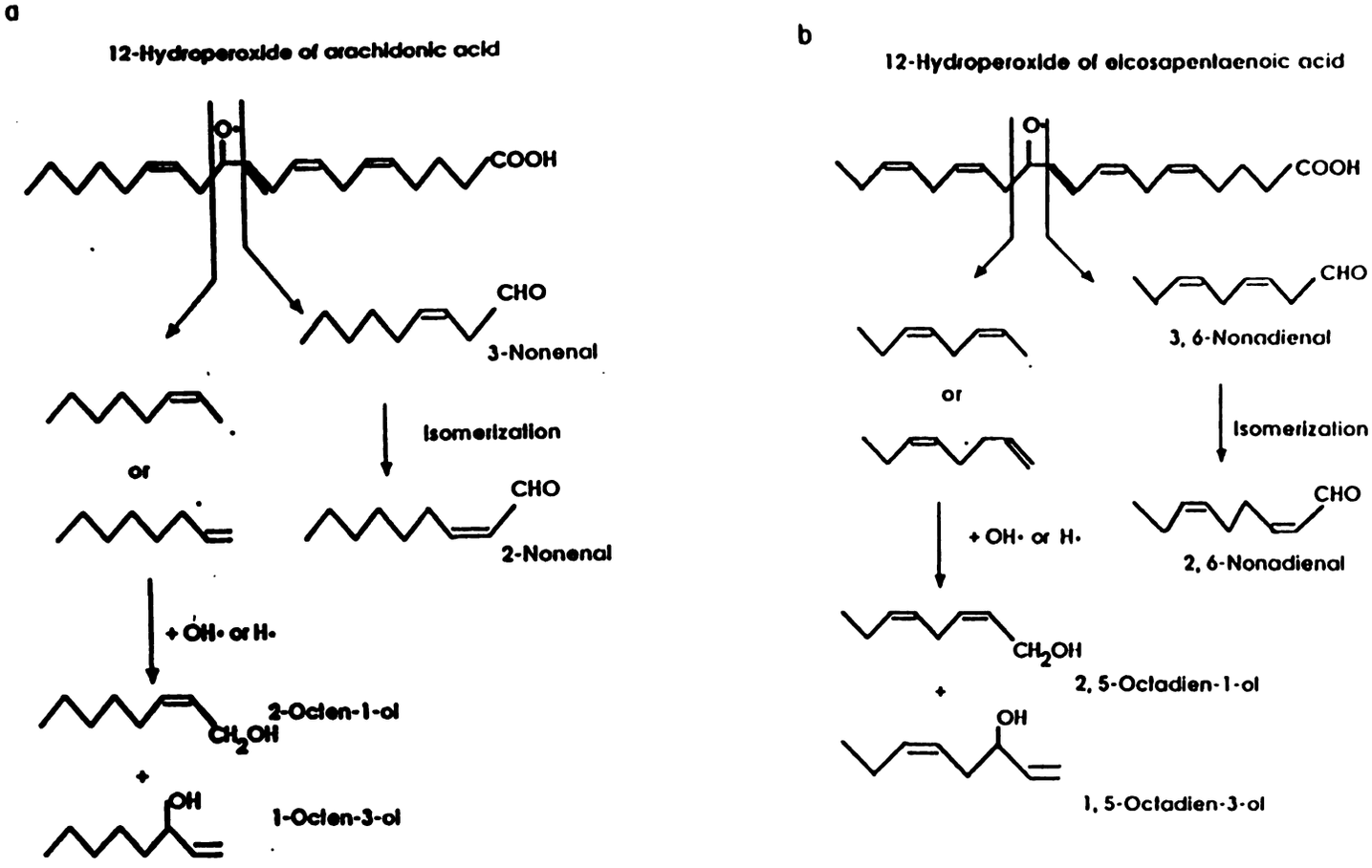


Figure 3. The proposed reaction scheme of lipoxygenase-catalyzed oxidation of arachidonic acid (a) and eicosapentaenoic acid (b), and the subsequent generation of oxidative flavor compounds (Hsieh and Kinsella, 1989a).

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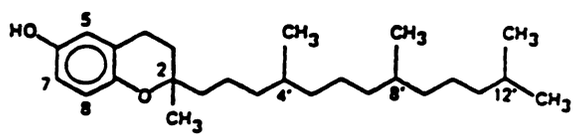
by trapping a radical. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant (Niki, 1993). It prevents membrane peroxidation by scavenging the peroxy radicals involved in the chain reactions (Packer and Landvik, 1990). Vitamin E quenches singlet oxygen by both physical and chemical means and also reacts with the superoxide radical (Roberfroid and Buc Calderon, 1995). Chain-breaking and preventive antioxidants interfere at different points in the oxidation process and thus synergistically reinforce each other (Scott, 1965).

## 1. Chemistry of Vitamin E

Vitamin E is an essential lipid-soluble antioxidant present in mammalian cells and blood (Ingold et al., 1987; Cheeseman et al., 1988). Vitamin E is a generic term for a group of naturally occurring tocopherols (α, trimethyl-; β and γ, dimethyl-; and δ, monomethyl-) and tocotrienols (α, trimethyl-3; β and γ, dimethyl-3; and δ, monomethyl-3) which possess varying degrees of vitamin activity (Figure 4). All share a common 6-chromanol ring, but both types of compounds differ in the degree of unsaturation of the side chain. The tocopherols possess a phytyl side chain ($C_{16}H_{33}$) which favors its insertion into the lipid bilayer region. The trienols have a similar structure with double bonds at the 3′, 7′, and 11′ positions. The presence and position of methyl groups on the chromanol ring plays a key role in determining the biological activity of tocopherols and tocotrienols.

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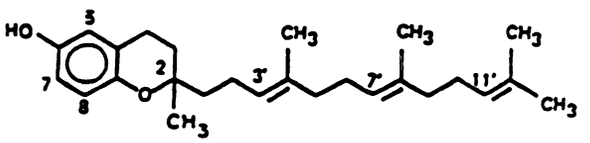


Trienol Structure

| Position of Methyls | Trivial Name (Abbreviations) Tocol Structure | Trienol Structure |
|---|---|---|
| 5,7,8 | α-tocopherol (α-T) | α-tocotrienol (α-T-3) |
| 5,8 | β-tocopherol (β-T) | β-tocotrienol (β-T-3) |
| 7,8 | γ-tocopherol (γ-T) | γ-tocotrienol (γ-T-3) |
| 8 | δ-tocopherol (δ-T) | δ-tocotrienol (δ-T-3) |

**Figure 4. Structures of tocopherols and tocotrienols (Machlin, 1984).**

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## 2. Mechanism of Antioxidant Activity of Vitamin E

Vitamin E functions as a chain terminator by reacting with lipid peroxy radicals. It donates a hydrogen atom to hydroxy radicals and other free radical species, thus preventing the formation of lipid hydroperoxide (Halliwell, 1987; Davies, 1988). Biologically, α-tocopherol is the most active form of vitamin E (Diplock, 1985; Farrell, 1988) and accounts for approximately 90% of the vitamin E found in tissues (Cohn et al., 1992). It functions as an antioxidant by rapidly transferring its phenolic hydrogen atom to a lipid peroxyl radical. This reaction results in the formation of a lipid hydroperoxide and the α-tocopheroxyl radical.

$$\alpha\text{-T} + \text{LOO}^{\bullet} \text{ ---> } \text{LOOH} + \alpha\text{-T}^{\bullet}$$

The radical chain of peroxidation reactions is effectively interrupted. Subsequently, the lipid hydroperoxide must be removed before it becomes a source of free radicals via transition metal-catalyzed decomposition. The tocopheroxyl radical may react with an other tocopheroxyl radical or a peroxyl radical to produce non-radical products. Both these radicals are relatively unreactive towards polyunsaturated lipids (Burton et al., 1993; Roberfroid and Buc Calderon, 1995).

Roberfroid and Buc Calderon (1995) reported that vitamin E radicals are stable because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure. On the other hand, it has been reported that the

α-tocopheroxyl radical can become a chain carrying radical in the heterogeneous micro-environment typical of lipoproteins and lipid membranes by reacting with polyunsaturated fatty acids (Bowry et al., 1992; Bowry and Stocker, 1993; Ingold et al., 1993). The entry of radicals into the low-density lipoprotein (LDL) particles accelerates lipid peroxidation. This reaction occurs more rapidly in the absence of vitamin E (Bowry and Stocker, 1993). Under these conditions, vitamin E can act as a pro-oxidant rather than an antioxidant. Thus, the tocopheroxyl radical must be reduced back to tocopherol in order to continue its chain breaking function. This can be done by other biological donors such as ascorbate (AH) and thiol systems.

$$\alpha\text{-}T^{\bullet} + AH \longrightarrow \alpha\text{-}T + A^{\bullet}$$

It has been reported that ascorbate in the aqueous phase can directly interact with membrane tocopherols whose oxidizable chromanol entity is located near the surface of membrane (Stoyanovsky et al., 1989).

The site of free radical formation is important in determining the antioxidant activity of selected compounds. It is generally believed that lipid oxidation in meats is initiated in the phospholipid fraction of subcellular membranes which contain substantial quantities of polyunsaturated fatty acids (Love and Pearson, 1971; Igene and Pearson, 1979; Gray and Pearson, 1987). It has been reported that the higher concentrations of polyunsaturated fatty acids cause an increased requirement of vitamin E in fish

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Diplock and Lucy (1973) proposed that vitamin E is primarily located within the biological membranes and physicochemical forces involving lipid-lipid interactions between vitamin E and polyunsaturated phospholipids help to stabilize the entire system. Results of a number of studies also suggested that lipid peroxidation in cells and membranes can be reduced by supplementation with high levels of vitamin E (Asghar et al., 1989; Monahan et al., 1990; Buckley and Morrissey, 1992; Sheehy et al., 1994).

Machlin (1984) reported that α-tocopherol in cells is concentrated in membrane-rich fractions such as mitochondria and microsomes. Apparently, cytosolic proteins facilitate the transport of α-tocopherol into the mitochondrial and microsomal membrane fractions (Mowri et al., 1981; Murphy and Mavis, 1981). The membrane hypothesis has not been accepted by all investigators. Takahashi et al. (1989) showed that mobility, especially vertical mobility, of vitamin E in the membranes is much reduced and hence the efficiency for radical scavenging is reduced in the membranes. It is further argued that the localization of vitamin E in membranes makes it impossible for vitamin E to protect directly non-membrane components (Burton, 1994). Thus, its antioxidant activity depends on the functional presence of other co-operative antioxidants such as vitamin C or ubiquinol.

The action of ubiquinol, a reduced form of coenzyme Q, in the membranes and low density lipoproteins has been the

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subject of extensive studies (Beyer, 1990). In the inner mitochondrial membrane, ubiquinol act as an electron carrier of the electron transport chain where it undergoes continuous addition and loss of an electron. Cabrini et al. (1991) reported that ubiquinol probably regenerates tocopherol by donating two aqueous reducing equivalents and thus functions as an inhibitor of lipid oxidation.

It has been suggested that there is a close relationship between dietary vitamin E and selenium (Figure 5). Combs and Scott (1977) concluded that both nutrients have cooperative roles in the maintenance of cell membrane integrity. Vitamin E functions as a membrane-bound chain-breaking antioxidant due to its free radical quenching ability, whereas selenium functions as an antioxidant via glutathione peroxidase, a Se-containing enzyme, that reduces hydroperoxides to more stable compounds without the production of free radicals. Within the cells, hydrogen peroxide is distributed in both the membrane and aqueous phases (McCay and King, 1980). In the cell membrane, α-tocopherol donates its phenolic hydrogen atom to hydroxyl radicals and other free radical species and stops the chain reaction. In the aqueous phase, glutathione peroxidase prevents the formation of hydroxyl radicals by eliminating hydrogen peroxide and thus functions as a preventive antioxidant.

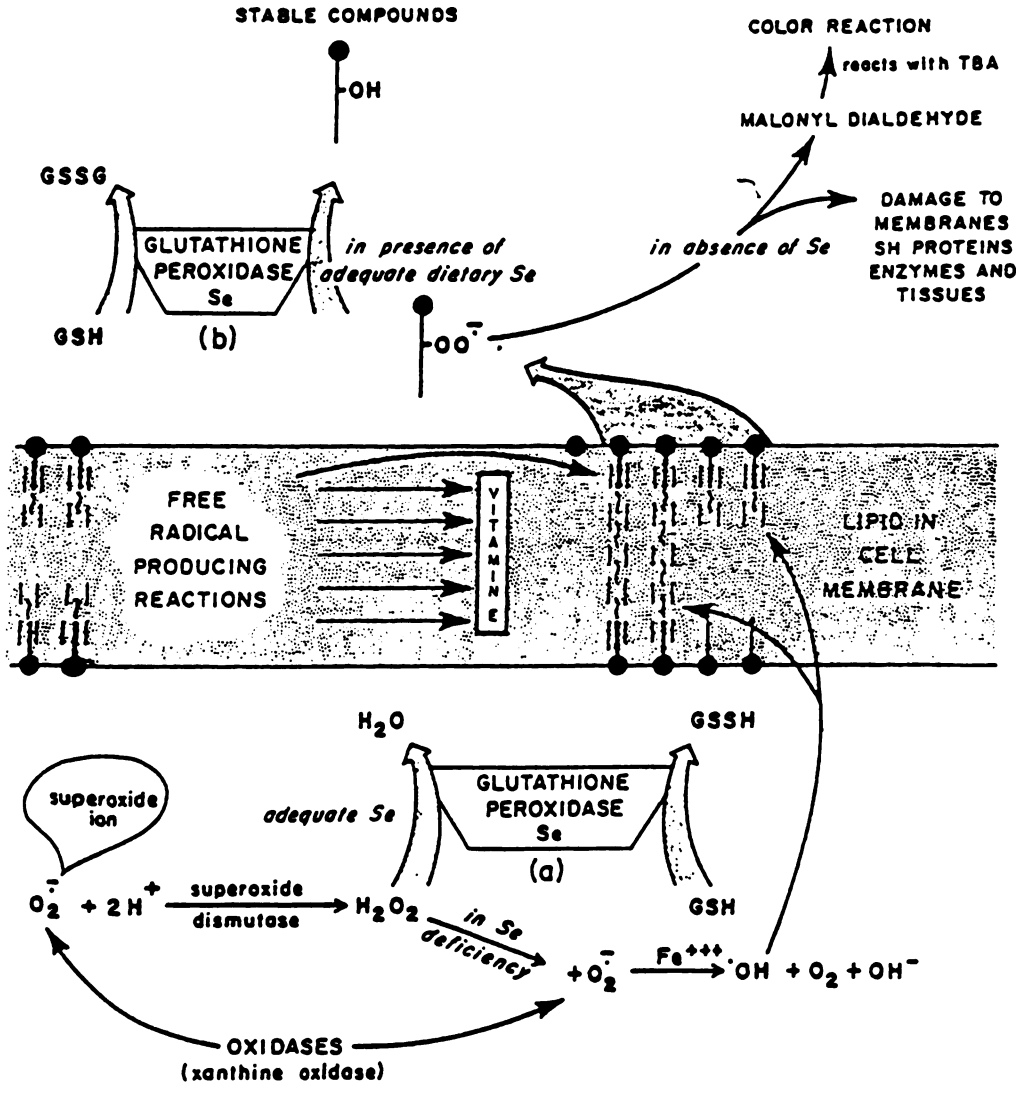


Figure 5. Interrelationships of selenium and vitamin E in protecting membranes (Machlin, 1984).

3. Role of Vitamin E in Fish Lipid and Color Stability

Considerable interest has been expressed in the use of antioxidants and their effects on meat quality (Gray and Pearson, 1984). Dietary supplementation with vitamin E has been shown to improve the oxidative stability of postmortem muscle and adipose tissue in a variety of meats such as beef (Faustman et al., 1989a,b; Arnold et al., 1993; Liu et al., 1994,1995; Sherbeck et al., 1995), chicken (Webb et al., 1972a; Brekke et al., 1975; Lin et al., 1989a,b; Frigg et al., 1991; Sheehy et al., 1994), fish (O'Keefe and Noble, 1978; Frigg et al.,1990; Gatlin et al., 1992; Erickson, 1993; Sigurgisladottir et al., 1994), lamb (Wulf et al., 1995), pork (Astrup, 1973; Tsai et al., 1978; Asghar et al., 1991; Monahan et al., 1994; Buckley et al., 1995), turkey (Webb et al., 1972b; Marusich et al., 1975; Sante and Lacourt, 1994), and veal (Shortland et al., 1981; Engeseth et al., 1993).

Tocopherol plays an important role in maintaining fish flesh quality, including color and the oxidative stability of lipids. Foote et al. (1974), Fahrenholtz et al. (1974, and McCay and King (1980) have suggested that tocopherol may play a role in the prevention of rancidity in fish tissues during frozen storage and marketing. It has been demonstrated that the vitamin E concentrations in the rainbow trout muscle are increased by dietary supplementation. Boggio et al. (1985) fed rainbow trout different diets supplemented with either fish oil or swine fat and α-tocopheryl acetate

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(0, 50, 500, or 1500 mg/kg of diet) for 4 months. The fish fed diets containing the two higher levels of α-tocopheryl acetate had lower levels of malonaldehyde after frozen storage at -80°C for 4 months, but not after frozen storage at -20°C for 10 months.

Frigg et al. (1990) also evaluated the effects of dietary vitamin E (0, 50, 100 or 200mg/kg feed) on the oxidative stability and organoleptic characteristics of trout fillets. Their results clearly demonstrated that dietary treatments had a major impact on the oxidative stability of fish fillets. Fillets from trout fed the higher levels of vitamin E were considerably more stable than those from the control group. Organoleptic evaluation also established significant differences between the dietary treatments. Similarly, WaagbØ et al. (1993) reported that a high vitamin E content improved sensory rancid flavor scores in fillets with increasing contents of ω-3 polyunsaturated fatty acids.

Carotenoids appear to be likely co-factors in the postmortem antioxidant process. Carotenoids are not only conventional chain-breaking antioxidants, but astaxanthin in particular is also superior to α-tocopherol as a reagent for reducing the concentration of the chain-carrying peroxyl radicals as well as quenching singlet oxygen (Miki, 1991). Sigurgisladottir et al. (1994) fed four different diets containing either astaxanthin or a mixture of natural tocopherols (Covi-ox T-70, 70% total tocopherols) or their combination to Atlantic salmon (*Salmo salar*) for 15 weeks.

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They observed that feeding natural tocopherols (α-, β-, γ-, and δ-tocopherols) to Atlantic salmon enhanced retention of astaxanthin. They further observed that incorporation of tocopherols and astaxanthin in fish diets did not affect the lipid content or fatty acid composition of Atlantic salmon.

On the other hand, a number of investigators reported that dietary α-tocopherol supplementation did not improve the storage stability of fish muscle. Hung and Slinger (1982) fed diets supplemented with α-tocopheryl acetate ranging from 33mg/kg to 750mg/kg to rainbow trout for 24 weeks. The effect of muscle α-tocopherol on the storage stability was measured by changes in thiobarbituric acid-reactive substances (TBARS) numbers in samples stored for one year at -17°C and for one year at -17°C followed by 7 days at 2°C. Changes in TBARS numbers between frozen and frozen-refrigerated storage were not significantly affected by dietary treatments. The expected improvement in storage stability of muscle of fish fed high levels of dietary vitamin E was not observed. Pozo (1988) reported that increasing the levels of α-tocopherol (50mg α-tocopheryl acetate/100g feed) in the diet of rainbow trout increased the deposition of canthaxanthin in the flesh. The deposited α-tocopherol did not seem to either protect the pigment from subsequent fading during cold storage or the oxidation of white muscle lipids. A little protection of dark muscle lipids was observed.

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## Oxidation of Cholesterol in Fish

Cholesterol (cholest-5-en-3β-ol) is the major sterol in mammalian tissues and the obligate precursor of steroid hormones and bile salts. Cholesterol spontaneously undergoes autoxidation when it comes in contact with air (Smith, 1981; Maerker, 1987), or photoxidation when exposed to fluorescent light (Herian and Lee, 1985; Bekbolet, 1990). More than 70 oxidized derivatives of cholesterol have been identified (Smith, 1981). Cholesterol oxidation products (COPS) may possibly cause disturbance of arachidonic acid metabolism (Seillan, 1990), inhibition of cholesterol synthesis (Kandustsch and Chen, 1978; Peng et al., 1979; Baranowski et al., 1982), carcinogenesis (Wrensch et al., 1989; Kendall et al., 1992), mutagenicity (Smith et al., 1979; Peterson et al., 1988), atherosclerosis (Guyton et al., 1990), and cytotoxicity (Jacobson et al., 1985; Peng et al., 1991).

The possible adverse biological effects of COPS have intensified interest in investigating cholesterol oxidation and its magnitude in food products such as baby food (Sanders et al., 1989a; Sarantinos et al., 1993), baked foods containing eggs (Zunin et al., 1995), butter (Luby et al., 1986; Pie et al., 1990), cookie mix (Pie et al., 1990), egg products (Hurst et al., 1985; Tsai and Hudson., 1985; Nourooz-zadeh and Appelqvist, 1987; Morgan and Armstrong, 1992; Huber et al., 1995; Lai et al., 1995), french fries (Zhang et al., 1991), ghee (Kumar and Singhal, 1992), marine

products (Ohshima et al., 1993; Osada et al., 1993; Chen and Yen, 1994), meat products (Sanders et al., 1989a; Pie et al., 1991; Monahan et al., 1992; Monahan et al., 1993; Engeseth et al., 1993; Engeseth and Gray, 1994), irradiated meat products (Maerker and Jones, 1992; Hwang and Maerker, 1993), milk and dairy products (Nourooz-Zadeh and Appelqvist, 1988a,b; Sanders et al., 1989b; Chan et al., 1993), and heated tallow (Bascoul et al., 1986; Park and Addis, 1986).

## 1.Chemistry of Cholesterol and its Oxidation Products

Cholesterol, $C_{27}$ $H_{46}$ O, has a cyclopentanophenanthrene ring structure with an eight-carbon aliphatic side chain at $C_{17}$, two angular methyl groups at $C_{10}$ and $C_{13}$, and a double bond between carbons 5 and 6 (Figure 6). Most of the cholesterol in the body is unesterified, except in plasma and skin where a greater portion exits in the ester form.

Similar to the oxidation of unsaturated fatty acyl moieties of other lipids, the initiation events in cholesterol oxidation remain unresolved. Among the potential initiators of cholesterol oxidation considered by Smith (1981) are preformed hydroperoxides, transition metals, activated oxygen species and radiation. The schemes for cholesterol oxidation are depicted in Figure 7. Cholesterol oxidation is initiated by abstraction of an allylic C-7 hydrogen and followed by reaction with triplet oxygen to form two epimeric cholesterol 7-hydroperoxides (2a, 2b) (Smith, 1981). Kulig

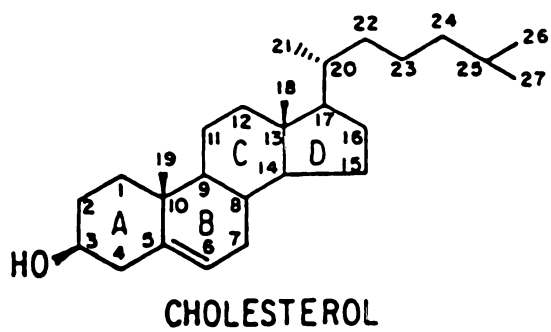
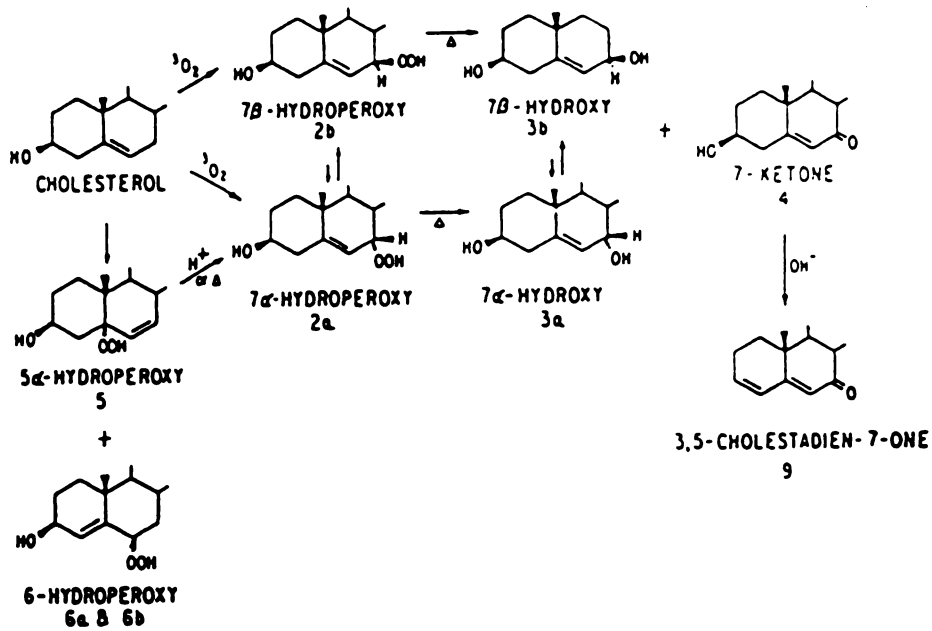



**Figure 6. Structure of cholesterol**

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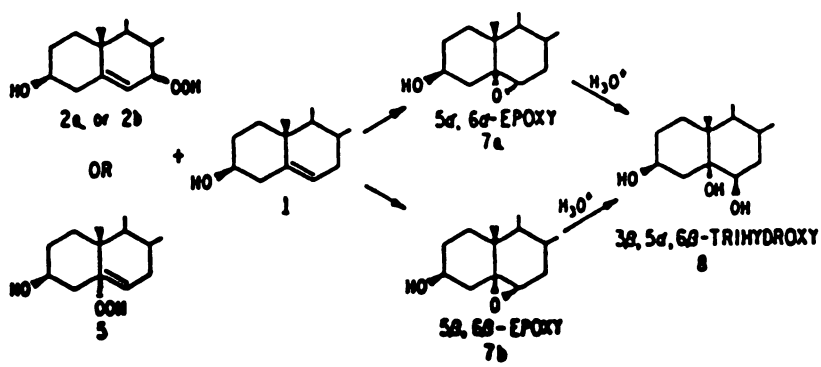


**SCHEME 2**

**Figure 7. Schemes showing cholesterol oxidation (Maerker, 1987).**

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and Smith (1973) reported that attack by singlet oxygen ($^1O_2$) on cholesterol results in the formation of (74-75%) of 5α-hydroperoxide (5) that is readily isomerized to 7α-hydroperoxide in the presence of acid or heat. The hydroperoxides (2a, 2b) are subsequently converted to more stable products such as the epimeric 7-hydroxycholesterols (3a, 3b) and 7-ketocholesterol (4) because of their thermal decomposition (Van Lier and Smith, 1970).

Smith and Kulig (1975) further reported that the epoxides (7a, 7b) are the products of attack by 7-hydoperoxides (2a, 2b) or by 5α-hydroperoxide (5) on the 5,6-double bond of cholesterol and therefore are secondary oxidation products. The hydration of the epoxides (7a, 7b) results in the formation of cholestan-3β, 5α, 6β-triol (Smith and Kulig, 1975). The α-epimers of cholesterol oxidation products such as 2a, 3a or 7a are thermodynamically less stable than the β-forms (2b, 3b or 7b), and interconversion occurs readily (Maerker, 1987). Side chain reactions can also occur and yield a variety of oxidation products (Korahani et al., 1982; Maerker,1986).

## 2.Cholesterol Oxidation in Fish and Fish Products

Ohshima et al. (1993) studied the oxidative decomposition of cholesterol in fish products including salted and dried, boiled and dried and smoked products. The concentration of total COPS ranged from 8.3mg/kg in boiled and dried shrimp to 188.0mg/kg in boiled and dried anchovy. Smoked

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salmon contained 26.8mg/kg of total COPS. The predominant COPS were 7β-hydroxycholesterol and 7-ketocholesterol.

To elucidate a mechanism for cholesterol oxidation during fish processing and subsequent preservation, Ohshima et al. (1993) conducted a model study using four systems consisting of a mixture of purified cod liver triglycerides (2.8g) plus cholesterol (1.1g), a mixture of triolein (2.8g) plus cholesterol (1.1g), triolein (2.8g) alone and cholesterol (1.1g) alone. These mixtures were placed in petri dishes and allowed to stand in a desiccator at 25°C over phosphorus pentoxide for 104 days. COPS were not found in the model system containing only cholesterol or in the mixture containing triolein and cholesterol over the entire storage period. However, the mixture of cod liver triglycerides and cholesterol produced several COPS, including 7β-hydroxycholesterol, 7-ketocholesterol, epimeric epoxides and the cholestane-triol after 24 days of storage. Their results strongly suggested that cholesterol oxidation in fish products proceeded simultaneously with the oxidative decomposition of the co-existing polyunsaturated fatty acids of fish oil.

Osada et al. (1993) also observed that COPS were generated shortly after heating cholesterol with various fats such as tristearin, beef tallow, triolein, soybean oil, safflower oil, linseed oil and sardine oil at 100°C for up to 24 hrs. Their observations indicated that lipid oxidation precedes cholesterol oxidation.

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The concentrations of oxidized cholesterol in uncooked and processed marine products (air-dried sardine, air-dried squid, canned squid, and pickled and spiced Alaskan pollack roe) were measured by Osada et al. (1993). Raw fish contained essentially no oxidized cholesterol, the processed products examined contained total COPS (11.0-28.7mg/100g), 7α-hydroxycholesterol (2.7-3.8mg/100g), 5α-epoxycholesterol (0.2-5.8mg/100g), 5β-epoxycholesterol (0.7-4.9mg/100g),7β-hydroxycholesterol (2.8-9.8mg/100g) and 7-ketocholesterol (1.9-5.3mg/100g). Chen and Yen (1994) studied the formation of COPS in small sun-dried fish (*Spratelloides gracilis* and *Decapterus maruadsi*) which were stored at ambient temperature without packaging for 3 months under air. Four major COPS, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, and 5,6α-epoxycholesterol, were identified. The total concentration of COPS ranged from 4.8μg/g to 65.7μg/g, with 7α-hydroxycholesterol being the predominant product.

## Natural Pigments and their Role in Salmonid Pigmentation

Nature relies on a variety of compounds for pigmentation of living organisms. The main groups of coloring substances in food are chlorophylls, anthocyanins, porphyrins and carotenoids which occur in both the animals and plant kingdoms. Within nature's own food colors, carotenoids play a particular interesting role due to their varied biological functions and are the most numerous and widespread group of

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plant-synthesized polyene pigments (Tacon, 1981). A wide variety of foods owe their color mainly to carotenoids. Approximately 600 naturally occurring carotenoids have been characterized. Two major families of carotenoids are xanthophylls (oxygenated) and hydrocarbons. Less than 10% of them are hydrocarbons containing a β-ionone ring and are potential sources of provitamin A activity (Olson, 1989; Thurnham, 1994).

However, none of them is as effective or as important as β-carotene as a source of vitamin A (Thurnham, 1994). β-Carotene not only act as an excellent quencher of singlet oxygen but also scavenges reactive oxygen species (Roberfroid and Buc Calderon, 1995). Terao (1989) reported that xanthophyll carotenoids such as zeaxanthin, astaxanthin and canthaxanthin appear to be more efficient antioxidants than β-carotene and are active at ambient oxygen pressures. Although it is generally assumed that animals are not able to synthesize carotenoids *de novo*, they are apparently able to modify plant carotenoids taken up in their food (Goodwin, 1984; Storebakken and No, 1992; Choubert and Heinrich, 1993).

## 1. Pigmentation of Salmonids

Carotenoids are the main pigments of many aquatic animals (Foss et al., 1984). One of the most striking characteristics of salmonid fishes (*Salmo* spp., *Oncorhynchus* spp. and *Salvelinus* spp) is the pink or red pigmentation of their

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flesh and eggs, and the more varied hues of their skin (Withler, 1986). The pink to red pigment in the flesh and other tissues of salmonids is due to oxygenated carotenoids of dietary origin (Torrissen, 1989; Gentles and Haard, 1990). Since fish are not able to synthesize these pigments *de novo*, they must be derived from the diet (Goodwin, 1984; Storebakken and No, 1992; Choubert and Heinrich, 1993). Carotenoids are widespread and their bright color is due to a chromophore consisting of conjugated double bonds. Carotenoids are labile to light, heat and oxygen (Krinsky, 1989) and their oxygenation, isomerization and rearrangement occur easily (Torrissen et al., 1989). This may result in the fading of flesh during storage and processing. However, carotenoids can also be degraded through enzymic activity (Krinsky, 1989).

Torrissen et al (1989) reviewed the carotenoid sources used for pigmenting salmonids, including crustaceans (krill, red crab), crustacean waste (shrimp), crustacean meat (crab, crawfish, krill), crustacean extract (crawfish, cupepode, red crab, shrimp), fish oil (capelin oil, krill oil, mackerel oil, shrimp oil), marigold flowers, squash flowers, paprika extract, algae, and yeast. Astaxanthin, the main carotenoid found in wild salmonids, is derived from ingested crustaceans (Torrissen, 1989). In farmed salmonids, carotenoids such as astaxanthin and canthaxanthin, are supplemented in the feed to achieve the desired coloration of the flesh (Pozo et al., 1988; Torrissen et al., 1989). About 90%

of the carotenoids found in the tissue are located in the flesh in the free form. Large amounts are also found in the skin and ovaries in maturing fish (Torrissen et al., 1989). Hydroxy-carotenoids in the skin are present mainly as esters (Hata and Hata, 1975). Generally, there are great individual differences in the ability of salmonids to absorb or deposit carotenoids in their flesh. Other factors such as fish size, growth rate, sexual maturation and genetic background also influence muscle pigmentation (Abdul-Malak et al., 1975; Torrissen, 1986,1989; Torrissen and Torrissen, 1985; Torrissen and Nævdal, 1988).

## 2. Factors Affecting Carotenoid Deposition

### (a) Carotenoid sources

Various dietary sources capable of providing oxygenated carotenoids have been evaluated for the pigmentation of salmonids. Major carotenoid sources include crustacea and crustacean-byproducts, yeast, plants, algae and pigments (Schmidt and Baker, 1969; Satio and Regier, 1971; Johnson et al., 1980; Torrissen et al., 1982; Tidermann et al., 1984; Torrissen et al., 1989). During the last decade, carotenoids (astaxanthin and cathaxanthin) have become the dominant pigments used to color cultured salmonids (Torrissen, 1989).

Several studies have indicated that rainbow trout utilize dietary astaxanthin 1.3 to 1.5 times more efficiently than canthaxanthin (Foss et al., 1984; Tidemann et al., 1984; Choubert and Storebakken, 1989; Torrissen,

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1985,1986,1989). However, these two carotenoids appear to have a synergistic effect when they are fed in a mixture (Foss et al., 1987a; Torrissen, 1989). Both free astaxanthin and canthaxanthin are deposited without modification in the flesh of rainbow trout (Hata and Hata,1975; Foss et al., 1984,1987a). The two carotenoids are metabolized by different routes in the skin of Atlantic salmon (Schiedt et al., 1985,1988) and rainbow trout (Schiedt et al., 1985).

A distinctive red color is of prime importance to consumer acceptance of salmon and trout (Ostrander et al., 1976; Sceurman et al., 1979). Rainbow trout (*Oncorhynchus mykiss*) reared in fresh water have been sold traditionally as unpigmented portion-size fish (0.2-0.4kg), whereas the trout produced in sea pens are brought to market at a larger size (2-4kg) and with a salmon-like pink coloring of the flesh. The main carotenoid in wild rainbow trout is astaxanthin, particularly (3S, 3'S)-astaxanthin (Schiedt et al., 1986).

Foss et al. (1984) reported that rainbow trout use three optical isomers of astaxanthin to the same extent. Choubert and Storebakken (1989) observed that the optimal concentrations of astaxanthin and canthaxanthin in rainbow trout are between 50 and 100mg/kg. Bjerkeng et al. (1990) found high carotenoid concentrations in the flesh after 16 weeks of feeding and demonstrated that rainbow trout reared in the sea are able to utilize dietary carotenoids efficiently. In this experiment, rainbow trout (*Oncorhynchus mykiss*), with

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an average initial weight of 460g, were fed diets containing 25, 50, and 100mg/kg of racemic astaxanthin (1:2:1 mixture of the three optical isomers (3R,3'R; 3R,3'S and 3S,3'S)), and canthaxanthin, respectively. The trout showed an average body weight increase of 450g during the 16 weeks of feeding in sea pens. There was a tendency for astaxanthin to be more efficiently utilized than canthaxanthin for flesh pigmentation, and a final maximum concentration of 11mg/kg was obtained when the highest dietary concentration of astaxanthin was fed. The benefit of increasing the dietary carotenoid concentration from 50 to 100mg/kg was minimal compared with the increase from 25 to 50mg/kg. The trout fed canthaxanthin tended to accumulate more carotenoids in the skin as metabolites. In the skin of the astaxanthin-fed trout, astaxanthin esters predominated. The dietary carotenoids were deposited unchanged in the flesh. No epimerization took place at C-3 and C-3' of astaxanthin in the flesh, and the isomeric composition resembled closely that of the feed.

These data are consistent with previous findings for rainbow trout (Foss et al., 1984,1987b; Schiedt et al., 1985; Katsuyama et al., 1987) and Atlantic salmon (Storebakken et al., 1985). However, slight deviations in the distribution of the three optical isomers of astaxanthin in the skin were noticed from that found in flesh. There was a preferred accumulation of the (3R,3'S) and (3S,3'S) isomers relative to that of the (3R,3R') isomer. The preferred accumulation contradicts the results obtained in other

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studies (Foss et al., 1984,1987b; Katsuyama et al., 1987; Schiedt et al., 1985,1988; Storebakken et al., 1985,1987) when astaxanthin was fed in the free form or as diesters to rainbow trout and Atlantic salmon. However, these results support the findings of Kamata (1986) who observed no epimerization in the flesh of rainbow trout. There was a preference for the deposition of the 3S,3'S isomer of astaxanthin in the skin.

In most of the studies, a linear relationship between visual score and carotenoid level in farmed fish was observed only at the lower carotenoid levels (Spinelli and Mahnken, 1978; Foss et al., 1987a; Storebakken et al., 1987). It has been reported that the human eye seems to be less sensitive to carotenoid concentrations over 3 to 4mg/kg compared with lower concentrations (Torrissen et al., 1989). Additionally, unpigmented intermuscular fat may mask the impression of color. Based on visual color impression, a level of 3 to 4mg/kg can be regarded as an acceptable carotenoid concentration in marketable farmed salmon (Torrissen et al., 1989).

(b) Initial Weight of Fish and Carotenoid Concentration

Trout initially weighing 0.1 to 0.5kg can obtain satisfactory flesh pigmentation if they double their body weight when fed a carotenoid-containing diet (Storebakken and No, 1992). Torrissen (1989) confirmed earlier results that rainbow trout weighing below 80 to 100g deposit only low amounts

of carotenoids. A clear effect of growth rate on pigment deposition was found. A rapid increase in flesh carotenoid concentrations with increasing growth for slow and medium slow-growing fish was observed, with a slower increase for fast-growing fish. The results also clearly showed that astaxanthin is deposited in the flesh more efficiently than canthaxanthin. A combination of astaxanthin and canthaxanthin in the diet gave a higher total carotenoid deposition in the flesh compared to supplementation with astaxanthin or canthaxanthin individually. A higher total carotenoid concentration was found in the flesh when the astaxanthin level was between 72% and 35% and the corresponding canthaxanthin levels were 28% and 65%, with a maximum at about 60% astaxanthin and 40% canthaxanthin. This shows that absorption and/or deposition of astaxanthin and canthaxanthin are, to some extent, interdependent. However, the carotenoid levels used in this experiment were high, and lower levels might give less pronounced effects.

In another study conducted by Choubert and Storebakken (1989), rainbow trout (*Oncorhynchus mykiss*) with a mean initial weight of 135g were fed diets supplemented with astaxanthin or canthaxanthin in concentrations ranging from 0 to 200mg/kg for 6 weeks. Fish pigmentation increased with increasing dietary carotenoid concentrations up to 3.7mg/kg flesh in the best pigmented groups. The fish were pigmented faster with astaxanthin than with canthaxanthin. For both pigments, the retention coefficients decreased as the

pigment dose in the diet increased. The mean retention coefficients for astaxanthin were 1.3 times higher than for canthaxanthin.

Studies on rainbow trout in freshwater (Choubert and Storebakken, 1989) and saltwater (Bjerkeng et al., 1990) showed no increase in the color of flesh of immature trout when the dietary intake was increased above 50 mg/kg. This lack of response was similar for both astaxanthin and canthaxanthin, mainly because carotenoid digestibility is depressed when the dietary concentrations are increased (Choubert and Storebakken, 1990; Torrissen et al., 1990). However, one exception to this generalization has been seen for sexually maturing fish since they have a reduced rate of depigmentation when they are fed diets with 100 mg astaxanthin/kg compared to 50 mg/kg (Helland et al., 1990).

(c) Feeding Rate

It has been reported that feeding rate does not significantly affect the digestibility of protein and energy when the trout are fed several meals a day (Storebakken et al., 1991). Storebakken and Choubert (1991) observed that feeding at the rate of 0.5% of the body weight/day (restricted feeding) in comparison with 1.0% of body weight/day (satiation, less than 15% overfeeding) to 0.1kg trout at 7°C resulted in a tendency for lower carotenoid concentrations in the flesh. However, the effect of both treatments on carotenoid pigment digestibility and retention of carotenoids

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(d) Water Temperature and Salinity

No and Storebakken (1991a) investigated the effect of water temperature (5°C and 15°C) on the pigmentation of rainbow trout by feeding a test diet supplemented with 57mg astaxanthin/kg feed. After feeding the test diet for 6 weeks, the flesh contained more carotenoid at 15°C than at 5°C. No significant temperature effects on the carotenoid concentrations in the skin, liver and gut were observed.

A number of studies conducted to determine the effect of salinity on pigmentation showed no significant differences in pigmentation between trout held in freshwater and saltwater (Storebakken and Choubert, 1991; No and Storebakken, 1992), in spite of the fact that there was a tendency for higher pigment availability in trout held in freshwater than those held in saltwater (Choubert and Storebakken, 1990).

(e) Sexual Maturation and Genetic Effect

It has been reported that fry and fingerlings deposit carotenoids mainly in the skin while post-juvenile fish mainly deposit carotenoids in the flesh (Storebakken and No, 1992). A number of studies indicated that salmonids undergoing sexual maturation mobilize carotenoids from the flesh and selectively transfer them to the skin and gonads (Crozier, 1970; Sivtseva and Dubrovin, 1981; Kitahara,

1983). Normally, sexually mature rainbow trout are not marketed. However, a high proportion of trout regain suitable flesh quality after spawning. Helland et al. (1990) suggested the use of relatively high carotenoid levels in the diet prior to spawning to minimize the depletion of carotenoids from the flesh.

Several studies showed the impact of genetic factors on carotenoid deposition in the flesh of rainbow trout (Torrissen and Naevdal, 1984; Blanc and Choubert, 1985). At present, there is no metabolic explanation for such responses. Choubert and Blanc (1985) observed that immature diploid and triploid trout have similar flesh pigmentation. This observation stresses the need for utilizing properly defined genetic stocks in order to obtain a desired flesh color.

3. Paprika Carotenoids as a Source of Pigmentation

Oleoresin paprika is prepared from the dried ground fruits of *Capsicum annuum* or *Capsicum frutescens*, often referred to as red papper. Most of the carotenoids in paprika oleoresins are esterified with fatty acids which makes them oil-soluble (Philip et al., 1971; Gregory et al., 1987). Fisher and Kocis (1987) separated paprika carotenoids by high performance liquid chromatography (HPLC), and reported the presence of hypophasic and epiphasic compounds. The hypophasic compounds including capsorubin, capsanthin and zeaxanthin eluted first. The epiphasic compounds eluted

later and included monoesters of carotenoids, β-carotene and diesters of carotenoids. These investigators also indicated that the diesters of carotenoids are the principal pigments in oleoresin paprika, but did not identify the individual peaks for the esters of carotenoids. However, Biacs et al. (1989) found that the majority of the carotenoid esters are mono- and diesters of capsanthin. These investigators further observed that these carotenoid esters were more stable toward lipoxygenase-catalyzed oxidation of linoleic acid in comparison to free pigments.

Reeves (1987) reported that red paprika color is primarily caused by capsanthin and capsorubin, while β-carotene and violaxanthin give the yellow-orange color. Capsanthin esters account for 44% of the total carotenoids in paprika oleoresin (Klaui and Bauernfeind, 1981). Esterification reduces the absorption properties of capsanthin, but does not significantly change the hue.

The inclusion of paprika in the diet of salmonids for flesh pigmentation is well documented. Tunison et at. (1944) and Bitzer (1963) observed optimum color in trout on feeding 2% dietary paprika for 6 months and 4-6 months, respectively. However, dietary paprika at a level of 10% produced a brilliant color when fed for 24 weeks (Tunison et al., 1944). Peterson et al. (1966) fed paprika xanthophylls (237 mg/kg) to brook trout for various periods of time. The colors began to appear in the skin after two weeks of feeding and were found to be fairly prominent after four weeks.

These investigators reported that the colors appeared to be natural except for a small amount of an undesirable yellow.

The mechanism of carotenoid oxidation in processed foods is very complex because of the complicated interactions of the various carotenoids present in foods with the surrounding media (Francis, 1985). Therefore, model systems have been frequently used to elucidate the mechanism of carotenoid oxidation. Philip and Francis (1971) investigated the oxidation of capsanthin by molecular oxygen at 40°C in the solid state. They reported that the oxidation process involves primarily the conversion of hydroxyl groups to keto groups, followed by scission of the chain at the carbon-carbon bond 'a' to the in-chain carbonyl group. A number of keto-carotenoids such as capsanthin, 3-keto-kryptocapsone and 3-keto-β-apo-8′-carotenal were identified among the oxidation products.

## Color Stability of Pigmented Rainbow Trout under Various Packaging and Processing conditions

The color of salmonid flesh may be assessed by various analytical methods including sensory analysis using trained panelists for descriptive tests (Ostrander et al., 1976; Skrede and Storebakken, 1986a), comparison of fish samples with standardized colors (Francis and Clydesdale, 1975; McCallum et al., 1987; Skrede et al., 1990), instrumental analysis based on light reflected from flesh samples

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(Francis and Clydesdale, 1975; Little et al., 1979; Skrede and storebakken, 1986 a,b), and quantitative carotenoid analysis of salmonid flesh (Schiedt et al., 1981; Yamazaki et al., 1983; Torrissen et al., 1989).

1.Color Stability under Various Packaging Conditions

Carotenoids are labile to light, heat and oxygen, and may fade in salmonid flesh during storage (Krinsky, 1989). Chen et al. (1984) and Pozo et al. (1988) reported fading of astaxanthin and canthaxanthin in vacuum-packed rainbow trout fillets during cold storage.

Chen et al. (1984) reported that carotenoids (astaxanthin and canthaxanthin) deposited in rainbow trout were stable (approximately 90% retention) in air-packed samples stored at 1-2°C. However, maximal TBARS numbers were observed in the air-packed samples. Oxygen-evacuated and $CO_2$-enriched packaging gave adverse color stability. Lowest pigment levels were found in the frozen samples, with 53% of the absorbed pigment degraded in 14 days. In contrast, No and Storebakken (1991b) found that vacuum packed-fillets of rainbow trout during frozen storage (3 and 6 months at -20 and -80°C) were stable (up to 5% loss) regardless of carotenoid source and rearing conditions. Frozen storage resulted in increased $L^*$ (lightness), $a^*$ (redness) and $b^*$ (yellowness), and decreased $H(^\circ)_{ab}$ (hue) values. Color characteristics from different parts of the fillet differed significantly.

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2. Color Stability under Various Processing Conditions

Stability of the deposited carotenoids during transportation, storage, and cooking of fish is critical to assure optimal acceptance of aquaculturally raised products. Skrede and Storebakken (1986a) studied the color characteristic of raw, baked and smoked wild and farmed Atlantic salmon and reported that the color of baked salmon flesh contained less redness and had a more yellowish hue than raw flesh. The decrease in redness was more extensive in the farmed salmon (41%) than in the wild salmon (23%). As in raw fish, the redness and hue in baked salmon were better correlated with the carotenoid concentration of raw salmon than were lightness and yellowness.

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## CHAPTER ONE

## DIETARY PIGMENTATION AND DEPOSITION OF α-TOCOPHEROL AND CAROTENOIDS IN RAINBOW TROUT MUSCLE AND LIVER TISSUE.


### ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) were fed diets supplemented with canthaxanthin, oleoresin paprika and α-tocopherol in order to study their deposition in the muscle tissue. Fish weight increased approximately four fold over the 7 month feeding period, with the composition of diets having little or no growth-enhancing effect. Canthaxanthin was more efficiently absorbed than the carotenoids in the oleoresin paprika. The average total carotenoid concentration in the flesh of rainbow trout fed a commercial canthaxanthin diet was 7.9 mg/kg. As the dietary level of α-tocopheryl acetate increased, muscle and liver concentrations of α-tocopherol correspondingly increased. Concentrations of α-tocopherol in the muscle tissue of fish fed 500mg α-tocopheryl acetate/kg feed ranged from 54.1 to 57.1mg/kg. Liver contained 1300 to 1456.1mg/kg. With the increased pigmentation, decrease in lightness ($L^*$) and hue angle, and increase in redness ($a^*$) was observed. The dietary level of α-tocopherol had no effect on muscle fatty acid composition.

## INTRODUCTION

Carotenoids are the main pigments of many fish species. The distinctive red color of salmonids such as trout, salmon and charr is due to the incorporated carotenoids of dietary origin (Foss et al., 1984). Salmonids like other animals are unable to synthesize carotenoids *de novo* (Torrissen et al., 1989). The carotenoids are absorbed from the diet (Tanaka, 1978) and are deposited in the unesterified form in the flesh where they bind to actomyosin (Henmi et al., 1987, 1989).

Astaxanthin (β,β-carotene-3,3′-dihydroxy-4,4′-dione) is the dominant pigment of wild salmonid muscle (Khare et al., 1973; Schiedt et al., 1981). Wild salmonids obtain astaxanthin and its esters by ingesting either zooplankton or fish that have zooplankton in their digestive tract. The reddish pink color is very important for consumer acceptance of salmon and trout (Choubert, 1982; Ostrander et al., 1976; Sceurman et al., 1979; Simpson, 1982). The economic importance of pigmented flesh in farmed salmonids makes it important to find new sources of pigments that could be incorporated into salmonid diets. The relevant literature concerning pigmentation in salmonids has been reviewed by many scientists (Foss et al., 1984; Tacon, 1981; Torrissen et al., 1989; Storebakken and No, 1992). Recent trends show the use of astaxanthin or canthaxanthin, or their combination, as the sole source of pigment in the diet. This has resulted in a renewed interest to explore other sources to be used as

pigments in the salmonid diet. Biological pigments such as shrimp waste (Schiedt et al., 1985; Tidemann et al., 1984), krill meal (Kotik et al., 1979), paprika (Tunison et al., 1944; Peterson et al., 1966), crayfish extracts (Peterson et al., 1966), red yeast, *Phaffia rhodozyma* (An et al., 1989; Gentles and Haard, 1990; Johnson et al., 1980), yeast, *Rhodotorula sanneii* (Jouko et al.,1970) *tagetes erecta* flowers (Lee et al., 1978), *Adonis aestivalis* flowers (Kamata, 1986) and green algae, *Haematococcus pulvialis* (Choubert and Heinrich, 1993; Sommer et al., 1991) have been investigated.

Fish flesh is the major site of carotenoid deposition and 90% of the carotenoids accumulated there are found in the free form (unesterified). Carotenoids are also located in the skin and ovaries of maturing fish (Torrissen et al., 1989). Hydroxy-carotenoids found in the skin are mainly in the ester form (Hata and Hata, 1975). Generally, it has been observed that there are individual differences in the ability of salmonids to absorb or deposit carotenoids in their flesh (Torrissen and Nævdal, 1984, 1988). Other factors which may influence muscle pigmentation include fish size, growth rate, sexual maturation, water temperature and genetic background (Abdul-Malak et al., 1975; No and Storebakken, 1991; Torrissen, 1986, 1989; Torrissen and torrissen, 1985; Torrissen and Nævdal, 1988).

The ability of vitamin E to act as a free radical chain-breaking antioxidant *in vivo* is well recognized. Its function in preventing lipid peroxidation makes it an essential

nutrient in the diets of humans and other animals (Horwitt, 1980; Parker, 1989; Drevon, 1991). Vitamin E is an essential nutrient in salmonid dietary formulations (Poston et al., 1976; Watanabe, 1990; Watanabe and Takeuchi, 1989) and is usually supplied as α-tocopherol in the ester form.

The objectives of the present study were to investigate the incorporation of dietary carotenoid pigments (synthetic canthaxanthin and paprika) and α-tocopherol in rainbow trout flesh, to monitor color development in fish flesh as a function of time, and to evaluate the fatty acid composition of fish flesh as it might be influenced by dietary components.

## MATERIALS AND METHODS

### Diet preparation and composition

Rainbow trout (*Oncorhynchus mykiss*) were fed five different diets for a period of 7 months.

The diets are as follows, the values cited being target levels:

Diet I : Control commercial diet (Martin Mills Inc., Elmira, ONT).

Diet II : Commercial canthaxanthin diet (Martin Mills Inc., Elmira, ONT).

Diet III : Diet supplemented with α-tocopheryl acetate (500mg /kg) and canthaxanthin (15mg/kg).

Diet IV : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (175mg/kg).

Diet V : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (350mg/kg).

Diets I and II were commercial rainbow trout diets (floating 40% trout feed grower pellets) and contained 10% herring oil by weight. These diets also contained 100mg α-tocopheryl acetate/kg feed (target level). Diet I served as the control diet in this study. A commercial rainbow trout diet without added herring oil was also purchased from Martin Mills Inc. and used to prepare three supplemented diets (diets III, IV and V). The supplemented diets were prepared by adding α-tocopheryl acetate (500 mg/kg) to herring oil (10% based on weight of diet) which was carefully coated on the surface of the feed. Additionally, diet III and diets IV and V contained canthaxanthin (Martin Mills Inc.) and oleoresin paprika (Kalsec Inc.,Kalamazoo, MI), respectively. Diets III, IV and V were prepared by Kalsec Inc.

According to the manufacturer's specifications given on the packaging label, all diets contained 40% crude protein (min), 3% crude fiber (max), 1% calcium, 0.80% phosphorus, 0.15% sodium, 0.15% magnesium, 0.75% potassium, 35 mg/kg copper, 8.50 mg/kg iodine, 125 mg/kg manganese, 215 mg/kg Zinc, 0.10 mg selenium/kg, 5000 IU (international units)/kg vitamin A (min), 2500 IU/kg vitamin $D_3$ (min), 200 IU/kg vitamin C (min) and 100 IU vitamin E (min). Diets I and II also contained 11% crude fat (min).

Feeding trial

The study was carried out with 180 rainbow trout (*Oncorhynchus mykiss*). Rainbow trout (8-10 inches in length) were purchased from Spring Valley Trout Farm (Dexter, MI) and reared in 15 fiberglass tanks (containing 60 gallons water) at the Aquaculture Research Laboratory, Michigan State University (East Lansing, MI). The fish were raised under a continuous flow of fresh water (54°-56°F water temperature) and the water flow was adjusted to accommodate two changes per tank per hr. The water was continuously aerated using air pumps (10-11 ppm dissolved oxygen).

The fish were weighed individually and sub-divided into their weight groups as low weight (95 to 140g), medium weight (140 to 185g) and high weight (185 to 230g). The fish within each weight group were then randomly assigned to five tanks. Subsequently, dietary treatments were randomly assigned to one of the five tanks within each weight group. Twelve fish were housed in each tank.

The fish were fed at the rate of 1% of their body weight per day (Leitritz and Lewis, 1980), the feed being given manually two times per day. Initially, all fish were fed the control diet (diet I) for two months. The feeding trial was conducted for 7 months. Periodically, three fish from each treatment group were randomly caught and used for the various analyses conducted during the feeding trial.

Weight measurement

The weight of fish was periodically monitored during the feeding trial by taking the collective wet weight of all fish from each tank and then calculating the average weight.

Determination of α-tocopherol concentrations in the diets and fish tissues

Extraction of α-tocopherol: The α-tocopherol in the diet, muscle and liver was extracted using the method of Buttriss and Diplock (1984). A 5g sample was homogenized with 15ml distilled water using a polytron homogenizer. A 1ml aliquot of the sample homogenate and 2ml 1% ethanolic pyrogallol were added to a screw cap test tube. The sample was saponified by blending with 0.3ml 50% potassium hydroxide and holding at 70°C in a water bath for 30 min. On cooling, 4ml hexane (containing 0.005% butylated hydroxytoluene as an antioxidant) were added to the sample and the contents of the test tube shaken vigorously for 1 min. The hexane layer was removed and the extraction procedure was repeated two more times with 2ml aliquots of hexane. The extracts were pooled and 200µl of an α-tocopheryl acetate solution (100µg/ml ethanol) was added as an internal standard in order to adjust results according to its recovery. The contents were evaporated to dryness under nitrogen. The residue obtained was redissolved in ethanol (200µl, 1ml and 1.5ml for muscle, diet and liver samples).

The recovery rates were established by adding a known quantity of α-tocopherol (100µg/ml ethanol) to freshly

homogenized diet, muscle and liver samples before extraction. The resulting peak area on the chromatograms were compared with the peak area obtained by direct injection of standard α-tocopherol onto the column. The average recoveries of α-tocopherol in the diet, muscle and liver samples were 89.7%, 88.9% and 90.1% with coefficients of variation (CV) of 2.9%, 3.7% and 2.5%, respectively. Each recovery experiment was repeated three times and each extract was analyzed in duplicate.

Quantitation of α-tocopherol by high performance liquid chromatography: Concentrations of α-tocopherol were determined using a Symmetry™ $C_8$ reverse phase column (Water Associates, Milford, MA) with a length of 150mm and an internal diameter of 3.9mm. A Sentry™ integrated guard column (Waters Associates) was attached to the column. Separation was achieved using a mobile phase containing acetonitrile, methanol and water (25:25:1 v/v/v) with a flow rate of 1.0ml/min. A high performance liquid chromatograph (Model 501, Waters Associates) equipped with dual pumps was used. Detection was achieved with an absorbance detector (Model 486, Waters Associates) set at 280nm. Chromatograms were recorded on a NEC printer (Model Pinwriter P5200, NEC Information Systems, Inc. Boxborough, MA). The quantitation was done using a standard curve based on the peak areas obtained by injecting known amounts of an α-tocopherol standards (5-120ng/μl for muscle samples, 20-150ng/μl for diet samples and 50-300ng/μl

for liver samples).

Determination of total carotenoids in the diet and fish muscle

A 10g muscle sample was finely ground with 20g anhydrous magnesium sulfate using a mortar and pestle. The mixture was transferred to a 250ml round bottom flask and acetone (50ml) was added. The contents were stirred for 1 hr prior to filtering through Whatman No.4 filter paper. The filtrate was collected in a 100ml volumetric flask. Two 20ml aliquots of acetone were used to rinse the flask and re-extract the residue. The extracts were pooled together and the volume was adjusted to 100ml. The absorbance was measured at 460nm with a Bausch and Lomb Spectronic 2000 spectrophotometer (Bausch and Lomb, Rochester, NY). To quantitate total carotenoids in the samples containing oleoresin paprika, an experimentally determined absorptivity at 1% in acetone of 1922 was used (Fisher, 1993, personal communication). The absorptivity value of 1900 was used to calculate the total carotenoid concentrations in samples containing canthaxanthin (Skrede et al., 1989).

To determine the total carotenoid contents in the diet samples, a 5g sample was extracted with a total volume of 100ml acetone. The sample was heated at 60°C for 1 hr during stirring. The rest of the procedure was similar to that used for the muscle sample.

High performance liquid chromatographic analysis of carotenoids

Sample preparation: To the carotenoid extract prepared as described previously, an internal standard, β-apo-8´-carotenal (20mg/l acetone) was added in order to adjust results according to its recovery. The carotenoid extract was evaporated to dryness in a rotary evaporator (BÜchi Rotavapor, Postfach, Switzerland). The contents were redissolved in 2ml hexane and passed through a prewetted Superclean™ LC-Si SPE tube (Supelco Inc., Bellefonte, PA) filled with 1g silica packing material at a flow rate of 1.0ml/min using a Visiprep solid phase extraction vacuum manifold (Supelco Inc., Bellefonte, PA). The triglycerides were removed using 8ml hexane. The carotenoids were eluted out of the SPE tube with four 1ml aliquots of acetone. The combined acetone aliquots were evaporated under nitrogen and the carotenoids redissolved in 200μl acetone.

Carotenoid analysis: A Hitachi liquid chromatograph (Model 655A-11, EM Science, Gibbstown, NJ) equipped with a Hitachi controller (Model L-5000LC, EM Science), a Waters 712 WISP pump (Waters Associates, Milford, MA) and a 250mm x 4.6mm (i.d.) Supelcosil LC 18™ column (Supelco Inc.) was used. The separation of carotenoids was established with gradient elution at a flow rate of 1ml/min. Eluant A contained acetone: methanol (75:25 v/v), while eluant B was composed of acetone:water (75:25 v/v). The gradient was programmed as follows: 0% A increased to 65% in 10 min, then to 80% A in 30

min, and finally, to 100% A in 60 min.

A Waters Model 990 photodiode array detector was used to record the absorbance (440 to 550nm) and the retention time of each peak as the carotenoid was eluted. After all peaks were eluted, the absorbance of each peak was calculated as the relative area at any given wavelength (i.e., 460nm). The identification of the major carotenoids was based on the spectra and the elution order of these compounds (Fisher and Kocis, 1987).

## Color measurement

The color attributes for trout flesh were measured using a HunterLab optical sensor colorimeter (Model D25-PC2Δ, Hunter Associates Laboratory, Inc., Reston, VA). Results were recorded as $L^*$, $a^*$, and $b^*$ values. Two measurements were made on each sample by rotating 90° between each measurement. The mean of these readings was reported. From the determined $a^*$, and $b^*$ values, the hue ($H^\circ_{ab}=\tan^{-1} b^*/a^*$; $H^\circ_{ab}$= 0° for red and 90° for yellow) and the chroma [$C^*_{ab}=(a^{*2} + b^{*2})^{1/2}$] values were calculated (Francis and Clydesdale, 1975).

## Extraction of lipids

Lipids were extracted using the dry column method of Marmer and Maxwell (1981). A dichloromethane:methanol (9:1 v/v) solvent system was used to elute the lipids from the column. The solvent was evaporated to dryness on a rotary evaporator. The lipids were redissolved in hexane containing

0.005% butylated hydroxytoluene and transferee to tared vials. Hexane was evaporated under nitrogen and the vials were capped after flushing with nitrogen. Samples were stored at -20°C until required.

Preparation of fatty acid methyl esters

Methyl esters of the total lipids were prepared following the boron trifluoride-methanol method of Morrison and Smith (1964).

Gas chromatographic analysis of fatty acid methyl esters

Fatty acid methyl esters were separated and quantitated using a Hewlett Packard gas chromatograph (Model 5890A, Hewlett Packard, Avondale, PA) equipped with a flame ionization detector. A fused silica capillary column (DB-225, J & W Scientific, Folsom, CA) with a length of 30m and an inside diameter of 0.25mm was used for separating the fatty acid methyl esters. Helium was used as the carrier gas and a split ratio of 20:1 was maintained. The oven temperature was initially held at 175°C for 10 min, then increased at a rate of 1.5°C/min to a final temperature of 200°C and held for 47 min. The injector and detector temperatures were maintained at 275°C and 300°C, respectively.

Identification of the fatty acid methyl esters was based on comparison of retention times of samples to those of standard fatty acid methyl esters (Supelco, Bellefonte, PA). Peak area of each fatty acid was computed by an integrator

(Model 3392A, Hewlett Packard, Avondale, PA) and reported as percent relative area.

## Statistical Analysis

The data were analyzed using a split-plot design with factors A (treatment) and B (replication) applied to the whole plot (fish in rearing tanks) and factor C (time) to the sub plot. The MSTAT-C microcomputer statistical program (Michigan State University, 1991) was employed to analyze data. Tukey's test was applied to separate the mean values.

# RESULTS AND DISCUSSION

## Weight gain

The average wet weights of rainbow trout were recorded monthly for up to 5 months and then at the time of slaughter Figure 1). The average wet body weight of rainbow trout assigned to all treatment groups (228.2 to 246.9g) at the start of experiment was not significantly different ($p>0.05$). The fish remained in good health during the feeding trial and the mortality rate remained very low in fish on all the diets.

Difference in growth rates between fish fed the various dietary treatments were observed after the first month of feeding. Rainbow trout fed diet II had a significantly ($p<0.05$) lower weight gain throughout the feeding period starting from the second month. Fish fed oleoresin paprika (diets IV and V) gained significantly ($p<0.05$) more weight

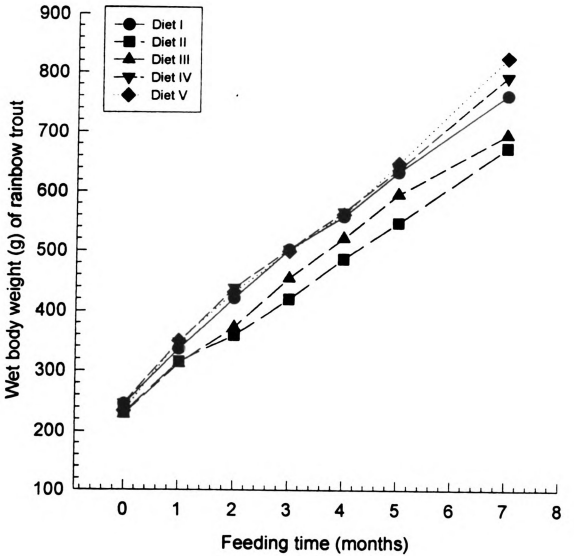


**Figure 1. Increase in wet weight of rainbow trout during feeding trial.**

**(See Table 1 for description of dietary treatments)**

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compared to the fish on the diets containing canthaxanthin (diets II and III). Statistical analysis revealed that there was no significant effect of using higher levels of α-tocopheryl acetate supplementation on the rate of growth of fish.

These results agree with the findings of Boggio et al. (1985) who also reported that there was no significant difference in the final average weights of fish fed diets containing either fish oil or swine fat and supplemented with 0, 50, 500, or 1500mg of α-tocopheryl acetate per kg of diet. However, Frigg et al. (1990) found that rainbow trout fed a diet without vitamin E supplementation showed a significantly lower growth than those ingesting a diet with 200mg vitamin E per kg.

The higher weight gain observed in fish fed dietary oleoresin paprika may be due to its favorable effect on the acceptability of the feed. Tacon (1981) speculated that carotenoids can act as fertilization hormones and enhance growth. However, any biological function of the carotenoids in fish remains unconfirmed (March et al., 1990).

Concentration of α-tocopherol in diet, muscle and liver tissue

The α-tocopherol contents of the various dietary treatments are presented in Table 1. The control diet (I) and the commercial canthaxanthin diet (II) had α-tocopherol concentrations of 98.9mg/kg and 84.7mg/kg, respectively. The concentrations of α-tocopherol in the higher supplemented

Table 1. Concentrations of α-tocopherol in the diets.

| Diets | α-tocopherol concentration (mg/kg) |
|---|---|
| I | 98.9 ± 1.0[b] |
| II | 84.7 ± 3.6[b] |
| III | 474.4 ± 19.2[a] |
| IV | 498.1 ± 64.4[a] |
| V | 483.1 ± 10.9[a] |

All values represent the mean (± standard deviation) of duplicate analyses.

Diet I : Commercial control diet (supplemented with 100mg α-tocopheryl acetate/kg feed).

Diet II : Commercial canthaxanthin diet (supplemented with 40mg canthaxanthin and 100mg α-tocopheryl acetate/kg feed).

Diet III: Supplemented diet I (supplemented with 15mg canthaxanthin and 500mg α-tocopheryl acetate/kg feed).

Diet IV : Supplemented diet II (supplemented with 175mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

Diet V : Supplemented diet III (supplemented with 350mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

[ab] Means bearing the same superscript are not significantly different from one another ($p>0.05$).

diets (III, IV and V) ranged from 474.4mg/kg to 498.1mg/kg. These concentrations are close to the targeted concentrations of 100 and 500mg/kg, respectively.

Differences of opinion exist among scientists regarding an adequate level of vitamin E in the diet of rainbow trout. It has been established that the vitamin E requirement and the tissue storage of α-tocopherol in rainbow trout is greatly affected by the degree of unsaturation of the fat in the diet (Watanabe et al., 1981). The dietary levels of vitamin E required to prevent deficiency signs are higher in diets containing unsaturated fatty acids than in diets containing more saturated fatty acids. Cowey et al. (1981) reported that 20-30mg vitamin E/kg diet provided a safe level for diets containing 10% saturated fats. These investigators later reported that 50mg vitamin E/kg diet was a safe level in diets containing unsaturated fat (Cowey et al., 1983). On the other hand, Hung et al. (1981) observed that a dietary level of 24mg vitamin E/kg diet was adequate to prevent deficiency signs in trout fed diets containing 7.5% good quality herring oil.

Watanabe et al. (1981) recommended a dietary vitamin E level of 100mg per kg in trout diets containing 15% pollock liver oil methyl esters. They further recommended that dietary levels greater than 50mg vitamin E/kg diet should be added to diets containing more than 10% fish oil, especially when the dietary oil may not be of good quality. Preliminary results obtained by Hamre and Lie (1995) indicated that the optimal dietary vitamin E level for Atlantic salmon is 120mg

dl-α-tocopheryl acetate per kg dry diet, or more. However, our study was not designed to determine the precise vitamin E requirements of rainbow trout based on the nature of the fatty acids incorporated in the diet.

The concentrations of α-tocopherol in the muscle and liver tissues of rainbow trout are reported in Table 2 and 3, respectively. Analysis of these data reveal that as dietary levels of α-tocopheryl acetate increased, muscle and liver concentrations of α-tocopherol correspondingly increased. These results agree with the findings of other scientists (Boggio et al., 1985; Frigg et al., 1990; WaagbØ et al., 1991) who also observed that vitamin E concentration deposited in fish are influenced by the dietary level.

The concentration of α-tocopherol in the muscle samples of rainbow trout fed the control and commercial canthaxanthin diets appeared to reach their maximal level after four months. However, it is possible that this could be even sooner because of the sampling procedure, i.e., analyses were performed at 2 month intervals. The concentrations of α-tocopherol deposited in the muscle of rainbow trout fed the control and commercial canthaxanthin diets at the termination of the experiment were 10.6mg/kg and 10.5mg/kg, respectively. These results were consistent with the findings of Frigg et al. (1990) who reported α-tocopherol concentrations averaging 11.2±1.1mg/kg in the muscle of rainbow trout fed a diet supplemented with 100mg vitamin E/kg feed for 85 days. Hamre (1995) reported that juvenile Atlantic salmon adjust their body concentration

Table 2. Concentrations of α-tocopherol in rainbow trout fillets.

| Dietary[1] treatment | α-Tocopherol concentrations (mg/kg)[2] Month 2 | Month 4 | Month 7 |
|---|---|---|---|
| I | 4.9±0.1[b] | 9.7±0.2[b] | 10.6±0.7[b] |
| II | 8.0±0.3[ab] | 11.1±0.3[b] | 10.5±1.1[b] |
| III | 23.2±1.6[a] | 33.6±4.1[a] | 54.1±8.5[a] |
| IV | 22.0±1.9[a] | 36.2±7.1[a] | 56.3±9.9[a] |
| V | 15.9±0.8[ab] | 47.3±1.9[a] | 57.1±6.5[a] |

[1] See Table 1 for description of dietary treatments.

[2] All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[ab] Means bearing the same superscript in a column are not significantly different from one another ($p>0.05$).

Table 3. Concentrations of α-tocopherol in rainbow trout liver after 7 months of feeding.

| Dietary treatment[1] | α-Tocopherol concentrations (mg/kg)[2] |
|---|---|
| I | 178.4 ± 47.7[b] |
| II | 136.7 ± 22.7[b] |
| III | 1300.0 ± 144.5[a] |
| IV | 1456.1 ± 200.2[a] |
| V | 1340.7 ± 282.9[a] |

[1] See Table 1 for description of dietary treatments.

[2] All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[ab] Means bearing the same superscript in a column are not significantly different from one another ($p>0.05$).

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of α-tocopherol to the dietary α-tocopherol level during the first three months of feeding. After this period, the body concentrations remain essentially constant, and the amount of α-tocopherol metabolized and excreted equals the amount absorbed.

Concentrations of α-tocopherol in the muscle of fish fed the higher level of vitamin E supplementation (500mg/kg) ranged from 54.1mg/kg to 57.1mg/kg. WaagbØ et al. (1993) reported an α-tocopherol concentration range of 23.4 to 33.8mg/kg in the muscle of Atlantic salmon (*Salmo salar*) fed experimental diets containing 300mg α-tocopheryl acetate/kg for 18 months.

The overall concentrations of α-tocopherol in the liver samples were, as expected, much higher then those in the muscle samples. A similar trend was reported by Watanabe et al. (1981), Hung and Slinger (1982) and Frigg et al. (1990). The concentrations of α-tocopherol in the liver samples of rainbow trout fed the control and commercial canthaxanthin diets were not significantly different ($P>0.05$) and ranged from 136.7mg/kg to 178.4mg/kg. A range of 1300mg/kg to 1456.14mg/kg was observed in fish fed the higher supplemented diets. Watanabe et al. (1981) reported an α-tocopherol concentration of 27.1mg/kg in liver of rainbow trout after 12 weeks of feeding an experimental diet containing vitamin E (100mg/kg). Hung and Slinger (1982) reported concentrations of α-tocopherol in the liver of rainbow trout fed experimental diets containing vitamin E (111 and 119mg/kg) for 24 weeks in

the range of 136.0±14.0 and 198.0±14.0mg/kg, respectively. Frigg et al. (1990) indicated concentrations 201.9±44.1mg/kg after feeding 100 mg vitamin E/kg for 85 days.

Deposition of carotenoids

Flesh pigmentation of salmonid is generally influenced by individual differences in the ability of salmonids to absorb or deposit carotenoids. However, other factors contribute considerably to the pigmentation process. Responses to the composition and concentration of dietary carotenoids are not well established. The usual dietary levels of added carotenoids vary from 50 to 100mg/kg diet (Choubert and Storebakken, 1989). The carotenoid contents of the dietary treatments are given in Table 4. The control diet (I) contained 10.4mg/kg of total carotenoids which probably came from dietary components and added vitamin A. The commercial canthaxanthin diet (II) and diet III in which canthaxanthin was incorporated, contained 38.2mg/kg and 11.7mg/kg of total carotenoids, respectively. The oleoresin paprika-supplemented diets (diets IV and V) had average total carotenoid concentrations of 163.2mg/kg and 339.0mg/kg, respectively. Statistical analysis revealed that the levels of carotenoids in the control and the supplemented diet III were not significantly different ($P>0.05$). However, the remaining diets (II, IV and V) had total carotenoid concentrations significantly different from each other ($p<0.05$).

Table 4. Total carotenoid concentrations in the diets fed to rainbow trout.

| Dietary treatment[1] | Total carotenoid concentrations[2] (mg/kg) |
|---|---|
| I | 10.4 ± 0.5[d] |
| II | 38.2 ± 0.8[c] |
| III | 11.7 ± 0.4[d] |
| IV | 163.2 ± 0.3[b] |
| V | 339.0 ± 1.1[a] |

[1] See Table 1 for description of dietary treatments.

[2] All values represent the mean (± standard deviation) of duplicate analyses and were determined by UV absorption.

[abcd] Means bearing the same superscript are not significantly different from one another ($p>0.05$).

The total carotenoid concentrations of muscle tissue from fish harvested after 2, 4 and 7 months of feeding are reported in Table 5. Results reveal that among all the treatment groups, rainbow trout fed the commercial canthaxanthin diet (diet II) exhibited the greatest deposition of total carotenoids throughout the entire feeding period. After 7 months of feeding, these fish samples contained 7.9mg/kg of total carotenoids. Torrissen and Nævdal (1984) reported that rainbow trout contained concentrations of 5.5 to 6.5mg/kg after about 5 to 6 months of feeding with a diet supplemented with canthaxanthin. No and Storebakken (1992) found a canthaxanthin concentration of 6.36mg/kg in rainbow trout fed 100mg canthaxanthin/kg for 12 weeks. Torrissen et al. (1989) reported that flesh carotenoid concentrations of about 6mg/kg are sufficient to give a satisfactory pink pigmentation for marketable fresh trout.

The second highest deposition of total carotenoids (3.8mg/kg after 7 months of feeding) was observed in fish receiving the prepared supplemented diet containing canthaxanthin (diet III). Fish receiving diet V, which contained the higher level of oleoresin paprika, contained 3.1mg/kg. This concentration was not significantly ($p>0.05$) different from that for diet III.

A number of studies have shown a lower retention rate of pigment deposition when the level of carotenoid in the diet increases (Spinelli and Mahnken, 1978; Kotik et al., 1979; Torrissen, 1985). The canthaxanthin-supplemented diet (III),

Table 5. Total carotenoid concentrations in rainbow trout fillets during feeding trial.

| Dietary treatment[1] | Total carotenoid concentrations (mg/Kg)[2] | | |
|---|---|---|---|
| | Month 2 | Month 4 | Month 7 |
| I | 0.7±0.3$^{b}$ | 1.2±0.2$^{c}$ | 1.5±0.2$^{c}$ |
| II | 3.3±0.5$^{a}$ | 5.9±0.7$^{a}$ | 7.9±1.3$^{a}$ |
| III | 1.9±0.5$^{ab}$ | 2.9±0.3$^{b}$ | 3.8±0.2$^{b}$ |
| IV | 1.1±0.1$^{b}$ | 1.9±0.4$^{bc}$ | 2.4±0.1$^{bc}$ |
| V | 1.6±0.2$^{b}$ | 3.1±0.2$^{b}$ | 3.1±0.2$^{b}$ |

[1] See Table 1 for description of dietary treatments.

[2] All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate. All values were determined by UV absorption.

$^{abc}$ Means bearing the same superscript in a column are not significantly different from one another ($p>0.05$).

in spite of having a lower dietary level of canthaxanthin, deposited relatively high levels of carotenoid in the fish flesh. This trend may be due to the high concentrations of α-tocopherol in the diet (Pozo et al., 1988). These latter investigators reported that increasing the level of α-tocopherol in the diet of rainbow trout increased the deposition of canthaxanthin in the flesh of rainbow trout. Choubert and Luquet (1979) found that carotenoids are extensively degraded, probably in the intestines, during digestion. Thus, Pozo et al. (1988) suggested that it is likely that reduction of oxidative degradation in the gut due to the antioxidative action of supplementary α-tocopherol may have increased the flesh concentration of canthaxanthin. However, Sigurgisladottir (1994) observed that deposition of astaxanthin in the muscle was not effected by the presence of vitamin E in the diet.

March et al. (1990) concluded that poor flesh pigmentation results from the rapid metabolism of the absorbed pigments to colorless derivatives rather than from failure of the fish to absorb pigments. There are extreme variations reported in the literature regarding pigment retention (Torrissen et al., 1989). Hardy et al. (1990) reported that retention of canthaxanthin averaged 6.5%, while apparent digestibility was between 39 and 49.5%.

The inclusion of paprika in the diet of salmonids to pigment their flesh was studied by Peterson et al. (1966) who fed paprika xanthophylls (237mg/kg) to brook trout for various

periods of time. The colors began to appear in the skin after two weeks of feeding and were found to be fairly prominent after four weeks. The flesh color appeared to be natural except for a small amount of an undesirable yellow. Reeves (1987) reported that red paprika color is primarily caused by capsanthin and capsorubin, while β-carotene and violaxanthin contribute to the yellow-orange color.

Carotenoid composition of diet and muscle

The carotenoid composition of the five diets is presented in Table 6. The major carotenoids in the control diet were zeaxanthin (33.66%) and cis-capsanthin/lutein (47.97%). Canthaxanthin (75.30%-92.44%) was the major carotenoid in canthaxanthin diets (diets II and III). The predominant carotenoids in the diets supplemented with oleoresin paprika were capxanthin (50.56%-51.60%), zeaxanthin (12.36%-13.11%) and β-carotene (10.33%-10.52%).

The rainbow trout muscle was analyzed for carotenoid composition after 2, 4 and 7 months of feeding. The results are reported in Tables 7, 8 and 9. The muscle tissue from the fish fed the control diet was analyzed only after 7 months of feeding. Canthaxanthin (93.8% to 95.4%) was the predominant carotenoid present in the muscle of rainbow trout fed diets containing added canthaxanthin. No and Storebakken (1992) found 92.3% canthaxanthin deposited in the flesh of rainbow trout fed 100 mg canthaxanthin/kg for 12 weeks. Capsanthin (35.44%-40.72%) and canthaxanthin (25.87%-37.22%) were the

Table 6. Carotenoid composition of diets[1].

| Carotenoids | Relative area percents of absorbance at 460nm | | | | |
|---|---|---|---|---|---|
| | Diet I | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | 0.60 | 0.06 | 1.08 | 51.60 | 50.56 |
| Cis-capsanthin | 3.50 | 0.25 | ND | 7.59 | 7.25 |
| Zeaxanthin | 33.66 | ND | ND | 13.11 | 12.36 |
| Cis-capsanthin/Lutein | 47.97 | 5.70 | ND | 6.60 | 6.21 |
| Antheraxanthin | 1.51 | 0.13 | 2.88 | 0.33 | 2.14 |
| Canthaxanthin | 4.58 | 92.44 | 75.30 | 2.24 | 2.48 |
| β-Cryptoxanthin | 6.91 | 1.36 | 13.68 | 8.20 | 8.48 |
| β-Carotene | 1.27 | 0.06 | 7.06 | 10.33 | 10.52 |

[1] See Table 1 for description of dietary treatments.

*Table* 7. Carotenoid composition of fillets from rainbow trout after 2 months of feeding.

| Carotenoids | Relative area percents of absorbance at 460 nm | | | | |
|---|---|---|---|---|---|
| | Diet I[1] | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | - | ND | ND | 26.11±4.10 | 43.63±5.51 |
| Zeaxanthin | - | 0.39±0.34 | 1.47±1.38 | 12.02±4.31 | 7.43±0.60 |
| Cis-capsanthin/Lutein | - | 0.66±0.36 | 2.22±1.40 | 12.79±4.20 | 21.42±2.38 |
| Canthaxanthin | - | 74.09±4.00 | 79.72±4.80 | 36.83±7.14 | 20.91±5.17 |
| Canthaxanthin isomer | - | 24.86±4.64 | 16.59±7.30 | 12.25±2.86 | 6.61±2.61 |

All values represent the mean of three replications and each replication analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

Table 8. Carotenoid composition of fillets from rainbow trout after 4 months of feeding.

| Carotenoids | Relative area percents of absorbance at 460 nm | | | | |
|---|---|---|---|---|---|
| | Diet I[1] | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | - | ND | ND | 42.10±7.18 | 44.41±2.20 |
| Zeaxanthin | - | 0.57±0.37 | 0.44±0.01 | 16.35±2.49 | 20.66±3.64 |
| Cis-capsanthin/Lutein | - | 0.67±0.26 | 0.99±1.00 | 21.55±1.33 | 21.90±1.16 |
| Canthaxanthin | - | 77.68±8.50 | 71.79±1.25 | 17.00±4.77 | 10.63±3.32 |
| Canthaxanthin isomer | - | 21.08±9.14 | 26.78±2.40 | 3.00±2.20 | 2.40±0.88 |

All values represent the mean of three replications and each replication analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

Table 9. Carotenoid composition of fillets from rainbow trout after 7 months of feeding.

| Carotenoids | Relative area percents of absorbance at 460 nm | | | | |
|---|---|---|---|---|---|
| | Diet I[1] | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | 22.01±7.36 | 2.54±2.04 | 3.88±2.54 | 35.44±1.39 | 40.72±0.67 |
| Cis-capsanthin | 3.96±3.96 | 0.18±0.11 | 0.11±0.08 | 7.48±1.41 | 10.57±2.42 |
| Zeaxanthin | 16.66±5.26 | 0.02±0.02 | ND | 8.96±4.53 | 5.45±4.28 |
| Cis-capsanthin/Lutein | ND | 0.32±0.32 | 0.26±0.21 | 9.32±6.82 | 15.68±2.02 |
| Antheraxanthin | 0.51±0.01 | 0.59±0.32 | 1.00±0.90 | 0.68±0.28 | 0.82±0.05 |
| Canthaxanthin | 50.00±4.02 | 95.42±3.50 | 93.80±3.69 | 37.22±4.64 | 25.87±5.38 |
| β-Cryptoxanthin | 6.86±5.20 | 0.94±0.65 | 0.95±0.54 | 0.90±0.17 | 0.89±0.86 |

All values represent the mean of three replications and each replication analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

predominant carotenoids after 7 months of feeding in the muscle of rainbow trout fed oleoresin paprika-supplemented diets. Analyses of the oleoresin paprika-supplemented diets showed the presence of only small amounts of canthaxanthin (2.24%-2.48%), thus the high amounts of canthaxanthin found in the muscle of rainbow trout fed these diets either came through its absorption or through its metabolic synthesis from other carotenoids. The results presented by Jouko et al. (1970) indicated that except for α-carotene, the specific carotenoids of rainbow trout can be synthesized metabolically in the fish from other types of carotenoids. These investigators revealed that lutein and β-carotene and to a lesser extent canthaxanthin can be synthesized from precursors provided by non-specific carotenoids. On the other hand, α-carotene occurs only in fish when the food used contains this compound.

Lai (1993) reported that zeaxanthin and capsanthin were deposited approximately 43% and 13% as efficiently as lutein, respectively, in egg yolk of hens fed diets containing from 0-16mg paprika carotenoids/kg feed. Our results indicated that deposition efficiencies for zeaxanthin and capsanthin were 48% and 49%, respectively, in fish fed diets containing the lower level of oleoresin paprika (diet IV). Similarly, deposition efficiencies for zeaxanthin and capsanthin were 18% and 32% in fish fed the diet containing the higher level of oleoresin paprika (diet V). The lower deposition efficiency observed in fish fed diet V may be due to the increased level of the

paprika carotenoids in the diet.

Color characteristics of fish flesh

At the end of 7 months of feeding, fish fillets were visually assessed for color by an informal panel. Fillets from fish fed the control diet (diet I) had a tan color, while the flesh of fish fed the commercial canthaxanthin diet (diet II) had a reddish-pink color. Fish fed the diet supplemented with the lower level of canthaxanthin (diet III) also had a reddish pink color, but the color was not as intense as that observed in fish fed diet II. The flesh of a fish fed diet containing the lower level of oleoresin paprika (diet IV) had a peach color. However, a more intense peach color was observed in fish fed the diet containing the higher level of oleoresin paprika (diet V). The color of the fish flesh had similar trends after 2 and 4 months of feeding as that observed after 7 months of feeding, but the color intensity was not as great.

Paprika carotenoids did not provide the highly desirable pink to red color associated with wild salmonid species or that obtained on feeding canthaxanthin at the commercial level (diet II). However, the peach color achieved by the higher dietary level of oleoresin paprika was considered more desirable than the light tan color of the flesh of the fish fed the control diet (diet I) by an informal penal of cooperators in the laboratory.

It has been reported that the color attributes of the muscle of salmonids are strongly influenced by the type and

nature of the sample being presented to the instrument during reflectance measurements (Little and Mackinny, 1969; Choubert, 1982; Skrede and Storebakken, 1986). Saito (1969) measured the color of the back, head, belly and tail parts of Atlantic salmon flesh with a Gardner colorimeter and noticed a little variation in reflected color. Color characteristics such as $L^*$, $a^*$, $b^*$, hue angle and chroma of fillets after 2, 4 and 7 months of feeding are presented in Table 10. Increased pigmentation causes reduced hue angle and increased chroma values. The results showed that lightness and hue angle decreased successively until the end of the feeding period in all the treatment groups. Similarly, redness increased during the entire trial. Yellowness and chroma increased up to four months and then started to decline in all the treatment groups except for the chroma value of fish fed diet III. Fish fed the commercial canthaxanthin diet (diet II) had significantly ($p<0.05$) higher redness and chroma. Similarly, significantly lower lightness and hue angle values were observed in those fish. These results agree closely with those of Choubert et al. (1992) who reported that increased pigmentation caused an increase in chroma and reduced the hue and lightness in the corresponding muscle samples.

Fatty acid composition of diets and fish muscle

The percentage of saturated and unsaturated fatty acids in the diets were in the range of 32.0% to 36.8% and 63.2% to 68.0%, respectively (Table 11). The predominant saturated

Table 10. Changes in color characteristics of muscle of rainbow trout fed supplemented dietary treatments during feeding trial.

| Period (Months) | Color Parameter | Dietary treatment[1] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 2 | L-value | 34.6±1.2$^{a}$ | 31.0±0.6$^{c}$ | 31.7±0.8$^{bc}$ | 33.7±1.4$^{ab}$ | 34.0±1.1$^{ab}$ |
| 4 | | 38.8±0.7$^{a}$ | 30.9±1.3$^{c}$ | 32.1±0.6$^{bc}$ | 34.1±2.0$^{b}$ | 32.8±0.8$^{bc}$ |
| 7 | | 27.3±0.5$^{a}$ | 23.9±1.0$^{b}$ | 26.7±0.3$^{a}$ | 26.7±0.1$^{a}$ | 27.3±0.3$^{a}$ |
| 2 | a-value | 1.6±0.2$^{c}$ | 6.6±0.6$^{a}$ | 4.7±0.8$^{ab}$ | 3.2±0.4$^{bc}$ | 4.5±0.9$^{ab}$ |
| 4 | | 1.9±0.2$^{c}$ | 11.3±1.2$^{a}$ | 5.3±0.8$^{b}$ | 5.3±1.2$^{b}$ | 6.5±1.2$^{b}$ |
| 7 | | 4.3±0.5$^{c}$ | 11.5±0.5$^{a}$ | 7.7±0.2$^{b}$ | 6.8±0.8$^{bc}$ | 6.1±0.5$^{bc}$ |
| 2 | b-value | 7.9±0.3$^{a}$ | 8.4±0.7$^{a}$ | 7.8±0.5$^{a}$ | 8.0±0.3$^{a}$ | 8.6±0.8$^{a}$ |
| 4 | | 9.1±0.2$^{b}$ | 11.3±0.3$^{a}$ | 8.6±0.9$^{b}$ | 9.1±1.2$^{b}$ | 11.0±0.3$^{a}$ |
| 7 | | 5.8±0.4$^{b}$ | 7.3±0.4$^{ab}$ | 7.3±0.5$^{ab}$ | 7.3±0.4$^{ab}$ | 7.9±0.2$^{a}$ |
| 2 | Hue angle | 78.6±1.0$^{a}$ | 51.9±2.3$^{c}$ | 59.2±3.0$^{bc}$ | 68.2±7.7$^{b}$ | 62.5±5.4$^{b}$ |
| 4 | | 77.9±1.5$^{a}$ | 45.2±3.2$^{c}$ | 58.4±2.1$^{b}$ | 60.9±3.5$^{b}$ | 58.5±2.7$^{b}$ |
| 7 | | 53.6±3.0$^{a}$ | 32.5±2.7$^{c}$ | 43.3±1.5$^{b}$ | 47.1±2.4$^{ab}$ | 52.2±2.4$^{ab}$ |
| 2 | Chroma | 8.1±0.3$^{a}$ | 10.6±0.8$^{a}$ | 9.1±0.7$^{a}$ | 8.6±0.2$^{a}$ | 9.8±0.9$^{a}$ |
| 4 | | 9.3±0.2$^{c}$ | 16.0±0.9$^{a}$ | 10.2±1.2$^{bc}$ | 10.5±1.7$^{bc}$ | 12.8±0.9$^{b}$ |
| 7 | | 7.8±0.9$^{c}$ | 13.6±0.4$^{a}$ | 10.6±0.4$^{b}$ | 10.0±0.8$^{bc}$ | 10.0±0.3$^{bc}$ |

[1] See Table 1 for description of dietary treatments.

All Values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

$^{abc}$ Means bearing the same superscript in a row are not significantly different ($p>0.05$).

Table 11. Fatty acid profile of diets used for feeding rainbow trout.

| Fatty acid | Percent relative area[1] | | | | |
|---|---|---|---|---|---|
| | Diet I[2] | Diet II | Diet III | Diet IV | Diet V |
| C 10:0 | 0.64 | 0.57 | 0.68 | 0.63 | 0.58 |
| C 12:0 | 0.21 | 0.17 | 0.17 | 0.22 | 0.19 |
| C 14:0 | 7.37 | 9.82 | 8.18 | 8.10 | 7.65 |
| C 15:0,i | 0.26 | 0.29 | 0.26 | 0.27 | 0.25 |
| C 15:0 | 0.47 | 0.50 | 0.45 | 0.44 | 0.41 |
| C 16:0 | 19.63 | 20.57 | 18.83 | 18.34 | 17.90 |
| C 16:1 | 13.85 | 10.96 | 13.12 | 12.89 | 12.08 |
| C 17:0 | 0.21 | 0.24 | 0.18 | 0.18 | 0.08 |
| C 17:0 ▲ | 1.41 | 1.31 | 1.51 | 1.48 | 1.39 |
| C 18:0 | 1.64 | 1.60 | 1.40 | 1.41 | 1.46 |
| C 18:1 ω9 | 12.07 | 11.42 | 9.99 | 9.95 | 10.14 |
| C 18:1 ω7 | 2.82 | 2.25 | 2.37 | 2.37 | 2.36 |
| C 18:2 ω6 | 9.14 | 9.95 | 10.21 | 9.95 | 11.25 |
| C 18:3 ω6 | 0.20 | 0.19 | 0.20 | 0.20 | 0.19 |
| C 18:3 ω3 | 1.14 | 1.38 | 1.40 | 1.32 | 1.52 |
| C 19:0 ▲ | 1.35 | 1.60 | 2.02 | 2.01 | 1.94 |
| C 20:0 | - | 0.10 | 0.03 | 0.10 | 0.13 |
| C 20:1 | 9.65 | 9.32 | 10.11 | 10.51 | 11.00 |
| C 18:4 ω3 | 0.76 | 0.42 | 0.65 | 0.69 | 0.74 |
| C 20:4 | 0.19 | 0.15 | - | 0.13 | - |
| C 20:5 | 3.42 | 3.83 | 3.77 | 3.92 | 3.98 |
| C 22:1 ω11 | 10.59 | 10.58 | 11.39 | 11.72 | 12.55 |
| C 22:1 ω9 | 1.22 | 0.88 | 1.16 | 1.18 | 0.13 |
| C 22:6 ω3 | 1.76 | 1.90 | 1.92 | 1.99 | 2.08 |
| Saturated | 33.19 | 36.77 | 33.71 | 33.18 | 31.98 |
| Unsaturated | 66.81 | 63.23 | 66.29 | 66.82 | 68.02 |
| Monounsaturated | 50.20 | 45.41 | 48.14 | 48.62 | 48.26 |
| Diunsaturated | 9.14 | 9.95 | 10.21 | 9.95 | 11.25 |
| Polyunsaturated | 7.47 | 7.87 | 7.94 | 8.25 | 8.51 |

[1] Fatty acid relative area percentages are the mean values of each diet analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

fatty acids were myristic (C14:0) and palmitic (C16:0), while the major unsaturated fatty acids were palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), eicosaenoic (C20:1) and docosaenoic (C22:1).

The fatty acid profile of the muscle samples of rainbow trout after seven months of feeding is shown in Table 12. The relative area percentages of C16:0, C18:1 and C22:6 in muscle samples were greater than those in the diets. The relative area percentage of C16:1, C20:1 and C22:1 decreased. However, the overall ratio of saturated fatty acids to unsaturated fatty acids in muscle samples remained the same as that in the diet samples.

There was no marked effect of α-tocopherol levels on the fatty acid composition of the muscle samples. These findings agree with the results of other investigators (Boggio et al., 1985; Frigg et al., 1990; Waagbo et al., 1993; Sigurgisladottir et al., 1994) who reported that neither the absence/presence of α-tocopherol nor its different levels in the diet affect the fatty acid composition of salmonids. Watanabe et al. (1981) also reported that there was no marked difference in fatty acid distribution in either liver or muscle due to differences in the tocopherol levels in the diets, although the lipids in both the muscle and liver of rainbow trout fed a control diet contained a high proportion of C18:2ω6. Boggio et al. (1985) found that the fatty acid composition of muscle was influenced by dietary fat, most markedly in the level of n-3 and n-6 fatty acids.

Table 12. Fatty acid profile of rainbow trout muscle samples after 7 months of feeding.

| Fatty acid | Percent relative area | | | | |
|---|---|---|---|---|---|
| | I[2] | II | III | IV | V |
| C 13:0 | 0.69 | 0.21 | - | 0.21 | - |
| C 14:0 | 5.05 | 5.10 | 5.53 | 4.79 | 4.61 |
| C 15:0,i | 0.15 | 0.17 | 0.18 | 0.12 | 0.14 |
| C 15:0 | 0.33 | 0.29 | 0.38 | 0.31 | 0.30 |
| C 16:0,i | 0.44 | 0.39 | 0.40 | 0.35 | 0.29 |
| C 16:0 | 24.81 | 22.68 | 24.34 | 21.88 | 20.49 |
| C 16:1 | 9.02 | 9.90 | 11.08 | 10.18 | 10.14 |
| C 17:0 | 0.17 | 0.17 | 0.16 | 0.15 | 0.16 |
| C 17:0 ▲ | 0.22 | 0.16 | 0.35 | 0.34 | 0.28 |
| C 18:0 | 3.34 | 3.36 | 2.94 | 2.74 | 3.59 |
| C 18:1 ω9 | 17.85 | 21.13 | 17.95 | 18.24 | 16.86 |
| C 18:1 ω7 | 2.91 | 2.73 | 2.85 | 2.74 | 2.97 |
| C 18:2 ω6 | 8.66 | 9.65 | 9.17 | 10.65 | 10.21 |
| C 18:3 ω6 | 0.32 | 0.33 | 0.36 | 0.38 | 0.35 |
| C 18:3 ω3 | 0.97 | 1.11 | 1.00 | 1.13 | 1.20 |
| C 19:0 ▲ | 1.59 | 1.00 | 1.25 | 1.53 | 1.17 |
| C 20:1 | 5.37 | 5.91 | 6.49 | 6.85 | 7.78 |
| C 18:4 ω3 | 0.34 | 0.30 | 0.42 | 0.45 | 0.61 |
| C 20:3 | 0.38 | 0.32 | 0.30 | 0.32 | 0.40 |
| C 20:4 | 0.45 | 0.32 | 0.32 | 0.33 | 0.26 |
| C 20:5 | 3.11 | 2.53 | 2.65 | 3.04 | 3.34 |
| C 22:1 ω11 | 4.23 | 4.86 | 4.76 | 4.97 | 5.14 |
| C 22:1 ω9 | 0.55 | 0.63 | 0.59 | 0.65 | 0.78 |
| C 22:6 ω3 | 9.05 | 6.75 | 6.53 | 7.65 | 8.93 |
| Saturated | 36.79 | 33.53 | 35.53 | 32.42 | 31.03 |
| Unsaturated | 63.21 | 66.47 | 64.47 | 67.58 | 68.97 |
| Monounsaturated | 39.93 | 45.16 | 43.72 | 43.63 | 43.67 |
| Diunsaturated | 8.66 | 9.65 | 9.17 | 10.65 | 10.21 |
| Polyunsaturated | 14.62 | 11.66 | 11.58 | 13.30 | 15.09 |

[1] Fatty acid relative area percentages are the mean values of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

In conclusion, canthaxanthin was more efficiently absorbed than the carotenoids in the oleoresin paprika and produced reddish pink color in the fish flesh. The increase in dietary level of α-tocopheryl acetate helped to increase the α-tocopherol concentration in fish muscle and liver tissue.

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## CHAPTER TWO

# EFFECT OF FEED COMPONENTS AND SURFACE APPLICATION OF OLEORESIN ROSEMARY ON THE COLOR AND LIPID STABILITY OF RAINBOW TROUT MUSCLE DURING REFRIGERATED STORAGE.

## ABSTRACT

The influence of dietary supplementation of α-tocopherol and surface application of oleoresin rosemary on lipid and color stability of rainbow trout muscle during refrigerated storage (4°C) was investigated. A significant ($P<0.05$) interaction between dietary treatments and the time of storage was observed. Fish receiving the higher level of α-tocopherol supplementation (500mg/kg diet) had lower thiobarbituric-acid reactive substance (TBARS) numbers. The oxidative stability of the lipids was further improved by applying oleoresin rosemary to the surface of the fish fillets.

There was no apparent inhibitory effect by either α-tocopherol or oleoresin rosemary on the oxidative degradation of muscle carotenoids except in the fish receiving additionally higher levels of canthaxanthin (38mg/kg diet). Dietary α-tocopherol supplementation and surface application of oleoresin rosemary, however, maintained color stability during storage under fluorescent light as indicated by Hunter

colorimetry measurements.

## INTRODUCTION

Standards of success in the fish industry are measured in terms of quality. The industry strives to provide products with the greatest quality at a cost that maintains a level of profitability. Lipid oxidation is one of the most important factors responsible for quality deterioration of fish during refrigerated storage. Changes occurring from the oxidation of lipids negatively affect functional and sensory properties and the nutritive value of meat products (Pearson and Gray, 1983; German, 1990). Several scientific reports have also suggested that different by-products of lipid oxidation may be angiotoxic, carcinogenic, cytotoxic and/or mutagenic (Sevanian and Peterson, 1986; Addis and Park, 1989; Peng et al., 1991).

The nature, proportion, and degree of unsaturation of fatty acids present in a food indicate the approximate susceptibility of that product to oxidative deterioration. The fatty acid composition of the intracellular phospholipid fractions of the muscle cell membranes is especially important in determining oxidative stability of meat as oxidative changes are initiated mainly in the membranes (Buckley et al., 1989, 1995). Fish lipids are characterized by the presence of polyunsaturated fatty acids which make them susceptible to attack by molecular oxygen (Olcott, 1962).

The choice of antioxidants to stabilize fish lipids is restricted to a few substances. Recent attention has focused on the use of antioxidants such as tocopherols and tocotrienols to control oxidative deterioration of food. Tocopherols are mainly located in cell membranes and protect fatty acids from peroxidative damage caused by highly reactive free radicals such as the hydroxyl, peroxyl and superoxide radicals. Many studies have demonstrated the effectiveness of dietary supplementation with vitamin E and its derivatives, e.g., α-tocopheryl acetate, in retarding lipid oxidation in meat (Frigg et al., 1990; Asghar et al., 1991; Buckley and Morrissey, 1992; Monahan et al., 1992; Engeseth et al., 1993; Sigurgisladottir et al., 1994; Chan et al., 1995; Wulf et al., 1995). Results on the effectiveness of other components such as β-carotene and other oxycarotenoids as antioxidants during the processing and storage of meat are not as conclusive as those for α-tocopherol (Terao, 1989; Leibovitz et al., 1990).

The antioxidant properties of spices and herbs are well established and are attributed to their phenolic contents. Such compounds identified in rosemary include carnosol, rosmanol, rosmaridiphenol and rosmariquinone, and some possess antioxidant activity similar to or greater than butylated hydroxyanisole (Houlihan and Ho, 1985). The antioxidant properties of rosemary have been studied in a number of meat and meat products such as turkey sausage (Barbut et al., 1985), minced pork products (Korczak et al., 1988), menhaden mince (Hwang and Regenstein, 1988), chicken nuggets (Lai et

al., 1991), restructured beef steaks (Stoick et al., 1991), restructured pork steaks (Liu et al., 1992), frozen-crushed bonito (*Katsunoonus pelamis*) meat (Wada and Fang, 1992) and cooked gray trout (*Cynoscion regalis*) flakes (Boyd et al., 1993). Little work has been done to investigate the effect of rosemary on the quality of fish muscle during storage.

The primary objective of this study was to assess the effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on lipid oxidation and color stability of rainbow trout muscle during refrigerated storage.

## MATERIALS AND METHODS

### Diets and feeding trial

The experimental design and composition of dietary treatments are described in detail in Chapter 1. Essentially, rainbow trout (*Oncorhynchus mykiss*) were fed five different diets for a period of 7 months.

The diets are as follows, the values cited being target levels:

Diet I : Control commercial diet (Martin Mills Inc., Elmira, ONT).

Diet II : Commercial canthaxanthin diet (Martin Mills Inc., Elmira, ONT).

Diet III : Diet supplemented with α-tocopheryl acetate (500mg /kg) and canthaxanthin (15mg/kg).

Diet IV : Diet supplemented with α-tocopheryl acetate (500mg

/kg) and oleoresin paprika (175mg/kg).

Diet V : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (350mg/kg).

Diets I and II were commercial rainbow trout diets (floating 40% trout feed grower pellets) and contained 10% herring oil by weight. These diets also contained 100mg α-tocopherol acetate/kg feed (target level). Diet I served as the control diet in this study. A commercial rainbow trout diet without added herring oil was also purchased from Martin Mills Inc. and used to prepare three supplemented diets (diets III, IV and V). The supplemented diets were prepared by adding α-tocopheryl acetate (500mg/kg) to herring oil (10% based on weight of diet) which was carefully coated on the surface of the diet. Additionally, diet III and diets IV and V contained canthaxanthin (Martin Mills Inc.) and oleoresin paprika (Kalsec Inc.,Kalamazoo, MI), respectively. Diets III, IV and V were prepared by Kalsec Inc.

All fish were slaughtered according to standard commercial practices at the Aquaculture Research Laboratory, Michigan State University at the termination of the feeding trial. Fish were kept on ice and immediately transferred to the Meat Processing Laboratory, Michigan State University, for processing and packaging.

Sample preparation

After slaughtering, all fish were filleted and their skins were removed. One fillet from each fish was immediately

immersed in a 2% solution of Herbalox Seasoning Type P, oleoresin rosemary extract (Kalsec Inc., Kalamazoo, MI) in distilled water (w/v), until an approximate pick up of 1% was obtained. The gain in weight was measured by periodic weighing of the fillets after removing excess oleoresin rosemary solution from the surface of the fillets. The other fillet was used as the reference.

Storage of samples

Six fish fillets from each treatment were placed individually on polystyrene trays containing absorbent pads. The trays were overwrapped with an oxygen-permeable meat stretch wrap (Sysco Corporation, Houston, TX). The fish fillets were stored at 4°C under fluorescent light for 8 days. Color and lipid oxidation measurements were conducted immediately and after 2, 4, 6 and 8 days of storage.

Measurement of lipid oxidation

Lipid oxidation in the fish samples was determined in duplicate by the 2-thiobarbituric acid (TBA) method of Tarladgis et al. (1960), as modified by Crackel et al. (1988). The thiobarbituric acid-reactive substances (TBARS) were determined spectrophotometrically at 532nm and the results were expressed as mg malonaldehyde (MDA)/kg of sample.

Determination of total carotenoids in fish muscle

A 10g sample of rainbow trout muscle and 20g anhydrous magnesium sulfate were finely ground using a mortar and pestle. The sample was then transferred to a 250ml round bottom flask containing acetone (50ml). The contents were stirred for 1 hr before filtering through Whatman No.4 filter paper. The filtrate was collected in a 100ml volumetric flask. Two aliquots of acetone (20ml each) were used to rinse the flask and to further extract the residue. The combined acetone extracts were diluted to 100ml and the absorbance was measured at 460nm with a double beam Bausch and Lomb Spectronic 2000 spectrophotometer (Bausch and Lomb, Rochester, NY). Quantitation of total carotenoids in the samples containing oleoresin paprika was achieved using the experimentally determined absorptivity at 1% in acetone of 1922 (Fisher, 1993, Personal communication). The absorptivity value of 1900 was used to calculate the total carotenoid concentrations in samples containing canthaxanthin (Skrede et al., 1989).

Color measurement

Color changes in the fish muscle during storage were monitored by recording $L^*$, $a^*$, and $b^*$ values using a HunterLab optical sensor colorimeter (Model D25-PC2Δ, Hunter Associates Laboratory, Inc., Reston, VA). Duplicate measurements were made on each sample of trout muscle by rotating 90° between each measurement and then averaging the results. From the obtained $a^*$, and $b^*$ values, the hue ($H^\circ_{ab}=\tan^{-1} b^*/a^*$; $H^\circ_{ab}= 0^\circ$

for red and 90° for yellow) and the chroma [$C^*_{ab}=(a^{*2} + b^{*2})^{1/2}$] values were calculated (Francis and Clydesdale, 1975).

Statistical analysis

The data were analyzed using a split-plot design with factors A (treatment) and B (replication) applied to the whole plot (fish in rearing tanks) and factors C (storage time) and D (surface application of oleoresin rosemary) applied to the sub-plot (fish during processing and storage). An analysis of variance (ANOVA) was conducted using the MSTATC microcomputer program (Michigan State University, 1991). Tukey's test was applied to separate the mean values.

## RESULTS AND DISCUSSION

Effect of dietary treatments on lipid stability

The fish fillets were stored under fluorescent light in order to simulate the condition in retail shopping stores. TBARS numbers increased with storage time in all cases (Table 1). A significant interaction ($p<0.05$) between dietary treatments and storage time was observed.

Muscle samples from the rainbow trout fed the higher α-tocopherol levels (diets III, IV and V , 500mg α-tocopheryl acetate/kg) had α-tocopherol concentrations ranging from 54.1 to 57.1mg/kg (data previously reported in Chapter 1). The concentrations of α-tocopherol deposited in the muscle of rainbow trout fed the control and commercial canthaxanthin

Table 1. Effect of dietary supplementation and surface application of oleoresin rosemary on the oxidative stability (as measured by TBARS) of rainbow trout muscle during refrigerated storage (4°C).

| Dietary Treatments | Storage time, days 0 | 2 | 4 | 6 | 8 |
|---|---|---|---|---|---|
| **Samples without surface application** | | | | | |
| I | 0.21±0.04[a] | 0.20±0.07[a] | 0.37±0.11[a] | 0.90±0.02[a] | 2.05±0.17[a] |
| II | 0.18±0.02[a] | 0.27±0.12[a] | 0.47±0.07[a] | 0.99±0.17[a] | 1.56±0.13[b] |
| III | 0.16±0.02[a] | 0.16±0.03[a] | 0.16±0.05[b] | 0.17±0.02[b] | 0.33±0.12[c] |
| IV | 0.12±0.02[a] | 0.13±0.01[a] | 0.15±0.02[b] | 0.15±0.02[b] | 0.17±0.01[cd] |
| V | 0.14±0.02[a] | 0.15±0.05[a] | 0.14±0.03[b] | 0.14±0.02[b] | 0.25±0.03[cd] |
| **Samples with surface application** | | | | | |
| I | 0.09±0.01[a] | 0.16±0.02[a] | 0.16±0.03[b] | 0.14±0.01[b] | 0.19±0.02[cd] |
| II | 0.07±0.01[a] | 0.13±0.02[a] | 0.12±0.02[b] | 0.18±0.06[b] | 0.20±0.02[cd] |
| III | 0.09±0.02[a] | 0.13±0.04[a] | 0.13±0.02[b] | 0.13±0.04[b] | 0.19±0.11[cd] |
| IV | 0.11±0.01[a] | 0.11±0.01[a] | 0.10±0.03[b] | 0.10±0.01[b] | 0.12±0.01[d] |
| V | 0.13±0.02[a] | 0.12±0.03[a] | 0.12±0.03[b] | 0.14±0.03[b] | 0.15±0.02[cd] |

TBARS numbers are the mean values (± standard deviation) of three replications and each replication was analyzed in duplicate. Results are expressed as mg malonaldehyde per kg sample.

Diet I : Commercial control diet (supplemented with 100 mg α-tocopheryl acetate/kg feed).

Diet II : Commercial canthaxanthin diet (supplemented with 40 mg canthaxanthin and 100 mg α-tocopheryl acetate/kg feed).

Diet III: Supplemented diet I ( Supplemented with 15 mg canthaxanthin and 500 mg α-tocopheryl acetate/kg feed).

Diet IV : Supplemented diet II (supplemented with 175 mg oleoresin paprika carotenoids and 500 mg α-tocopheryl acetate/kg feed).

Diet V : Supplemented diet III (supplemented with 350 mg oleoresin paprika carotenoids and 500 mg α-tocopheryl acetate/kg feed).

[a,b,c,d] Means in the same column bearing the same superscript are not significantly different ($p>0.05$).

diets were 10.6mg/kg and 10.5mg/kg, respectively (Chapter 1).

Muscle samples from rainbow trout fed diets III, IV and V had significantly lower ($P<0.05$) TBARS numbers (0.33mg MDA/kg, 0.17mg MDA/kg and 0.25mg MDA/kg, respectively) after 8 days of storage as compared to those from dietary groups I and II (2.05mg MDA/kg and 1.56mg MDA/kg, respectively).

The TBARS numbers of muscle samples of rainbow trout fed diets containing canthaxanthin or oleoresin paprika and the higher level of α-tocopherol (500mg/kg diet) were not significantly ($p>0.05$) different. This may be due to lower levels of canthaxanthin (11.7mg/kg in diet and 3.8mg/kg in muscle) in dietary group III (Chapter 1). However, the commercial canthaxanthin diet having a higher level of canthaxanthin (38.2mg/kg in diet) significantly enhanced ($p<0.05$) the storage stability of the muscle tissue (1.56mg MDA/kg after 8 days of storage) compared to the control (2.05mg MDA/kg). According to Terao (1989) and Miki (1991), canthaxanthin is a more efficient singlet oxygen ($^1O_2$) quencher and radical scavenger than β,β-carotene and zeaxanthin in preventing lipid oxidation.

The concentrations of α-tocopherol in diets I and II were similar, thus the lower TBARS numbers of muscle samples from fish fed diet II indicate the antioxidant effect of canthaxanthin. These results agree with the findings of Ajuyah et al. (1993) who reported that the addition of mixed tocopherols plus canthaxanthin to the diet significantly reduced concentrations of malonaldehyde in cooked broiler

meats enriched with omega-3 fatty acids during storage at 4°C for 15 days.

The enhanced antioxidant activity of the α-tocopherol/canthaxanthin combination may be due to the difference in their mechanism of action. In general, α-tocopherol is a lipid-soluble chain-breaking antioxidant (Tappel, 1982) and donates a phenolic hydrogen from C-6 to the peroxyl free radical. On the other hand, canthaxanthin traps the chain-carrying peroxyl radical in its conjugated polyene system (Terao, 1989). The results of this study suggest that the concentrations of oleoresin paprika in the muscle samples from dietary groups IV and V were smaller than the canthaxanthin that may be required to show their antioxidant effect.

The observed beneficial effect of feeding supranutritional levels of α-tocopherol on lipid oxidation in rainbow trout muscle confirm the results of Frigg et al. (1990) and Sigurgisladottir et al. (1994). Frigg et al. (1990) observed a pronounced dependency of the measured TBARS numbers on the dietary vitamin E level as well as on the α-tocopherol concentrations in fish fillets. These investigators stimulated rapid oxidative using a forced oxidation test for 30 min. The TBARS numbers in fillets from rainbow trout fed a vitamin E-supplemented diet (200mg/kg) were significantly ($p<0.05$) lower (1.92mg MDA/kg) than those in fillets from the control group (8.00mg MDA/kg). Similarly, Sigurgisladottir et al. (1994), using a forced oxidation test, reported a significant ($p<0.05$)

inhibitory effect of vitamin E supplementation on lipid oxidation in Atlantic salmon (*Salmo salar*). These investigators reported TBARS numbers of 5.5mg MDA/kg in fillets of salmon fed mixed tocopherols (the concentrations of tocopherols (α-, β-, γ- and δ-) in the diet were determined to be 212.0mg/kg, 20.4mg/kg, 793.0mg/kg and 294.4mg/kg, respectively). The control group had a TBARS number of 8.9mg MDA/kg.

The beneficial effects of feeding supranutritional levels of α-tocopherol in suppressing lipid oxidation in muscle foods have been well established. Studies have shown the advantages of dietary vitamin E in beef (Faustman et al., 1989; Sherbeck et al., 1995), chicken (Sklan et al., 1983; Asghar et al., 1991; Sheehy et al., 1994), lamb (Wulf et al., 1995), pork (Monahan et al., 1990), turkey (Sante and Lacourt, 1994) and veal (Engeseth et al., 1993).

The surface application of oleoresin rosemary significantly ($p<0.05$) decreased TBARS numbers in all dietary groups. The TBARS numbers in fish fed diets containing the lower level of α-tocopherol (diets I and II) were 0.19mg and 0.20mg MDA/kg, respectively. Fish fed diets containing the higher level of α-tocopherol (diets III, IV and V) had TBARS numbers ranging from 0.12mg to 0.19mg MDA/kg. There was no significant difference among the dietary treatments.

The antioxidant activity of oleoresin rosemary is due to the presence of phenolic compounds. Their mode of action is similar to that of phenolic antioxidants and they act as

chain-breaking antioxidants. Alpha-tocopherol is a fat-soluble molecule that concentrates in the hydrophobic interior of biological membranes (Kanner et al., 1987). Its primary role in biological systems is that of a lipid-soluble chain-breaking antioxidant. The enhanced antioxidant activity of the oleoresin rosemary/α-tocopherol combination is probably due to the complementary action of both antioxidants. Another possible explanation may be the site of action. The α-tocopherol is effective in protecting biological membranes (Buckley et al., 1995), while oleoresin rosemary may protect initially the fatty acids in the surface layers of the fish fillets.

Synergism between oleoresin rosemary and α-tocopherol has been demonstrated in model studies as well as in fish muscle. Fang and Wada (1993) studied the antioxidant effect of rosemary extract and α-tocopherol on fish lipid oxidation catalyzed by $Fe^{2+}$ or hemoprotein. In a sardine oil model system, a mixture of α-tocopherol and rosemary extract (0.035% + 0.035%) increased the induction period by 10 and 16 days over that achieved by α-tocopherol and rosemary extract when used individually. These investigators also monitored lipid oxidation in the dark muscle of bonito fish (*Katsunoonus pelamis*) stored at 5°C. The mixture of α-tocopherol and rosemary extract produced a significantly ($p<0.05$) stronger antioxidant effect than did the control (containing no antioxidants) and the samples treated with α-tocopherol. In an earlier study, Wada and Fang (1992) observed that the

mixture of α-tocopherol and rosemary extract (0.05% + 0.02%) had a stronger antioxidant effect than either α-tocopherol or rosemary extract alone in frozen-crushed bonito fish meat.

Stability of muscle carotenoids during storage

A highly significant effect of treatment and storage time ($p<0.01$) on the stability of muscle carotenoids was observed (Table 2). An analysis of variance showed a significant interaction ($p<0.05$) between treatment and storage time indicating that the effects of both these variables were dependent upon each other. A significant change in carotenoid concentration was observed in muscle of rainbow trout fed diets III, IV and V. However, canthaxanthin concentration in the muscle of fish fed diet II did not change during storage. This was the only dietary treatment where carotenoid stability was observed. There is no ready explanation for this observation, although it may be related to the more efficient deposition of canthaxanthin in the fish muscle. Choubert et al. (1992) reported that muscle carotenoid contents in rainbow trout increased with increased carotenoid concentrations in the diet. However, March et al. (1990) concluded that poor flesh pigmentation of fish is due to the rapid metabolism of the absorbed pigment to colorless derivatives rather than from failure of the fish to absorb the pigment.

The change in the carotenoid concentrations in rainbow trout muscle during storage have been reported by number of researchers. Chen et al. (1984) observed the fading of

Table 2. Stability of carotenoids in rainbow trout muscle during refrigerated storage.

| Dietary Treatments[1] | Total carotenoid contents (mg/kg)[2] | | |
|---|---|---|---|
| | Day 0 | Day 8 | |
| | | Undipped[3] | Dipped[3] |
| I | 1.5±0.2[a] | 0.8±0.2[a] | 0.9±0.1[a] |
| II | 7.9±1.3[a] | 7.8±1.2[a] | 7.2±0.7[a] |
| III | 3.8±0.2[a] | 2.4±0.8[b] | 2.2±0.4[b] |
| IV | 2.4±0.1[a] | 1.8±0.3[ab] | 1.4±0.3[b] |
| V | 3.1±0.2[a] | 1.7±0.1[b] | 1.6±0.2[b] |

[1] See Table 1 for description of dietary treatments.

[2] All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[3] Undipped; dipped-oleoresin rosemary treated.

[a,b] Means in the same row bearing the same superscript are not significantly different ($p>0.05$).

astaxanthin during the storage of muscle at 1-2°C and also under frozen storage. Pozo et al. (1988) reported that increasing the level of α-tocopherol in the diet of rainbow trout did not prevent canthaxanthin from subsequent fading during cold storage. However, these investigators observed a consistently higher concentration of canthaxanthin in fish fed an α-tocopherol-supplemented diet (control diet supplemented with 500mg α-tocopheryl acetate/kg) compared to fish fed control diet (contained 26mg α-tocopheryl acetate/kg) during storage. It was also noticed that canthaxanthin faded more rapidly in vacuum-packed rainbow trout fillets were stored at -12°C than in fish stored at -30°C. However, No and Storebakken (1991) showed that astaxanthin and canthaxanthin in vacuum-packed fillets of rainbow trout were stable (up to 5% loss) during storage for six months at -20°C or -80°C. Thus there is much variability in the literature on carotenoid stability in fish during short- and long-term storage.

Effect of supplementation on color characteristics

It is apparent that redness ($a^*$), yellowness ($b^*$) and chroma decreased, while lightness ($L^*$) increased in fish samples without surface application of oleoresin rosemary during storage (Table 3). The changes in the color values were slow in fish fed diets containing higher levels of α-tocopherol (diet III, IV and V). Fish fed diet II maintained the highest level of redness and exhibited a more reddish hue throughout the storage period. Changes in the redness ($a^*$) of

Table 3. Change in color characteristics of rainbow trout muscle stored under refrigerated conditions.

| Storage (days) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | L-value | 27.3±0.5$^{a}$ | 23.9±1.0$^{b}$ | 26.7±0.3$^{a}$ | 26.7±0.1$^{a}$ | 27.3±0.3$^{a}$ |
| 2 | | 28.7±0.6$^{a}$ | 24.9±0.9$^{c}$ | 27.9±0.6$^{b}$ | 28.0±0.4$^{b}$ | 28.2±0.6$^{ab}$ |
| 4 | | 28.1±1.0$^{a}$ | 24.3±0.9$^{c}$ | 27.3±0.5$^{b}$ | 27.4±0.3$^{b}$ | 27.6±0.6$^{ab}$ |
| 6 | | 27.9±1.5$^{a}$ | 24.1±0.9$^{c}$ | 27.2±0.8$^{b}$ | 26.9±0.3$^{b}$ | 27.3±0.5$^{ab}$ |
| 8 | | 28.8±1.1$^{a}$ | 24.9±0.8$^{c}$ | 28.0±1.0$^{b}$ | 27.4±0.4$^{b}$ | 28.0±0.7$^{b}$ |
| 0 | a-value | 4.3±0.5$^{d}$ | 11.5±0.5$^{a}$ | 7.7±0.2$^{b}$ | 6.8±0.8$^{bc}$ | 6.1±0.5$^{c}$ |
| 2 | | 4.3±0.4$^{d}$ | 11.6±0.5$^{a}$ | 7.8±0.6$^{b}$ | 6.0±0.4$^{c}$ | 6.7±0.6$^{bc}$ |
| 4 | | 4.1±0.5$^{d}$ | 11.5±0.3$^{a}$ | 8.1±0.5$^{b}$ | 6.8±0.3$^{c}$ | 7.1±0.4$^{bc}$ |
| 6 | | 3.9±0.7$^{c}$ | 10.4±0.5$^{a}$ | 7.4±1.0$^{b}$ | 6.8±0.8$^{b}$ | 6.6±0.2$^{b}$ |
| 8 | | 3.5±1.0$^{d}$ | 10.2±0.3$^{a}$ | 6.9±1.4$^{b}$ | 6.4±0.7$^{bc}$ | 5.7±0.5$^{c}$ |
| 0 | b-value | 5.8±0.4$^{c}$ | 7.3±0.4$^{b}$ | 7.3±0.5$^{b}$ | 7.3±0.4$^{b}$ | 7.9±0.2$^{a}$ |
| 2 | | 6.3±0.2$^{b}$ | 7.8±0.5$^{a}$ | 7.9±0.4$^{a}$ | 7.8±0.5$^{a}$ | 8.1±0.4$^{a}$ |
| 4 | | 6.6±0.3$^{b}$ | 7.6±0.5$^{a}$ | 8.1±0.3$^{a}$ | 7.6±0.5$^{a}$ | 8.0±0.2$^{a}$ |
| 6 | | 6.4±0.3$^{c}$ | 7.4±0.5$^{b}$ | 7.9±0.1$^{ab}$ | 7.4±0.3$^{b}$ | 8.1±0.2$^{a}$ |
| 8 | | 5.8±0.3$^{d}$ | 6.2±0.4$^{cd}$ | 6.9±0.1$^{ab}$ | 6.5±0.5$^{bc}$ | 7.4±0.2$^{a}$ |
| 0 | Hue angle | 53.6±3.0$^{a}$ | 32.5±2.7$^{c}$ | 43.3±1.5$^{b}$ | 47.1±2.4$^{ab}$ | 52.2±2.4$^{a}$ |
| 2 | | 56.0±2.0$^{a}$ | 34.0±2.4$^{c}$ | 45.4±1.4$^{b}$ | 49.7±2.2$^{ab}$ | 50.5±3.6$^{ab}$ |
| 4 | | 58.1±3.9$^{a}$ | 33.5±1.4$^{c}$ | 45.0±0.7$^{b}$ | 48.0±0.7$^{b}$ | 48.6±1.9$^{b}$ |
| 6 | | 58.7±6.3$^{a}$ | 34.8±2.4$^{c}$ | 47.1±3.5$^{b}$ | 47.6±2.9$^{b}$ | 50.9±0.3$^{b}$ |
| 8 | | 59.2±8.3$^{a}$ | 31.4±1.8$^{c}$ | 45.7±6.3$^{b}$ | 45.5±2.3$^{b}$ | 52.4±3.3$^{ab}$ |

"Table 3 (Cont'd)"

| Storage (days) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | Chroma | 7.8±0.9$^{c}$ | 13.6±0.4$^{a}$ | 10.6±0.4$^{b}$ | 10.0±0.8$^{b}$ | 10.0±0.3$^{b}$ |
| 2 | | 7.6±0.4$^{c}$ | 14.0±0.4$^{a}$ | 11.1±0.7$^{b}$ | 10.2±0.5$^{b}$ | 10.5±0.2$^{b}$ |
| 4 | | 7.8±0.3$^{d}$ | 13.8±0.5$^{a}$ | 11.4±0.6$^{b}$ | 10.2±0.6$^{c}$ | 10.7±0.2$^{bc}$ |
| 6 | | 7.6±0.4$^{c}$ | 13.0±0.4$^{a}$ | 10.8±0.7$^{b}$ | 10.0±0.7$^{b}$ | 10.4±0.2$^{b}$ |
| 8 | | 6.9±0.3$^{c}$ | 11.9±0.4$^{a}$ | 9.8±0.9$^{b}$ | 9.1±0.7$^{b}$ | 9.4±0.2$^{b}$ |

[1] All values represent mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

[a,b,c,d] Means in the same row bearing the same superscript are not significant different ($p>0.05$).

after two days of storage. Fish fed dietary treatment V fish muscle were observed after four days of storage, whereas changes in the lightness ($L^*$) and yellowness ($b^*$) commenced maintained the highest levels of yellowness during the entire storage period.

On the other hand, samples which had a surface application of oleoresin rosemary showed no change in color values (Table 4). However, slight changes in lightness ($L^*$) were observed after two days of storage, whereas yellowness ($b^*$) and hue angle started to show change after four and six days of storage, respectively. The control samples showed decreased lightness ($L^*$) and hue angle, and increase in redness ($a^*$).

Pozo et al. (1988) judged the pigment (canthaxanthin) intensities in rainbow trout by visual examination during storage at -12°C for 7 months. Although the results showed decrease in visual scores in all treatments, the visual scores for fish fed dietary α-tocopherol supplemented-diet (500mg α-tocopheryl acetate) were higher than those of fish fed commercial control diet (26mg α-tocopheryl acetate). There is a paucity of information on the protective effect of oleoresin rosemary on color stability in carotenoid-pigmented fish. The co-oxidation of carotenoids can occur because of their presence in a highly oxidative environment. The inhibitory effect of oleoresin rosemary on lipid oxidation may provide the possible explanation for its stabilizing effect on color.

Table 4. Effect of surface application of oleoresin rosemary on color values of rainbow trout muscle stored under refrigerated conditions.

| Storage (days) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | L-value | 30.3±0.9$^{a}$ | 27.0±0.5$^{c}$ | 29.7±1.5$^{ab}$ | 29.8±0.2$^{b}$ | 28.9±0.2$^{b}$ |
| 2 | | 30.5±0.6$^{a}$ | 27.1±0.4$^{b}$ | 30.2±0.7$^{a}$ | 30.2±0.4$^{a}$ | 29.7±0.3$^{a}$ |
| 4 | | 30.1±0.5$^{a}$ | 26.8±0.4$^{b}$ | 29.3±0.9$^{a}$ | 29.5±0.5$^{a}$ | 29.2±0.2$^{a}$ |
| 6 | | 29.7±0.6$^{a}$ | 26.6±0.5$^{b}$ | 29.4±0.7$^{a}$ | 29.7±0.5$^{a}$ | 29.3±0.4$^{a}$ |
| 8 | | 28.9±1.1$^{b}$ | 26.8±0.5$^{c}$ | 29.8±0.5$^{ab}$ | 30.0±0.6$^{a}$ | 29.7±0.2$^{ab}$ |
| 0 | a-value | 2.7±0.4$^{d}$ | 11.1±0.5$^{a}$ | 6.4±0.5$^{b}$ | 4.6±0.4$^{c}$ | 5.1±0.7$^{c}$ |
| 2 | | 3.2±0.1$^{d}$ | 11.8±0.4$^{a}$ | 7.5±0.4$^{b}$ | 5.4±0.3$^{c}$ | 5.7±0.4$^{c}$ |
| 4 | | 3.6±0.3$^{d}$ | 12.2±0.2$^{a}$ | 7.4±0.3$^{b}$ | 6.1±0.1$^{c}$ | 6.2±0.8$^{bc}$ |
| 6 | | 3.4±0.6$^{d}$ | 12.1±0.5$^{a}$ | 7.6±0.6$^{b}$ | 5.8±0.1$^{c}$ | 6.1±0.9$^{c}$ |
| 8 | | 3.5±0.6$^{d}$ | 10.9±0.9$^{a}$ | 6.3±0.2$^{b}$ | 4.9±0.2$^{c}$ | 5.3±0.5$^{bc}$ |
| 0 | b-value | 6.8±0.7$^{c}$ | 8.9±0.2$^{a}$ | 8.0±0.2$^{b}$ | 8.0±0.3$^{b}$ | 7.8±0.1$^{b}$ |
| 2 | | 6.9±0.5$^{c}$ | 9.3±0.1$^{a}$ | 8.2±0.4$^{b}$ | 8.3±0.3$^{b}$ | 8.7±0.2$^{b}$ |
| 4 | | 7.2±0.4$^{c}$ | 9.4±0.1$^{a}$ | 8.5±0.3$^{b}$ | 8.3±0.3$^{b}$ | 8.8±0.3$^{b}$ |
| 6 | | 7.1±0.7$^{b}$ | 8.8±0.1$^{a}$ | 8.4±0.1$^{a}$ | 8.3±0.3$^{a}$ | 8.5±0.4$^{a}$ |
| 8 | | 6.8±0.6$^{b}$ | 8.3±0.2$^{a}$ | 8.2±0.2$^{a}$ | 8.0±0.3$^{a}$ | 8.3±0.4$^{a}$ |
| 0 | Hue angle | 68.2±5.0$^{a}$ | 38.9±0.9$^{d}$ | 51.4±2.9$^{c}$ | 60.0±3.1$^{b}$ | 57.0±4.0$^{bc}$ |
| 2 | | 65.3±0.9$^{a}$ | 38.5±1.2$^{d}$ | 47.4±2.5$^{c}$ | 56.9±0.9$^{b}$ | 56.8±2.2$^{b}$ |
| 4 | | 63.5±2.2$^{a}$ | 37.6±0.5$^{d}$ | 49.0±2.2$^{c}$ | 53.8±1.1$^{bc}$ | 54.9±4.3$^{b}$ |
| 6 | | 64.1±4.9$^{a}$ | 36.2±1.0$^{d}$ | 48.0±1.9$^{c}$ | 54.9±1.5$^{b}$ | 54.2±5.2$^{b}$ |
| 8 | | 62.9±6.2$^{a}$ | 37.4±2.3$^{c}$ | 52.2±0.4$^{b}$ | 58.3±1.2$^{a}$ | 57.4±2.3$^{ab}$ |

"Table 4 (Cont'd)"

| Storage (days) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | Chroma | 7.4±0.5$^{c}$ | 14.2±0.4$^{a}$ | 10.2±0.2$^{b}$ | 9.2±0.3$^{b}$ | 9.3±0.3$^{b}$ |
| 2 | | 7.6±0.5$^{d}$ | 15.0±0.3$^{a}$ | 11.1±0.4$^{b}$ | 9.9±0.4$^{c}$ | 10.4±0.2$^{bc}$ |
| 4 | | 8.1±0.4$^{c}$ | 15.4±0.2$^{a}$ | 11.2±0.2$^{b}$ | 10.3±0.2$^{b}$ | 10.8±0.2$^{b}$ |
| 6 | | 7.9±0.7$^{d}$ | 15.0±0.4$^{a}$ | 11.3±0.4$^{b}$ | 10.1±0.2$^{c}$ | 10.5±0.2$^{bc}$ |
| 8 | | 7.7±0.2$^{c}$ | 13.7±0.8$^{a}$ | 10.3±0.3$^{b}$ | 9.4±0.3$^{b}$ | 9.9±0.3$^{b}$ |

[1] All values represent means (± standard deviation) of three replications and each replication analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

$^{a,b,c,d}$ Means in the same row bearing the same superscript are not significant different ($p>0.05$).

The results also indicated that carotenoid concentrations were reflected in instrumental redness ($a^*$) values of fish tissue. The fish which contained higher carotenoid contents also showed higher $a^*$ values and lower hue angle values. Similar observations were reported by other researchers. No and Storebakken (1991) reported a decrease in lightness ($L^*$) and an increase in redness ($a^*$) and yellowness ($Y^*$) with increased carotenoid concentration in the muscle of rainbow trout. The increased carotenoid concentration also decreased hue values which resulted in a more reddish hue. Similarly, Choubert et al. (1992) observed that carotenoid concentration was strongly correlated with hue, chroma and lightness in muscle of rainbow trout fed either astaxanthin (50 or 100mg/kg diet) or canthaxanthin (100mg/kg diet).

Peterson et al. (1966) fed paprika xanthophylls (237mg/kg) to brook trout and reported that the color of muscle appeared to be natural except for a small amount of an undesirable yellow.

In conclusion, the combination of dietary vitamin E and surface application of oleoresin rosemary improved the oxidative stability of fish lipids during refrigerated storage. These observations demonstrate the potential benefit to the fish industry of the combination of dietary vitamin E and the post harvest application of oleoresin rosemary.

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## CHAPTER THREE

## EFFECT OF DIETARY VITAMIN E AND SURFACE APPLICATION OF OLEORESIN ROSEMARY ON IRON/ASCORBATE-STIMULATED LIPID OXIDATION IN MUSCLE TISSUE AND MICROSOMES FROM RAINBOW TROUT.


### ABSTRACT

The effect of dietary α-tocopherol and surface application of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in rainbow trout muscle and microsomes was investigated. The thiobarbituric acid-reactive substances (TBARS) numbers of muscle and microsomes from fish fed the higher concentration of α-tocopherol (500mg/kg feed) were smaller than those of fish fed the lower concentration (100mg/kg feed). The protective effect of oleoresin rosemary on lipid oxidation in fish muscle was also observed. Analysis of variance revealed a significant ($p<0.01$) interaction between dietary treatments and incubation time. Dietary α-tocopherol supplementation significantly increased the α-tocopherol concentration of muscle microsomal membranes, thereby increasing the oxidative stability of the membrane lipids.

# INTRODUCTION

It is generally accepted that lipid oxidation in meat is initiated at the membrane level (Gray and Pearson, 1987). The presence of relatively large amounts of polyunsaturated fatty acids in the membranes of mitochondria, microsomes and lipoproteins makes them highly vulnerable to peroxidative changes (Buege and Aust, 1978). The presence of highly unsaturated fatty acids in the fish tissue itself also makes it extremely susceptible to oxidative deterioration during storage. Lipid oxidation in muscle foods can directly affect many quality attributes such as color, flavor, texture, nutritive value and safety (Khayat and Schwall, 1983; Pearson et al., 1983).

Lipid peroxidation has been traditionally attributed to non-enzymic reactions (Castell, 1971; Rhee et al., 1987). However, it can also be promoted by enzymic reactions (McDonald and Hultin, 1987; Eun et al., 1992) initiated by microsomal enzymes, lipoxygenase and peroxidase. A microsomal lipid peroxidation system has been reported in various fish species such as red hake (McDonald et al., 1979), flounder (Shewfelt et al., 1981; McDonald and Hultin, 1987), Herring (Slabyj and Hultin, 1982), rainbow trout (Han and Liston, 1987) and channel catfish (Eun et al., 1992). Similarly, the presence of an enzymic lipid peroxidation system has also been identified in beef, chicken and pork muscle microsomes (Lin and Hultin, 1976; Rhee et al., 1984; Rhee and Ziprin, 1987).

However, the avian and mammalian muscle systems (Lin and Hultin, 1976; Rhee at al., 1984) differ in their co-factor requirements compared to microsomes of fish muscle (Apgar and Hultin, 1982).

The mechanisms underlying the initiation of lipid oxidation in fish were investigated by German and Kinsella (1985) who demonstrated lipoxygenase activity in trout skin extract. They suggested that the postmortem release of skin lipoxygenase could contribute significantly to the generation of initiating radicals leading to subsequent lipid oxidation in fish. Hultin (1980) suggested that non-enzymic processes occur concurrently with enzymic processes. Additionally, other endogenous factors such as fatty acid composition of lipids, pH value and water activity also contribute to lipid oxidation (Khayat and Schwall, 1983). Recently, the results of studies of lipid hydrolysis and lipid oxidation during the frozen storage of light and dark muscle of rainbow trout revealed that the major cause of lipid deterioration was hydrolysis (Ingemansson et al., 1995).

It is well established that vitamin E, a lipid-soluble antioxidant, protects biological membranes, lipoproteins and stored lipids against oxidation. As tocopherols are mainly located in cell membranes, they protect fatty acids from peroxidative damage caused by highly reactive free radicals such as hydroxyl, peroxyl and superoxide radicals. Many studies have demonstrated the effectiveness of dietary supplementation with vitamin E, in the form of α-tocopheryl

acetate, in retarding lipid oxidation in meat (Frigg et al., 1990; Asghar et al., 1991; Buckley and Morrissey, 1992; Monahan et al., 1992; Engeseth et al., 1993; Sigurgisladottir et al., 1994; Chan et al., 1995; Wulf et al., 1995).

The antioxidant properties of oleoresin rosemary are also well established and are attributed to its phenolic contents. These phenolic constituents possess antioxidant activity similar to or greater than butylated hydroxyanisole (BHA) (Houlihan and Ho, 1985). The antioxidant properties of rosemary have earlier been studied in a number of meat products (Barbut et al., 1985; Hwang and Regenstein, 1988; Korczak et al., 1988; Lai et al., 1991; Stoick et al., 1991; Liu et al., 1992; Wada and Fang, 1992; Boyd et al., 1993; Eun et al., 1993; Fang and Wada, 1993).

The purpose of this study was to investigate the effect of dietary vitamin E and oleoresin rosemary (in the form of a dipping solution) on iron/ascorbate-induced lipid oxidation in rainbow trout muscle and the microsomal fraction.

## MATERIALS AND METHODS

### Diets and feeding trial

Rainbow trout (*Oncorhynchus mykiss*) were fed five different diets for a period of 7 months. The composition of dietary treatments, concentration of carotenoids and details of the feeding trial have been reported previously (Chapter 1).

The diets are as follows, the values cited being target levels:

Diet I : Control commercial diet (Martin Mills Inc., Elmira, ONT).

Diet II : Commercial canthaxanthin diet (Martin Mills Inc., Elmira, ONT).

Diet III : Diet supplemented with α-tocopheryl acetate (500mg /kg) and canthaxanthin (15mg/kg).

Diet IV : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (175mg/kg).

Diet V : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (350mg/kg).

Diets I and II were commercial rainbow trout diets (floating 40% trout feed grower pellets) and contained 10% herring oil by weight. These diets also contained 100mg α-tocopheryl acetate (target level). Diet I served as the control diet in this study. A commercial rainbow trout diet without added herring oil was also purchased from Martin Mills Inc. and used to prepare three supplemented diets (diets III, IV and V). The supplemented diets were prepared by adding α-tocopheryl acetate (500mg/kg) to herring oil (10% based on weight of diet) which was carefully coated on the surface of the diet. Additionally, diet III and diets IV and V contained canthaxanthin (Martin Mills Inc.) and oleoresin paprika (Kalsec Inc.,Kalamazoo, MI), respectively. Diets III, IV and V were prepared by Kalsec Inc.

All fish were slaughtered according to standard commercial practices at the Aquaculture Research Laboratory, Michigan State University at the termination of the feeding trial. Fish were kept on ice and immediately transferred to the Meat Processing Laboratory, Michigan State University, for processing and packaging.

Sample preparation

The slaughtered fish were filleted and their skins were removed. One fillet from each fish was immediately immersed in a 2% solution of Herbalox seasoning type P, oleoresin rosemary extract (Kalsec Inc., Kalamazoo, MI) in distilled water (w/v), until an approximate pick up of 1% was obtained. The gain in weight was measured by periodic weighing of the fillets after removing excess oleoresin rosemary solution from the surface of the fillets. The other fillet was not dipped in the oleoresin rosemary solution and was used as a control. Both fillets were analyzed to compare the effect of the different dietary and processing treatments.

Isolation of the microsomal fraction

The microsomal fraction was isolated by a procedure similar to that reported by Kanner and Harel (1985). Rainbow trout muscle (200g) was blended with 800ml buffer (0.12M KCl, 5mM Histidine, pH 7.3) in the following manner. The sample was initially blended with 200ml buffer for 30 sec in a Waring blender with an explosion proof base at moderately high speed.

Three 100ml aliquots of buffer were added and between each addition, the sample was blended for 30 sec. The muscle homogenate was equally distributed into 4 polycarbonate centrifuge bottles (250ml capacity) and centrifuged at 600g for 10 min at 4°C in a Sorvall centrifuge (Model RC-2B, Ivan Sorvall Inc., New Town, CT). The supernatant was poured through 8 layers of cheese cloth and collected in a 1 L beaker kept on ice. An additional 300ml aliquot of buffer was used to rinse the blender jar and then equally distributed between the centrifuge bottles containing the pellets. The contents of each bottle were stirred gently with a spatula and then recentrifuged for 10 min at 600g. The supernatant was combined with that previously collected after passing through 8 layers of cheese cloth and centrifuged for 10 min at 1,000g to sediment the nuclear fraction and other cell debris. The pH of the supernatant was readjusted to 7.3, and centrifuged at 10,000g for 15 min to precipitate the mitochondrial fraction. The microsomes were isolated from the supernatant by adding $CaCl_2$ to give a final concent-ration of 8mM. The mixture was centrifuged at 16,500g for 30 min at 4°C. The supernatant was discarded and the microsomal fraction was collected in a tared vial. The vials were frozen under nitrogen until required for use.

Protein determination

The protein contents of both muscle and the microsomal fraction of rainbow trout were determined by the method of

Lowry et al. (1951).

Stimulation and measurement of lipid peroxidation

The extent of lipid oxidation in fish muscle samples and the microsomal fraction was determined in duplicate by the 2-thiobarbituric acid (TBA) method of Beuge and Aust (1978). The molar extinction co-efficient for malonaldehyde (MDA) is 1.56 x $10^5$ $M^{-1}$ $cm^{-1}$. The TBARS numbers were expressed as nmol malonaldehyde (MDA)/mg tissue protein using the following equation:

$$\text{MDA (nmol/mg protein)} = \frac{6.4102 \times 1000 \times 3 \times \text{absorbance}}{100 \times \text{Protein (mg/ml)}}$$

Both muscle and tissue homogenates were assayed separately for TBARS numbers before and after stimulation of lipid peroxidation with iron/ascorbate by a modification of the method of Kornburst and Mavis (1980) as reported by Sheehy et al. (1994). Muscle sample (1g) was homogenized with 10ml of tris-malate buffer (80mM, pH 7.4) using a polytron homogenizer (Kinematica AG, Littau, Switzerland) at speed setting 4 for 45 sec. The microsomal fraction (180mg) was homogenized with 1ml tris-malate buffer using a tissue homogenizer (Model 985-370, Biospec Products Inc., Racine, WI) set at speed 4 for 45 sec. To a screw-capped glass test tube, 500µl tris-malate buffer, 200µl $FeSO_4.7H_2O$ (5 mM), 200µl ascorbic acid solution (2mM) and 100µl of muscle or microsomes (100mg/ml or 180mg/ml, respectively) were added. The reaction mixture was incubated for various lengths of time in a shaking water bath at 37°C.

Following incubation, the reaction was terminated by adding TBA reagent and color was developed by holding in boiling water for 15 min before spectrophotometric (Bausch and Lomb double beam spectrophotometer, Rochester, NY) determination at 532 nm.

Determination of α-tocopherol concentrations in fish muscle and the microsomal fraction

Extraction of α-tocopherol: The α-tocopherol in rainbow trout muscle and the microsomal fraction was extracted using the method of Buttriss and Diplock (1984). Muscle sample (5g) was homogenized with distilled water (15ml) in a polytron homogenizer. One ml of the 25% sample homogenate and 2ml 1% ethanolic pyrogallol were added to a screw cap test tube. The sample was saponified by blending with 0.3ml 50% potassium hydroxide and holding at 70°C in a water bath for 30 min. On cooling, 4ml hexane (containing 0.005% butylated hydroxytoluene as an antioxidant) were added to the sample and the contents of the test tube shaken vigorously for 1 min. The hexane layer was removed and the extraction procedure was repeated two more times with 2ml aliquots of hexane. The extracts were pooled and 200μl α-tocopheryl acetate (100μg/ml ethanol) was added as an internal standard in order to correct the data for recovery. The contents were evaporated to dryness under nitrogen. The residue was redissolved in 200μl of ethanol.

For α-tocopherol extraction from the microsomal membrane fraction, 0.08g of sample was suspended in 1ml of water and the whole sample used in the analysis as described above.

The recovery rates were established by adding 200μl α-tocopherol (100μg/ml ethanol) to freshly homogenized muscle and microsomes samples before extraction. The resulting peak area on the chromatograms was compared with the peak area obtained by direct injection of standard α-tocopherol onto the column. The average recoveries of α-tocopherol in the muscle and microsomes samples were 88.9% and 91.7% with coefficients of variation (CV) of 3.7% and 3.1%, respectively. Each recovery experiment was repeated three times and each extract was analyzed in duplicate.

Quantitation of α-tocopherol by high performance liquid chromatography

Concentrations of α-tocopherol were determined using a 150 mm x 3.9 mm Symmetry™ $C_8$ reverse phase column (Water Associates, Milford, MA) with a 5μ particle size. A Sentry™ integrated guard column (Waters Associates) was attached to the column. Separation was achieved using a mobile phase containing acetonitrile, methanol and water (25:25:1 v/v/v) with a flow rate of 1.0ml/min. A high performance liquid chromatograph (Model 501, Waters Associates) equipped with dual pumps was used. Detection was achieved with an absorbance detector (Model 486, Waters Associates) set at 280nm. Chromatograms were recorded on a NEC printer(Model Pinwriter

P 5200, NEC Information Systems, Inc. Boxborough, MA).

The identification and quantitation of α-tocopherol was achieved by injecting known amounts of α-tocopherol standard (5-120ng/μl for muscle samples and 5-30ng/μl for microsomal fraction) and making the standard curve.

## Statistical Analysis

Data were analyzed using a split-plot design with factors A (treatment) and B (replication) applied to the whole plot, and factors C (time) and D (surface application of oleoresin rosemary) applied to the sub-plot. The MSTAT-C microcomputer statistical program (Michigan State University, 1991) was employed to analyse the data. Tukey's test was applied to separate the mean values.

# RESULTS AND DISCUSSION

## Concentrations of α-tocopherol in the muscle and microsomal fraction of rainbow trout

The concentrations of α-tocopherol in the muscles of rainbow trout are summarized in Table 1. The concentrations in rainbow trout fed the control and commercial canthaxanthin diets (diets I and II) were 10.6μg/g and 10.5μg/g, respectively. The concentrations of α-tocopherol in fish fed diets III, IV and V were 54.1μg/g, 56.3μg/g and 57.1μg/g, respectively. There was no significant difference ($p>0.05$) in the concentrations of α-tocopherol among fish fed the

Table 1. Concentrations of α-tocopherol in the muscle of rainbow trout fed five different dietary treatments.

| Dietary Treatments | α-tocopherol concentrations (μg/g) |
|---|---|
| I | 10.6 ± 0.7[b] |
| II | 10.5 ± 1.1[b] |
| III | 54.1 ± 8.5[a] |
| IV | 56.3 ± 9.9[a] |
| V | 57.1 ± 6.5[a] |

All values represent the mean of three replications and each replication was analyzed in duplicate.

Diet I : Commercial control diet (supplemented with 100mg α-tocopheryl acetate/kg feed).

Diet II : Commercial canthaxanthin diet (supplemented with 40mg canthaxanthin and 100mg α-tocopheryl acetate/kg feed).

Diet III: Supplemented diet I (supplemented with 15mg canthaxanthin and 500mg α-tocopheryl acetate/kg feed).

Diet IV : Supplemented diet II (supplemented with 175mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

Diet V : Supplemented diet III (supplemented with 350mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

[a,b] Means bearing same superscripts are not significantly different ($p>0.05$).

supplemented diets (III, IV and V). Similarly, α-tocopherol concentrations in the muscle of fish fed the control and commercial canthaxanthin diets were also not significantly ($p>0.05$) different with respect to each other.

The concentrations of α-tocopherol in the microsomal fraction of rainbow trout are summarized in Table 2. The concentrations of α-tocopherol in microsomes of fish fed the control and commercial canthaxanthin diets ranged from 98.8ng/mg to 104.8ng/mg protein. Statistical analysis revealed that these values were not significantly ($p>0.05$) different. The concentrations of α-tocopherol in the microsomes from fish fed the higher supplemented dietary treatments (diets III, IV and V) ranged from 306.2ng/mg to 353.6ng/mg protein. These values again were not significantly ($p>0.05$) different from each other. The concentrations of α-tocopherol in the microsomal fractions of muscles from rainbow trout fed diets containing the higher α-tocopheryl acetate level (500mg/kg feed) were 3.5 times higher than those in the fractions from fish fed the control and commercial canthaxanthin diets (100mg α-tocopheryl acetate/kg feed). These results closely agree with those of earlier studies with pigs (Monahan et al., 1990) and chickens (Asghar et al., 1989) which clearly demonstrated that dietary α-tocopherol supplementation significantly increased the α-tocopherol levels of muscle microsomal membranes. Monahan et al. (1990) reported that the α-tocopherol concentration in muscle microsomes from pigs receiving a vitamin E-supplemented diet (200mg/kg) was almost

Table 2. Concentrations of α-tocopherol in microsomes of rainbow trout fed five different dietary treatments.

| Dietary treatment[1] | α-tocopherol concentrations (ng/mg protein) |
|---|---|
| I | 104.8 ± 24.6[b] |
| II | 98.8 ± 55.8[b] |
| III | 315.8 ± 96.0[a] |
| IV | 353.6 ± 98.9[a] |
| V | 306.2 ± 27.1[a] |

All values represent the mean of three replications and each replication was analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

[a,b] Means bearing same superscripts are not significantly different ($p>0.05$).

2.6 times greater than that in microsomes from pigs fed the control (basal) diet. The concentrations of α-tocopherol in muscle microsomes of pig fed control and supplemented diets were 62.4ng/mg and 164.8ng/mg protein, respectively. Machlin (1984) also reported that α-tocopherol is most concentrated in cell fractions such as mitochondria and microsomes.

## Effect of antioxidants on the oxidative stability of muscle lipids

The effect of dietary supplementation of α-tocopherol and surface application of oleoresin rosemary on the oxidative stability of rainbow trout muscle lipids was studied by stimulating lipid peroxidation with iron/ascorbate. Ferrous ions have been reported to catalyze lipid oxidation in various biological systems (Gutteridge et al., 1985; Quinlan et al., 1988). Halliwell and Gutteridge (1988) reported that ferrous and ferric ions decompose lipid peroxides to alkoxy and peroxy radicals, respectively. These radicals can in turn abstract further hydrogen atoms which help in accelerating the rate of lipid oxidation.

Initially, fish fed the commercial canthaxanthin diet exhibited slightly higher TBARS numbers compared to the rest of the dietary treatments (Figure 1). However, analysis of variance revealed that the TBARS numbers of fish from all five dietary treatments were not significantly different ($p>0.05$).

Statistical analysis revealed highly significant ($p<0.01$) effects of dietary treatments and incubation time on TBARS

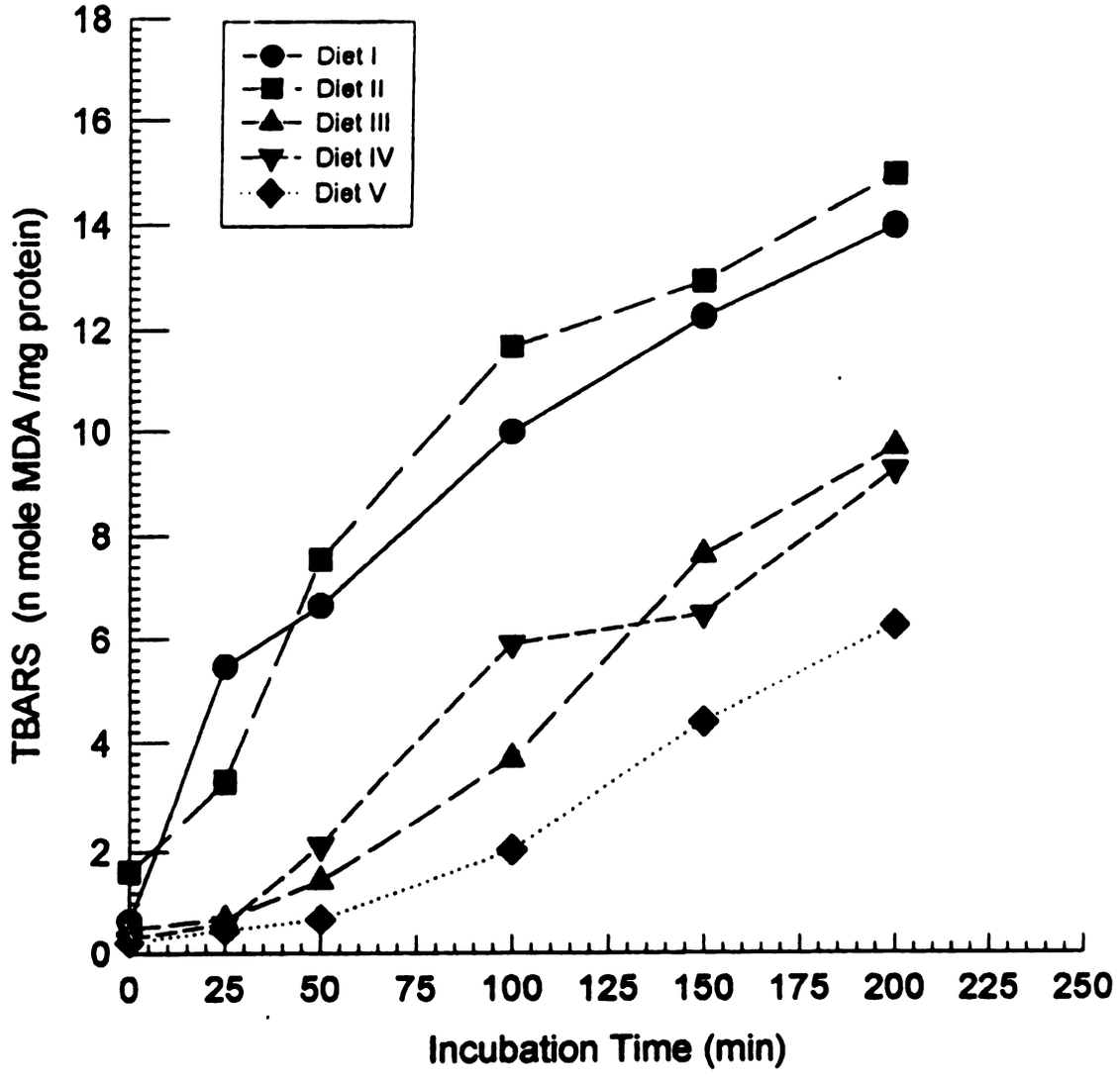


**Figure 1. Effect of dietary supplementation on iron/ascorbate-induced lipid oxidation in rainbow trout muscle.**

**(See Table 1 for description of dietary treatments)**

numbers. Analysis of variance also showed a highly significant interaction ($p<0.01$) between dietary treatments and incubation time which suggested that the effects of these variables are dependant upon each other. The first significant ($p<0.05$) effect of dietary treatment was observed after 25 min of incubation. The muscle samples from rainbow trout receiving the control and the commercial canthaxanthin diets (diets I and II) had higher TBARS numbers compared to muscle tissue from fish fed the diets containing the higher level of α-tocopherol (diets III, IV and V). It was further observed that TBARS numbers in muscle from fish fed the control and commercial canthaxanthin diets were significantly different ($p<0.05$) from those in the muscle from fish fed the remaining three diets.

These observations lead to the conclusion that α-tocopherol supplementation effectively inhibited lipid oxidation. This conclusion agrees with the findings of Frigg et al. (1990) who also stimulated lipid peroxidation in rainbow trout muscle with iron/ascorbate. They reported a pronounced dependency of the measured TBARS numbers on dietary vitamin E levels as well as on the muscle α-tocopherol levels. Rainbow trout weighing 150g each were fed one of the four dietary treatments (0, 50, 100 and 200mg supplemental vitamin E/kg feed). Results indicated that dietary supplementation with vitamin E had a major impact on the oxidative stability of fish muscle. Recently, Sheehy et al (1994) investigated the effects of feeding diets containing fresh or heated sunflower

oil, with or without α-tocopherol, on the stability of chick tissues toward iron/ascorbate-induced lipid oxidation. They concluded that supplementation with α-tocopheryl acetate inhibited lipid oxidation, the degree of inhibition depending on the kind of tissue analyzed.

The effect of surface application of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in muscle samples is shown in Figure 2. Dietary treatments and incubation time had a highly significant ($p<0.01$) effect on lipid oxidation. However, the significant ($p<0.01$) interaction between both these variables indicates that their effect was not independent of each other. The significant effect of dietary treatments was noticed after 50 min of incubation. Significantly ($p<0.05$) lower TBARS numbers in muscle samples of fish fed diets supplemented with the higher level of α-tocopherol were observed at the termination of the experiment. Fish fed the diet containing the higher level of oleoresin paprika (diet V) had the lowest TBARS numbers of all the treatments. On the other hand, fish fed commercial canthaxanthin diet (diet II) had the highest TBARS numbers. However, the TBARS numbers in muscle samples from fish fed the control diet and the commercial canthaxanthin diet were not significantly different ($p>0.05$) from each other. Similarly, no significant difference ($p>0.05$) in TBARS numbers was observed among the muscle of fish fed the higher vitamin E-supplemented diets (III, IV and V). Statistical analysis further revealed the strong inhibitory effect ($p<0.01$) of

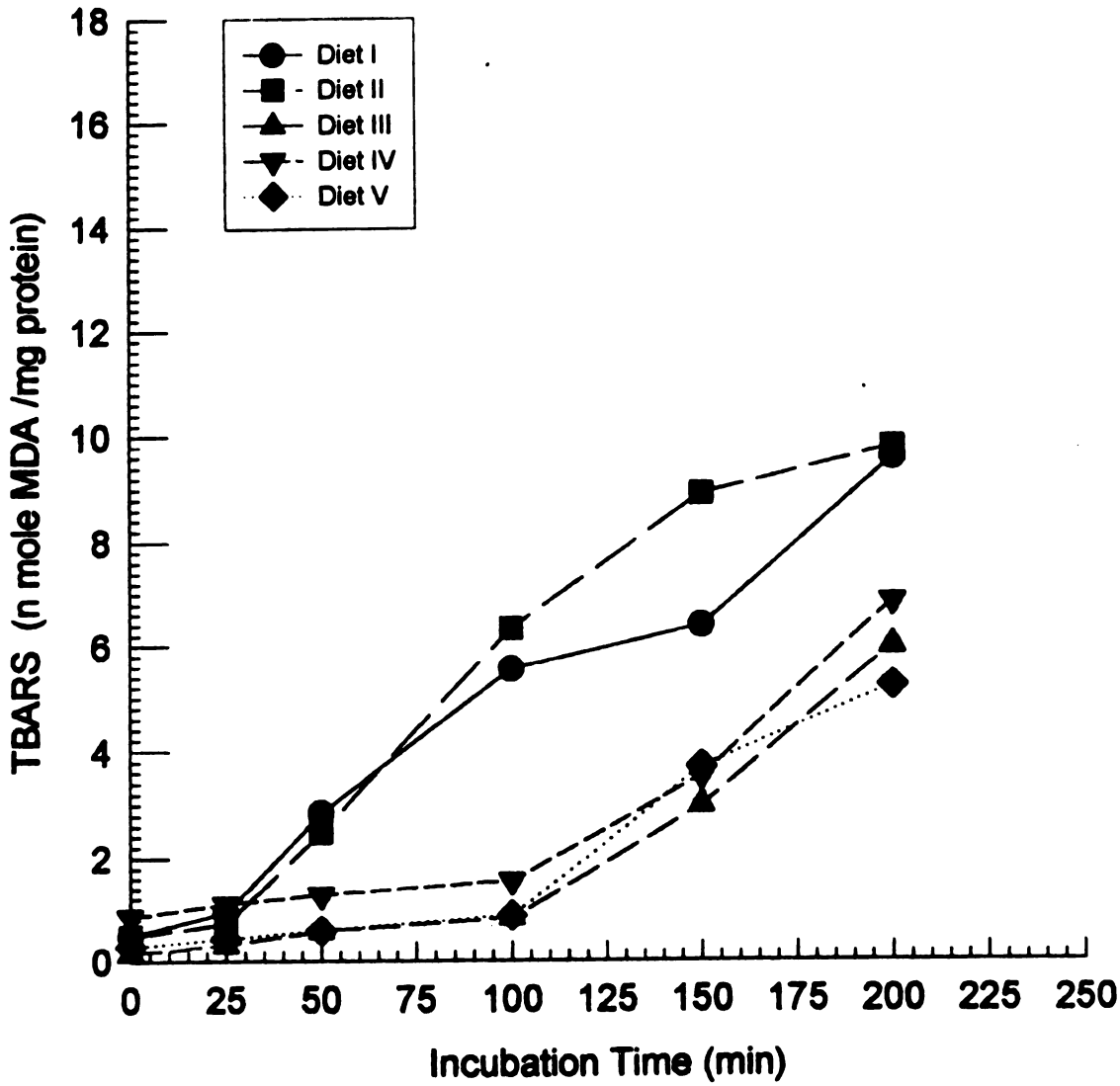


**Figure 2. Effect of surface application of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in rainbow trout muscle.**

**(See Table 1 for description of dietary treatments.**

surface application of oleoresin rosemary on lipid oxidation.

Recently, Fang and Wade (1993) observed a synergistic effect between α-tocopherol and rosemary extract in a sardine oil model system and in frozen-crushed fish meat. In the sardine oil model system, a mixture of α-tocopherol and rosemary extract (0.035% + 0.035%) extended the induction period by 10 and 16 days compared to α-tocopherol and rosemary extract when used individually. The lowest TBARS numbers were observed in the frozen-crushed fish meat α-tocopherol and rosemary extract. In an earlier study, Wada and Fang (1992) observed that the mixture of α-tocopherol and rosemary extract (0.05% + 0.02%) had a stronger antioxidant effect than either α-tocopherol or the rosemary extract alone in frozen-crushed bonito fish meat.

These results clearly demonstrated the protective effect of supranutritional levels of α-tocopherol supplementation (500mg/kg) and surface application of oleoresin rosemary on lipid oxidation. The results also confirm the trends observed for the whole fillets during refrigerated storage (Chapter 2). Frigg et al. (19990) also observed that the iron/ascorbate-induced lipid oxidation method gave reproducible results which clearly indicated the interrelationship between TBARS numbers and the concentrations of α-tocopherol in rainbow trout muscle.

Effect of dietary supplementation on the oxidative stability of microsomes

The effect of dietary treatment on iron/ascorbate-induced lipid oxidation in the microsomal fraction of rainbow trout muscle is shown in Figure 3. A highly significant ($p<0.01$) effect of dietary treatment and incubation time on TBARS numbers was observed. Additionally, analysis of variance showed the highly significant ($p<0.01$) interaction between dietary treatments and incubation time which suggested that the effects of these variables are not independent of each other. The first significant effect of dietary treatment was observed after 15 min of incubation. The microsomes from rainbow trout receiving the control diet had higher TBARS numbers than those from fish fed the commercial canthaxanthin diet (diet II) and the higher vitamin E-supplemented diets (III, IV and V) at the termination of the experiment. No significant difference ($p>0.05$) in TBARS numbers was observed among the microsomes from fish fed either supplemented diets (diets III and IV) or the commercial canthaxanthin diet (diet II). Results showed that the diet containing the higher level of oleoresin paprika (V) gave the lowest TBARS numbers which were significantly ($p<0.05$) different from the rest of the dietary treatments.

Eun et al. (1993) studied the relative effectiveness of different antioxidants on enzymic lipid peroxidation of catfish muscle microsomes. They reported that α-tocopherol and rosemary powder moderately inhibited enzymic lipid

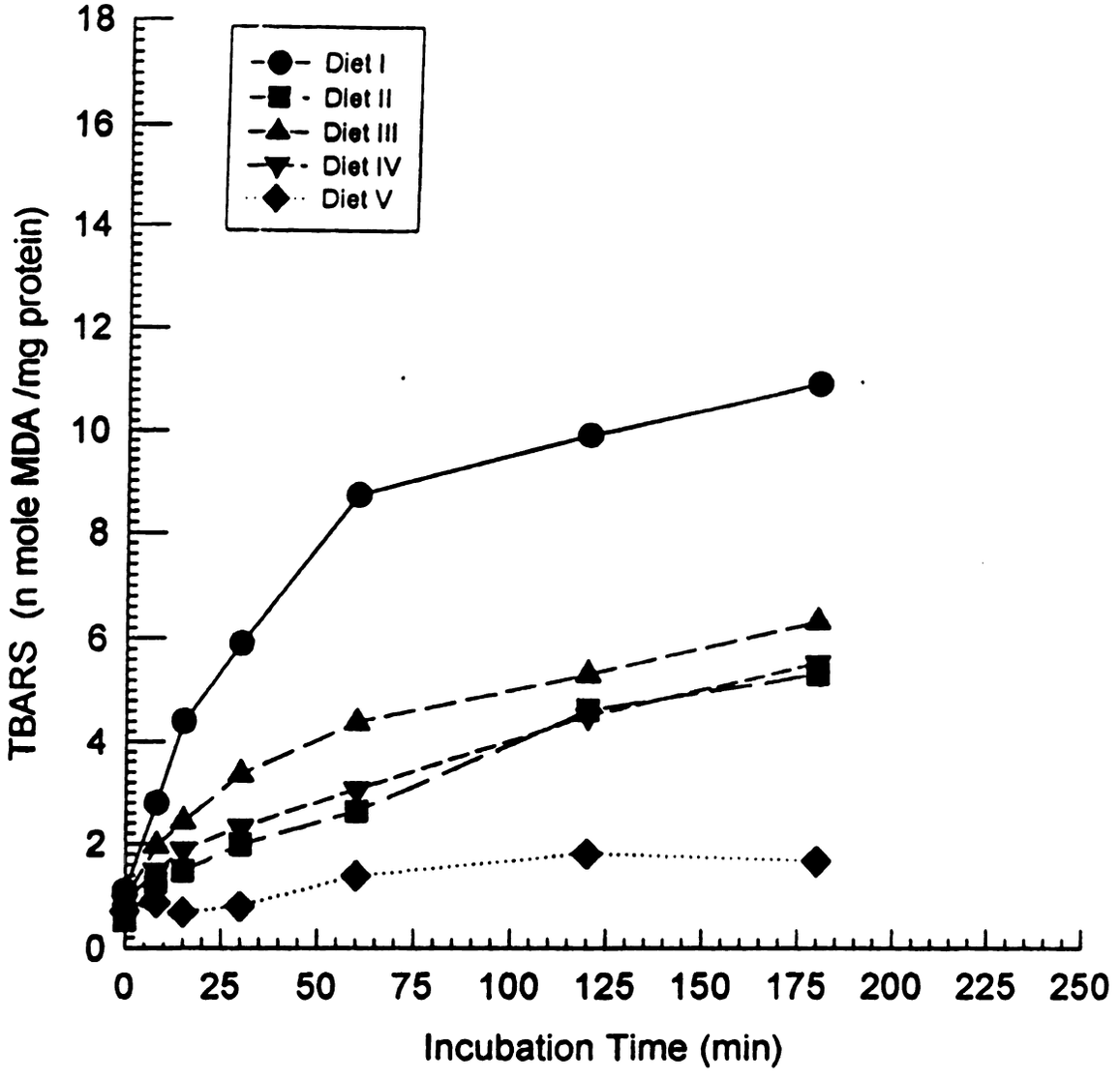


**Figure 3. Effect of dietary supplementation on iron/ascorbate-induced lipid oxidation in the microsomal fraction of rainbow trout muscle.**

**(See Table 1 for description of dietary treatments)**

peroxidation in catfish muscle microsomes. However, rosemary powder had a greater inhibitory effect than α-tocopherol.

Other researchers have also suggested that increasing the α-tocopherol content of tissues may offer an effective way of stabilizing membranal lipids. Asghar et al. (1990) reported that the microsomal fraction of muscle isolated from broilers receiving an α-tocopherol-supplemented diet had lower TBARS numbers than those from broilers receiving a control diet. Monahan et al. (1990) showed that iron-induced lipid peroxidation in the porcine microsomal fractions isolated from the basal (low α-tocopherol) group was significantly greater ($p<0.01$) than that in the α-tocopherol supplemented group. Engeseth et al. (1993) observed that the oxidative stability of veal mitochondrial and microsomal lipids was enhanced by dietary supplementation with α-tocopheryl acetate.

Schiedt et al. (1985) reported that canthaxanthin is metabolically reduced to β-carotene via echinenone in rainbow trout. It has been shown that β-carotene modestly inhibits lipid peroxidations conducted *in vitro* (Burton, 1989). However, the inhibitory effect is obtained only at low partial pressures of oxygen (e.g., 15 torr). This is the mode of action most likely to be considered in mammalian cells. The possible role of different concentrations of paprika carotenoids in inhibiting lipid oxidation has yet to be explored. The possible interaction between paprika carotenoids and α-tocopherol supplementation need to be investigated thoroughly

in model systems.

Results of this study indicate that the higher level of α-tocopherol supplementation had a protective effect on microsomal lipid stability. Cellular membranes host vitamin E where its chromanol ring is probably associated with the polar surface of the membrane. The phytol chain interacts with the polyunsaturated fatty acids of the phospholipids in the non-polar interior of the membrane (Machlin, 1984). It is well established that α-tocopherol effectively quenches free radicals generated by membrane-bound enzymes (Machlin, 1984). Additionally, α-tocopherol may stabilize membranes by a physico-chemical mechanism through a lipid-lipid interaction (Diplock, 1985).

In conclusion, dietary supplementation with α-tocopheryl acetate will increase the concentration of α-tocopherol in cell membranes. Greater concentrations of α-tocopherol in membranes in turn will help to stabilize the membrane-bound phospholipids. This will ultimately assist in delaying lipid oxidation in food and food products during refrigerated and frozen storage. Furthermore, surface application of oleoresin rosemary enhances the oxidative stability of the fish muscle tissue.

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## CHAPTER FOUR

## EFFECT OF FEED COMPONENTS AND SURFACE APPLICATION OF OLEORESIN ROSEMARY ON THE COLOR AND LIPID STABILITY OF RAINBOW TROUT MUSCLE DURING FROZEN STORAGE.


### ABSTRACT

The effect of dietary supplementation of α-tocopherol and surface application of oleoresin rosemary on the lipid and color stability of rainbow trout muscle during frozen storage (-20°C) was investigated. Changes in muscle α-tocopherol concentrations, carotenoid concentrations and composition and fatty acid composition were also studied. Significant ($P<0.05$) reductions in α-tocopherol and carotenoid concentrations were observed. The TBARS numbers of muscle samples, with or without surface application of oleoresin rosemary, increased during storage for all dietary groups. However, surface application of oleoresin rosemary significantly ($p<0.05$) reduced TBARS numbers. Dietary α-tocopherol had little protective effect on color stability. However, combination of oleoresin rosemary and α-tocopherol stabilized redness ($a^*$), yellowness ($b^*$) and chroma in fish fed diets containing 500mg α-tocopheryl acetate/kg feed. Little change in the fatty acid composition of the fish samples was observed during storage.

## INTRODUCTION

It is important to maintain the high quality of frozen fish and fish products as a considerable segment of commercial fish trading involve these foods. The main problem in the frozen storage of fish and fish products is the hydrolytic and oxidative deterioration of lipids and pigments (Ingemansson et al., 1995). Lipid oxidation adversely affects the quality attributes and functional properties of foods (Simic and Karel, 1980). Carotenoid degradation has long been recognized to proceed in an analogous fashion to lipid degradation (Frankel, 1985). Deterioration of carotenoids during the frozen storage of rainbow trout muscle has been reported by a number of researchers (Chen et al., 1984; Pozo et al., 1988; Anderson et al., 1990).

Lipid oxidation has been traditionally attributed to non-enzymic reactions (Castell, 1971; Rhee et al., 1987). However, it could also be promoted by enzymic reactions initiated by microsomal enzymes, lipoxygenase and peroxidase (McDonald and Hultin, 1987; Eun et al., 1993). It has been suggested that non-enzymic processes occur concurrently with enzymic processes (Hultin, 1980). German and Kinsella (1985) studied the mechanisms underlying the initiation of lipid oxidation in fish and found lipoxygenase activity in trout skin extract. They suggested that postmortem release of skin lipoxygenase may produce initiating radicals and subsequent lipid oxidation in fish.

Frozen storage not only inhibits microbial spoilage but also helps to slow down enzymic activity. However, it does not appear to inhibit lipid oxidation. The oxidation of lipids and development of rancidity in frozen herring is well documented (Banks, 1952). Recently, a link between phospholipid hydrolysis and lipid oxidation in low fat fish muscle during frozen storage was suggested by Han and Liston (1987). Hardy and Smith (1976) reported free fatty acids and glycerol as the major products of lipid breakdown arising from postmortem lipolysis which can occur in chilled and frozen fish. There is also evidence that under frozen conditions, oxidation of some fatty species is enhanced by long-term storage (Smith et al., 1980). The high activity of fish lipoxygenase at temperatures near freezing also suggests that it may be important in initiating fish lipid oxidation (Hsieh et al., 1988).

The main objectives of this study were to investigate the effect of feed components on lipid stability and color degradation of rainbow trout muscle under frozen conditions, and to observe changes in lipid composition, carotenoid composition and α-tocopherol concentrations as a result of storage.

## MATERIALS AND METHODS

### Diets and feeding trial

Rainbow trout (*Oncorhynchus mykiss*) were fed five different diets for a period of 7 months. The composition of

the dietary treatments, concentrations of α-tocopherol and carotenoids and details of the feeding trial have been reported previously (Chapter 1).

The diets are summarized as follows, the values cited being target levels:

Diet I : Control commercial diet (Martin Mills Inc., Elmira, ONT).

Diet II : Commercial canthaxanthin diet (Martin Mills Inc., Elmira, ONT).

Diet III : Diet supplemented with α-tocopheryl acetate (500mg /kg) and canthaxanthin (15 mg/kg).

Diet IV : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (175 mg/kg).

Diet V : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (350 mg/kg).

Diets I and II were commercial rainbow trout diets (floating 40% trout feed grower pellets) and contained 10% herring oil by weight. These diets also contained 100mg α-tocopheryl acetate/kg feed (target level). Diet I served as the control diet in this study. A commercial rainbow trout diet without added herring oil was also purchased from Martin Mills Inc. and used to prepare three supplemented diets (diets III, IV and V). The supplemented diets were prepared by adding α-tocopheryl acetate (500 mg/kg) to herring oil (10% based on weight of diet) which was carefully coated on the surface of the diet. Additionally, diet III and diets IV and V contained canthaxanthin (Martin Mills Inc.) and oleoresin paprika

(Kalsec Inc.,Kalamazoo, MI), respectively. Diets III, IV and V were prepared by Kalsec Inc.

All fish were slaughtered according to standard commercial practices at the Aquaculture Research Laboratory, Michigan State University at the termination of the feeding trial. Fish were kept on ice and immediately transferred to the Meat Processing Laboratory, Michigan State University for processing and packaging.

Samples preparation

The slaughtered fish were filleted and their skins removed. One fillet from each fish was immediately immersed in a 2% solution of Herbalox seasoning type P, oleoresin rosemary extract (Kalsec Inc., Kalamazoo, MI) in distilled water (w/v), until an approximate pick up of 1% was observed. The gain in weight was measured by periodic weighing of the fillets after removing excess oleoresin rosemary solution from the surface of the fillets. The other fillet was used as the reference.

Storage of samples

The fish fillets were kept in polyethylene-laminated nylon pouches (Koch, Kansas city, MO) and stored at -20°C (without vacuum) for 6 months. These pouches had an oxygen transmission rate of 9ml/$m^2$/day at 4°C. Color and lipid oxidation analyses were conducted immediately and after 2, 4 and 6 months of storage. Analyses for fatty acid composition, α-tocopherol and carotenoids were conducted immediately after

slaughtering and after 6 months of frozen storage.

Measurement of lipid oxidation

The distillation procedure of Tarladgis et al.(1960), as modified by Crackel et al. (1988), was used to determine TBARS (2-thiobarbituric acid-reactive substances). The measurements were done spectrophotometrically at 532nm and the results were expressed as mg malonaldehyde (MDA)/kg of sample.

Determination of total carotenoids in fish muscle

A 10g sample of rainbow trout muscle was finely ground with anhydrous magnesium sulfate (20g) using a mortar and pestle. The mixture was transferred to a 250ml round bottom flask containing acetone (50ml). The contents were stirred for 1 hr prior to filtering through Whatman No.4 filter paper. The filtrate was collected in a 100ml volumetric flask. Two 20ml aliquots of acetone were used to further extract the residue and rinse the flask. The extracts were pooled together and the volume was adjusted to 100ml. Absorbance was measured at 460 nm with a double beam Bausch and Lomb Spectronic 2000 spectrophotometer (Bausch and Lomb, Rochester, NY). To quantitate total carotenoids in the samples containing oleoresin paprika, an experimentally determined absorptivity at 1% in acetone of 1922 was used (Fisher, 1993, personal communication). An absorptivity value of 1900 was used to calculate the total carotenoid concentrations in samples containing canthaxanthin (Skrede et al., 1989).

High performance liquid chromatographic analysis of carotenoids

Sample preparation: To the carotenoid extract described previously, an internal standard, β-apo-8´-carotenal (20mg/L, w/v) was added. The carotenoid extract was evaporated to dryness in a rotary evaporator (BÜchi Rotavapor, Postfach, Switzerland). The contents were redissolved in hexane (2ml) and passed through a prewetted Superclean™ LC-Si SPE tube (Supelco Inc., Bellefonte, PA) filled with 1g silica packing material. A flow rate of 1.0ml/min was maintained using a Visiprep solid phase extraction vacuum manifold (Supelco Inc., Bellefonte, PA). The triglycerides were removed from the tube using 8ml hexane, while the carotenoids were eluted with four 1ml aliquots of acetone. The combined acetone eluates were concentrated under nitrogen and the carotenoids redissolved in 200μl acetone.

Carotenoid analysis: A Hitachi liquid chromatograph (Model 655A-11, EM Science, Gibbstown, NJ) equipped with a Hitachi controller (Model L-5000LC, EM Science), a Waters 712 WISP pump (Waters Associates, Milford, MA) and a 250mm x 4.6mm (i.d.) Supelcosil LC 18™ column (Supelco Inc.) was used. The separation of carotenoids was achieved by solvent gradient elution at a flow rate of 1 ml/min. Eluant A contained acetone and methanol (75:25 v/v), while eluant B was composed of acetone:water (75:25 v/v). The gradient was programmed as follows: 0% A increased to 65% in 10 min, then to 80% A in 30 min, and finally, to 100% A in 60 min.

A Waters Model 990 photodiode array detector was used to record the absorbance (440 to 550nm) and the retention time of each peak as the carotenoid was eluted. The identification of the major carotenoids was based on the spectra and the elution order of these compounds (Fisher and Kocis, 1987).

Color measurement

Color changes in the fish muscle during frozen storage were monitored by recording $L^*$, $a^*$, and $b^*$ values using a HunterLab optical sensor colorimeter (Model D25-PC2Δ, Hunter Associates Laboratory, Inc., Reston, VA). Two measurements were made on each sample by rotating 90° between each measurement. The mean of these readings was reported. From the determined $a^*$, and $b^*$ values, the hue ($H^\circ_{ab}=\tan^{-1} b^*/a^*$; $H^\circ_{ab}$= 0° for red and 90° for yellow) and the chroma [$C^*_{ab}=(a^{*2} + b^{*2})^{1/2}$] values were calculated (Francis and Clydesdale, 1975).

Determination of α-tocopherol concentrations in fish muscle

Extraction of α-tocopherol: The α-tocopherol in fish muscle was extracted using the method of Buttriss and Diplock (1984). The muscle sample (5g) was homogenized with distilled water (15ml) using a polytron homogenizer (Kinematica AG, Littau, Switzerland). A 1ml aliquot of the sample homogenate and 2ml 1% ethanolic pyrogallol were added to a screw cap test tube. The sample was saponified by blending with 0.3ml 50% potassium hydroxide and holding at 70°C in a water bath for 30 min. On cooling, 4ml hexane (containing 0.005% butylated hydroxy-

toluene, as an antioxidant) were added to the sample and the contents of the test tube shaken vigorously for 1 min. The hexane layer was removed and the extraction procedure was repeated two more times with 2ml aliquots of hexane. The extracts were pooled and 200μl of an α-tocopheryl acetate solution (100μg/ml ethanol) was added as an internal standard in order to correct the results for recovery. The contents were evaporated to dryness under nitrogen. The residue obtained was redissolved in 200μl ethanol.

The recovery rates were established by adding 200μl of a solution of α-tocopherol (100ng/μl) into freshly homogenized muscle samples before extraction. The resulting peak area on the chromatograms were compared with the peak area obtained by the direct injection of 10 μl of a standard ethanolic α-tocopherol solution (100ng/μl) onto the column. The average recovery of α-tocopherol in muscle samples was 88.9% with a coefficient of variation (CV) of 3.7%. The recovery experiment was repeated three times and each extract was analyzed in duplicate.

Quantitation of α-tocopherol by high performance liquid chromatography: A high performance liquid chromatograph (Model 501, Water Associates, Milford, MA) equipped with dual pumps and a 150mm x 3.9mm Symmetry™ $C_8$ reverse phase column (Water Associates), 5μ particle size, was used to determine the concentration of α-tocopherol. A Sentry™ integrated guard column (Waters Associates) was attached to the column. A

mobile phase containing acetonitrile, methanol and water (25: 25:1 v/v/v) with a flow rate of 1.0ml/min was used. Detection was achieved with an absorbance detector (Model 486, Waters Associates) set at 280 nm. Chromatograms were recorded on a printer (Model Pinwriter P5200, NEC Information Systems, Inc. Boxborough, MA). The quantitation was done using a standard curve generated by injecting known amounts of an α-tocopherol standard (5ng-120ng/μl).

Extraction of lipids

Lipids were extracted using the dry column method of Marmer and Maxwell (1981). A dichloromethane and methanol (9:1 v/v) solvent system was used to elute the lipids and the solvent was evaporated to dryness on a rotary evaporator. The lipids were redissolved in hexane containing 0.005% BHT and transferred to tared vials. Hexane was evaporated under nitrogen and the vials were capped after flushing with nitrogen. Samples were stored at -20°C until required.

Preparation of fatty acid methyl esters

Methyl esters of the total lipids were prepared following the boron trifluoride-methanol method of Morrison and Smith (1964).

Gas chromatographic analysis of fatty acid methyl esters

Fatty acid methyl esters were separated and quantitated using a Hewlett Packard gas chromatograph (Model 5890A,

Hewlett Packard, Avondale, PA) equipped with a flame ionization detector and a fused silica capillary column (DB-225, J & W Scientific, Folsom, CA) with a length of 30m and 0.25mm inside diameter. Helium was used as the carrier gas and a split ratio of 20:1 was maintained. The temperature of the gas chromatograph was programmed from 175°C (10 min) to 200°C at a rate of 1.5°C/min and held for 47 min. The injection port and detector temperatures were 275°C and 300°C, respectively.

Identification of the fatty acid methyl esters was based on comparison of the retention times of samples with those of standard fatty acid methyl esters (Supelco Inc.). The peak area of each fatty acid was computed by an integrator (Model 3392A) and reported as relative area percent of the total fatty acid methyl esters.

Statistical Analysis

The data were analyzed using a split-plot design with factors A (treatment) and B (replication) applied to the whole plot (fish in rearing tanks) and factors C (storage time) and D (surface application of oleoresin rosemary) to the sub-plot (fish during processing and storage). Analysis of variance (ANOVA) for data was calculated using the MSTATC microcomputer program (Michigan State University, 1991). Tukey's test was applied to separate the mean values.

## RESULTS AND DISCUSSION

### Change in α-tocopherol concentrations during storage

The concentrations of α-tocopherol in the muscle of rainbow trout from all dietary groups decreased 48% or more during 6 months of frozen storage (Table 1). Statistical analysis showed a significant ($p<0.01$) effect of dietary treatment and storage time on α-tocopherol concentrations.

The α-tocopherol concentrations of fish fed the lower level of α-tocopheryl acetate (diets I and II, 100mg/kg) decreased from the initial range of 10.5-10.6mg/kg of muscle to 4.2-5.5mg/kg at the expiration of the storage study, a 54.1% reduction. Similarly, the α-tocopherol concentrations in fish fed the higher level of α-tocopheryl acetate (diets III, IV and V, 500mg/kg) decreased from the range of 54.1-57.1 to 21.9-24.5mg/kg, a 58.2% reduction. These results indicated that α-tocopherol concentrations decreased at the same rate regardless of the 5-fold difference in concentrations of α-tocopherol in the diets. The decrease in α-tocopherol concentration during frozen storage may be due to its role as free radical chain terminator or oxygen scavenger.

These results closely agree with those reported by Pozo et al. (1988) who reported that α-tocopherol concentrations in the white and dark muscle of rainbow trout decreased markedly (66% and 68%, respectively) during frozen storage for 7 months. They showed that α-tocopherol concentrations were well maintained during the initial 45 days of storage at -12°C and

Table 1. Change in α-tocopherol concentrations in rainbow trout muscle during storage at -20°C.

| Dietary Treatment | α-tocopherol concentrations (mg/kg) 0 Month | 6 Month |
|---|---|---|
| I | 10.6 ± 0.7[b] | 5.5 ± 1.7[bc] |
| II | 10.5 ± 1.1[b] | 4.2 ± 0.3[c] |
| III | 54.1 ± 8.5[a] | 23.6 ± 2.9[ab] |
| IV | 56.3 ± 9.9[a] | 24.5 ± 8.1[a] |
| V | 57.1 ± 6.5[a] | 21.9 ± 6.8[abc] |

All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

Diet I : Commercial control diet (supplemented with 100mg α-tocopheryl acetate/kg feed).

Diet II : Commercial canthaxanthin diet (supplemented with 40mg canthaxanthin and 100mg α-tocopheryl acetate/kg feed).

Diet III: Supplemented diet I ( Supplemented with 15mg canthaxanthin and 500mg α-tocopheryl acetate/kg feed).

Diet IV : Supplemented diet II (supplemented with 175mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

Diet V : Supplemented diet III (supplemented with 350mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

[a,b,c] Means in the same column bearing the same superscript are not significantly different ($p>0.05$).

for 180 days at -30°C. Thereafter, significant decreases ($p<0.01$) in α-tocopherol concentrations took place.

On the other hand, WaagbØ et al. (1993) observed that α-tocopherol concentrations in Atlantic salmon muscle were not affected during frozen storage at -18°C for 5 weeks. The shorter storage time may be the reason for the better retention of α-tocopherol in contrast to that achieved in the present longer storage study.

Effect of dietary vitamin E supplementation on lipid stability of fish muscle

The TBARS in all samples, with and without surface application of oleoresin rosemary, increased with storage time (Table 2). Storage time and surface application of oleoresin rosemary had a highly significant ($p<0.01$) effect on TBARS numbers. Analysis of variance showed a significant ($p<0.01$) interaction between dietary treatment and surface application. A significant ($p<0.01$) interaction between storage time and surface application was also noticed.

The TBARS numbers of muscle samples of fish fed diets containing the higher level of α-tocopherol (diets III, IV and V) were smaller (14.8%-35.2%) than those in fish fed the control diet. The lowest TBARS numbers (average 32.8% less compared to the control) were found in fish fed diets containing oleoresin paprika (diets IV and V).

The beneficial effect of dietary α-tocopherol supplementation on fish lipid stability is well established.

Table 2. Effect of dietary supplementation and surface application of oleoresin rosemary on the oxidative stability (as measured by TBARS) of rainbow trout fillets during storage at -20°C.

| Dietary[1] Treatment | Storage time, Months 0 | 2 | 4 | 6 |
|---|---|---|---|---|
| **Samples without surface application** | | | | |
| I | 0.21±0.04$^{a}$ | 1.83±0.58$^{a}$ | 2.40±0.35$^{ab}$ | 4.12±0.66$^{a}$ |
| II | 0.18±0.02$^{a}$ | 0.59±0.24$^{ab}$ | 2.26±1.22$^{abc}$ | 3.16±1.49$^{abc}$ |
| III | 0.16±0.02$^{a}$ | 0.70±0.26$^{ab}$ | 2.72±0.26$^{a}$ | 3.51±0.55$^{ab}$ |
| IV | 0.12±0.02$^{a}$ | 1.31±0.32$^{ab}$ | 1.94±0.33$^{abc}$ | 2.67±0.21$^{bcd}$ |
| V | 0.14±0.02$^{a}$ | 1.24±0.41$^{ab}$ | 2.21±0.56$^{abc}$ | 2.87±0.60$^{abcd}$ |
| **Samples with surface application** | | | | |
| I | 0.09±0.01$^{a}$ | 0.70±0.15$^{ab}$ | 1.15±0.23$^{bc}$ | 0.86±0.04$^{f}$ |
| II | 0.07±0.02$^{a}$ | 0.48±0.07$^{b}$ | 1.74±0.67$^{abc}$ | 2.59±0.47$^{bcde}$ |
| III | 0.09±0.02$^{a}$ | 0.56±0.24$^{ab}$ | 1.58±0.44$^{abc}$ | 2.11±0.63$^{cdef}$ |
| IV | 0.11±0.01$^{a}$ | 0.49±0.06$^{b}$ | 1.11±0.45$^{c}$ | 1.65±0.45$^{def}$ |
| V | 0.13±0.02$^{a}$ | 0.58±0.17$^{ab}$ | 1.15±0.08$^{bc}$ | 1.36±0.29$^{ef}$ |

TBARS are the mean values (± standard deviation) of three replications and each replication was analyzed in duplicate. Results are expressed as mg malonaldehyde per kg sample.

[1] See Table 1 for description of dietary treatments.

$^{a,b,c,d,e,f}$ Means in the same column bearing same superscript are not significantly different ($p>0.05$).

O'Keefe and Noble (1978) found that an α-tocopherol concentration of 200 mg/kg protected channel catfish (*Ictalurus punctatus*) against lipid oxidation during frozen storage (-10°C) for 3 months. Comparison of their observations with those of the present study clearly indicated that dietary supplementation provided more stability to channel catfish lipids. This discrepancy may be due to the difference in efficiency of depositing dietary α-tocopherol in the muscle by these species (Channel catfish vs rainbow trout). Such a comparison is not possible as O'Keefe and Noble (1978) did not report α-tocopherol concentrations in muscle from channel catfish. Pozo et al. (1988) observed that peroxide values of white and dark muscle of rainbow trout fed diets containing 500 mg α-tocopherol (fish were fed the supplemented diet for 2 months) increased during storage at -12°C and -30°C for 7 months. He and his co-workers observed a slight but significant ($p<0.01$) reduction in peroxide values of dark muscle lipids from rainbow trout fed the supplemented diet. The peroxide values of white muscle of fish fed the supplemented diet were not significantly different from those of the control group. It was concluded that the concentration of α-tocopherol in the muscle (5.9±2.1μg/g wet white muscle; 34.6±10.6μg/g wet dark muscle) of trout fed the basal diet was sufficient to give maximum protection.

A number of studies have established that lipid oxidation in rainbow trout during frozen storage is not inhibited by supranutritional levels of α-tocopherol. Hung and Slinger

(1982) reported TBARS numbers of 3.3±0.4, 3.4±0.5 and 3.7±0.6 in frozen muscle (1 year at -17°C) of rainbow trout fed experimental diets containing 43, 111 and 119mg/kg vitamin E for 6 months. Fish fed a diet containing 769mg dietary vitamin E/kg had a TBARS number of 3.1±0.2. Similarly, Boggio et al. (1985) reported that the oxidative stability of rainbow trout fed diets (4 months prior to harvest) containing 500 and 1500 mg α-tocopheryl acetate were not significantly different ($p<0.05$) after frozen storage at -20°C for 10 months. However, a significant inhibitory effect of vitamin E supplementation on lipid oxidation was observed in fish samples kept at -80°C for 4 months.

There are number of factors which could possibly influence the lipid-stabilizing functions of dietary α-tocopherol during storage. Muscle and muscle membrane concentrations of α-tocopherol, the degree of unsaturation of fatty acids in the muscle and muscle membranes, storage temperature, packaging, and the length of storage could all play a vital role. The degradation of α-tocopherol during frozen storage, as reported previously, may significantly contribute to the oxidation of lipids during the frozen storage of fish. Hsieh et al. (1988) reported high activity (60%) of the lipoxygenase enzyme at 0°C. The activity of lipoxygenase near freezing temperatures may also contribute to lipid oxidation during frozen storage. The effectiveness of α-tocopherol towards lipoxygenase activity needs to be explored further.

Results clearly demonstrate the beneficial effect of surface application of oleoresin rosemary on the oxidative stability of rainbow trout muscle lipids during frozen storage. Surface application of oleoresin rosemary further enhanced the protective effect of α-tocopherol supplementation and the extent of lipid oxidation was significantly ($p<0.05$) reduced in all dietary groups. The TBARS numbers of muscle samples from fish fed diets I and II were 0.86mg MDA/kg and 2.59mg MDA/kg, respectively after 6 months of storage. The TBARS numbers of muscle samples from fish fed diets III, IV and V were 2.11mg MDA/kg, 1.65 mgMDA/kg and 1.36mg MDA/kg, respectively. Muscle samples containing oleoresin paprika were well protected against lipid oxidation and this inhibitory effect need to be explored further.

Synergism between oleoresin rosemary and α-tocopherol has been reported. Fang and Wada (1993) monitored lipid oxidation in the dark muscle of bonito fish (*Katsunoonus pelamis*) stored under refrigerated conditions. The mixture of α-tocopherol and rosemary extract (0.035% + 0.035%) produced a significantly ($p<0.05$) stronger antioxidant effect than α-tocopherol itself.

Changes in carotenoid concentrations during storage

Carotenoid concentrations decreased in fillets from all dietary treatments during frozen storage (Table 3). A highly significant ($p<0.01$) effect of surface application of oleoresin rosemary, dietary treatments and storage time on carotenoid concentrations was observed. A significant ($p<0.01$)

Table 3. Changes in total carotenoid concentrations in rainbow trout muscle during storage at -20°C.

| Dietary[1] Treatment | Total carotenoid concentrations (mg/Kg)[2] | | |
|---|---|---|---|
| | Month 0 | 6 Month | |
| | | Undipped[3] | Dipped[3] |
| I | 1.5±0.2[a] | 0.7±0.1[b] | 1.2±0.1[a] |
| II | 7.9±1.3[a] | 5.5±0.4[c] | 6.6±0.2[b] |
| III | 3.8±0.2[a] | 2.0±0.3[c] | 3.2±0.1[b] |
| IV | 2.4±0.1[a] | 1.5±0.2[c] | 2.0±0.1[b] |
| V | 3.1±0.2[a] | 1.5±0.1[c] | 2.2±0.1[b] |

[1] See Table 1 for description of dietary treatments.

[2] All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[3] Undipped; Dipped-oleoresin rosemary treated.

[a,b,c] Means in the same row bearing the same superscript are not significantly different ($p>0.05$).

interaction between dietary treatment and storage time indicated that both these variables were dependent on each other. A highly significant ($p<0.01$) interaction between storage time and surface application of oleoresin rosemary was also observed.

Carotenoid concentrations in fish fed control and commercial canthaxanthin diets decreased by 55.0% and 30.5%, respectively, during the storage period. Degradation of carotenoids in fish fed diets III, IV and V ranged from 37.6% to 51.3%. Carotenoid concentrations in fish fed control and commercial canthaxanthin diet decreased less (20.8% and 17.2%, respectively) after surface application of oleoresin rosemary. Degradation of carotenoids in fish fed diets III, IV and V was also reduced (15.5% to 31.1%.).

These results are consistent with other studies which demonstrated the degradation of carotenoids in fish flesh during storage. Chen et al. (1984) reported that 53% of the carotenoid (astaxanthin) in rainbow trout muscle degraded during frozen storage (-20°C) within 14 days. Pozo et al. (1988) observed that canthaxanthin faded more rapidly during storage at -12°C for 7 months then at -30°C. Supplementation with dietary α-tocopherol did not protect the pigment from fading during frozen storage. Ingemansson et al. (1993) reported that the carotenoid (astaxanthin) concentrations in both light and dark muscle of farmed rainbow trout fed α-tocopherol-supplemented and control diets decreased during frozen storage.

In contrast, No and Storebakken (1991) showed that astaxanthin and canthaxanthin were stable (a maximum 5% loss) in rainbow trout (*Oncorhynchus mykiss*) fillets during storage at -20°C or -80°C, regardless of carotenoid source and rearing conditions.

The degradation of paprika carotenoids may be due to the action of lipoxygenase as Biacs et al. (1989) observed that red xanthophylls are susceptible to oxidative processes such as lipoxygenase-catalyzed linoleic acid oxidation. Tsukuda and Amano (1967) reported that a lipoxygenase-type enzyme present in skin tissue homogenates of rockfish (*Sebastes thompsoni*) and guarnard (*Chelidonichthys kumu*) could convert astaxanthin, tunaxanthin and β-carotene into colorless compounds at refrigerated temperatures. Stone and Kinsella (1989) reported the bleaching of β-carotene by rainbow trout gill lipoxygenase using polyunsaturated fatty acids as a substrate.

Change in carotenoid composition during storage

Changes in the relative percentages of the individual carotenoids in fish muscle as affected by frozen storage were observed (Tables 4, 5 and 6). Carotenoids such as cis-capsanthin, zeaxanthin and β-cryptoxanthin degraded in muscle samples of rainbow trout fed the control diet. Degradation of capsanthin was detected in fish fed canthaxanthin-supplemented diets (diets II and III). In fish fed oleoresin paprika-supplemented diets (diets IV and V), degradation of cis-capsanthin and β-cryptoxanthin was observed. The surface

Table 4. carotenoid composition of rainbow trout at the time of slaughter and immediately before storage at -20°C.

| Carotenoids | Relative area percents of absorbance at 460 nm | | | | |
|---|---|---|---|---|---|
| | Diet I[1] | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | 22.01±7.36 | 2.54±2.04 | 3.88±2.54 | 35.44±1.39 | 40.72±0.67 |
| Cis-capsanthin | 3.96±3.96 | 0.18±0.11 | 0.11±0.08 | 7.48±1.41 | 10.57±2.42 |
| Zeaxanthin | 16.66±5.26 | 0.02±0.02 | ND | 8.96±4.53 | 5.45±4.28 |
| Cis-capsanthin/Lutein | ND | 0.32±0.32 | 0.26±0.21 | 9.32±6.82 | 15.68±2.02 |
| Antheraxanthin | 0.51±0.01 | 0.59±0.32 | 1.00±0.90 | 0.68±0.28 | 0.82±0.05 |
| Canthaxanthin | 50.00±4.02 | 95.42±3.50 | 93.80±3.69 | 37.22±4.64 | 25.87±5.38 |
| β-cryptoxanthin | 6.86±5.20 | 0.94±0.65 | 0.95±0.54 | 0.90±0.17 | 0.89±0.86 |
| β-carotene | ND | ND | ND | ND | ND |

All values represent the mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

Table 5. Carotenoid composition of rainbow trout muscle after six months of storage at -20°C.

| Carotenoids | Relative area percents of absorbance at 460 nm | | | | |
|---|---|---|---|---|---|
| | Diet I[1] | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | 24.68±04.59 | 0.16±0.15 | 0.18±0.17 | 38.53±1.81 | 48.48±2.13 |
| Cis-capsanthin | 12.28±12.27 | 1.45±1.40 | 0.10±0.10 | ND | ND |
| Zeaxanthin | ND | 1.06±0.99 | ND | 10.12±1.35 | 9.20±1.29 |
| Cis-capsanthin/Lutein | 7.60±07.59 | ND | 0.42±0.31 | 10.57±1.47 | 13.99±0.29 |
| Antheraxanthin | 20.53±20.52 | 0.14±0.10 | ND | ND | ND |
| Canthaxanthin | 38.73±16.64 | 96.49±3.00 | 98.36±0.67 | 40.78±4.06 | 28.33±1.91 |
| β-cryptoxanthin | ND | 0.40±0.34 | 0.95±0.63 | ND | ND |
| β-carotene | ND | 0.30±0.28 | ND | ND | ND |

All values represent the mean (± standard deviation) of three replicates analyzed in duplications and each replication was analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

Table 6. Carotenoid composition of rainbow trout muscle dipped in oleoresin rosemary after six months of storage at -20°C.

| Carotenoids | Relative area percents of absorbance at 460 nm | | | | |
|---|---|---|---|---|---|
| | Diet I[1] | Diet II | Diet III | Diet IV | Diet V |
| Capsanthin | 40.53±4.81 | 0.11±0.07 | 0.23±0.22 | 44.15±2.96 | 51.19±0.90 |
| Cis-capsanthin | ND | ND | ND | ND | ND |
| Zeaxanthin | ND | 0.51±0.21 | 1.33±1.32 | 9.78±1.57 | 9.45±1.10 |
| Cis-capsanthin/Lutein | ND | ND | ND | 10.92±1.21 | 13.66±3.27 |
| Antheraxanthin | ND | ND | ND | 0.56±0.55 | 0.87±0.71 |
| Canthaxanthin | 59.47±4.81 | 98.26±0.68 | 94.52±1.87 | 31.99±4.39 | 24.56±4.90 |
| β-cryptoxanthin | ND | 0.60±0.10 | 0.60±0.44 | 2.60±0.77 | 0.27±0.26 |
| β-carotene | ND | 0.52±0.40 | 3.32±2.96 | ND | ND |

All values represent the mean (± standard deviation) of three replicates analyzed in duplications and each replication was analyzed in duplicate.

[1] See Table 1 for description of dietary treatments.

application of oleoresin rosemary did not provide any protective effect.

There are some literature reports pertaining to the effect of lipoxygenase activity on carotenoid stability in fish (Tsukuda, 1970). Eskin et al. (1977) reported that lipoxygenase had carotenoid bleaching activity through its aerobic and anaerobic pathways. Stone and Kinsella (1989) speculated that the postmortem release of 12-lipoxygenase from skin tissue of salmon and trout during handling may contribute to their discoloration by bleaching of carotenoids. Biacs et al. (1989) reported that monoesters and diesters of capsanthin and capsorubin were more stable towards lipoxygenase-catalyzed linoleic acid oxidation than were the free pigments. Capsanthin esters containing saturated fatty acids tended to resist enzymic oxidation.

Change in color characteristics during storage

Results indicate that lightness ($L^*$), redness ($a^*$), yellowness ($b^*$) and chroma decreased and hue angle increased during frozen storage in all dietary groups (Tables 7 and 8). A significant ($P<0.05$) effect of dietary treatment and storage time on color attributes was observed. Analysis of variance indicated that the effect of both these variables was independent of each other. There was little protective effect of the supranutritional level of α-tocopherol supplementation (500mg/kg) on color stability. However, a combination of oleoresin rosemary and α-tocopherol provided stability to

Table 7. Change in color characteristics of rainbow trout muscle stored under frozen conditions.

| Storage (Months) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | L-value | 34.5±0.3$^{a}$ | 31.2±0.7$^{d}$ | 32.0±0.3$^{cd}$ | 33.6±0.2$^{ab}$ | 32.9±0.4$^{bc}$ |
| 2 | | 34.0±0.1$^{a}$ | 30.9±1.4$^{c}$ | 31.8±0.8$^{bc}$ | 32.9±0.6$^{ab}$ | 32.5±0.9$^{b}$ |
| 4 | | 32.2±0.2$^{a}$ | 29.9±1.2$^{b}$ | 31.7±0.8$^{a}$ | 32.0±0.6$^{a}$ | 31.4±0.4$^{a}$ |
| 6 | | 32.5±0.1$^{a}$ | 30.7±0.9$^{b}$ | 32.1±0.9$^{a}$ | 32.3±0.7$^{a}$ | 31.8±0.5$^{ab}$ |
| 0 | a-value | 4.1±0.2$^{e}$ | 11.0±0.5$^{a}$ | 8.7±0.8$^{b}$ | 6.1±0.6$^{d}$ | 7.4±0.5$^{c}$ |
| 2 | | 3.5±0.3$^{d}$ | 9.1±0.7$^{a}$ | 8.4±0.9$^{a}$ | 5.0±1.2$^{c}$ | 7.1±0.6$^{b}$ |
| 4 | | 2.4±0.9$^{d}$ | 7.2±0.5$^{a}$ | 6.6±0.7$^{a}$ | 3.9±1.1$^{c}$ | 5.3±0.9$^{b}$ |
| 6 | | 2.3±0.4$^{c}$ | 5.6±0.6$^{a}$ | 5.3±1.2$^{a}$ | 3.6±0.4$^{b}$ | 4.8±0.7$^{a}$ |
| 0 | b-value | 8.7±0.3$^{c}$ | 9.3±0.4$^{bc}$ | 10.3±0.4$^{a}$ | 9.9±0.3$^{ab}$ | 9.9±0.3$^{ab}$ |
| 2 | | 8.6±0.4$^{b}$ | 9.2±0.5$^{ab}$ | 10.0±0.2$^{a}$ | 9.6±0.5$^{a}$ | 9.7±0.5$^{a}$ |
| 4 | | 8.5±0.4$^{bc}$ | 8.2±0.7$^{c}$ | 9.4±0.3$^{a}$ | 9.1±0.2$^{ab}$ | 9.3±0.6$^{ab}$ |
| 6 | | 8.7±0.2$^{ab}$ | 8.5±0.4$^{b}$ | 9.5±0.4$^{a}$ | 9.1±0.6$^{ab}$ | 9.1±0.6$^{ab}$ |
| 0 | Hue angle | 64.8±1.3$^{a}$ | 40.3±1.6$^{d}$ | 49.9±2.8$^{c}$ | 58.6±3.1$^{b}$ | 53.4±1.1$^{bc}$ |
| 2 | | 67.6±2.2$^{a}$ | 45.2±3.3$^{c}$ | 49.9±2.7$^{bc}$ | 62.9±6.3$^{a}$ | 53.7±1.0$^{b}$ |
| 4 | | 74.5±5.9$^{a}$ | 48.7±3.1$^{d}$ | 54.9±2.3$^{c}$ | 66.9±5.5$^{b}$ | 60.5±2.3$^{c}$ |
| 6 | | 75.4±2.1$^{a}$ | 56.9±1.6$^{c}$ | 61.2±5.2$^{c}$ | 68.5±1.0$^{b}$ | 62.6±1.6$^{bc}$ |

"Table 7 (cont'd)"

| Storage (Months) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | Chroma | 9.7±0.3$^{d}$ | 14.5±0.4$^{a}$ | 13.4±0.6$^{b}$ | 11.7±0.3$^{c}$ | 12.4±0.5$^{c}$ |
| 2 | | 9.3±0.4$^{d}$ | 13.0±0.5$^{a}$ | 13.1±0.6$^{a}$ | 10.9±0.5$^{c}$ | 12.0±0.8$^{b}$ |
| 4 | | 8.8±0.4$^{c}$ | 10.9±0.6$^{a}$ | 11.4±0.7$^{a}$ | 9.9±0.5$^{b}$ | 10.8±0.9$^{a}$ |
| 6 | | 9.0±0.2$^{c}$ | 10.2±0.7$^{ab}$ | 11.0±0.8$^{a}$ | 9.8±0.7$^{bc}$ | 9.9±1.2$^{b}$ |

[1] All values represent mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

$^{a,b,c,d,e}$ Means in the same row bearing same superscript are not significantly different ($p>0.05$).

Table 8. Effect of surface application of oleoresin rosemary on color values of rainbow trout muscle stored under frozen conditions.

| Storage (Months) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | L-value | 34.6±0.2$^{a}$ | 31.9±1.3$^{b}$ | 34.3±0.4$^{a}$ | 34.1±0.8$^{a}$ | 32.9±0.9$^{ab}$ |
| 2 | | 34.4±0.3$^{a}$ | 31.7±1.4$^{c}$ | 33.9±0.6$^{ab}$ | 33.8±0.9$^{ab}$ | 32.5±1.1$^{bc}$ |
| 4 | | 34.1±0.4$^{a}$ | 30.9±1.4$^{c}$ | 33.1±0.7$^{ab}$ | 33.4±1.2$^{ab}$ | 32.0±1.1$^{bc}$ |
| 6 | | 34.0±0.7$^{a}$ | 31.3±1.4$^{b}$ | 33.6±0.8$^{a}$ | 32.8±0.2$^{ab}$ | 32.4±0.9$^{ab}$ |
| 0 | a-value | 2.9±0.6$^{d}$ | 9.5±0.7$^{a}$ | 8.4±0.5$^{ab}$ | 6.4±0.9$^{c}$ | 7.1±0.3$^{bc}$ |
| 2 | | 2.7±0.9$^{d}$ | 9.2±0.7$^{a}$ | 8.0±0.9$^{ab}$ | 6.1±1.0$^{c}$ | 6.7±0.6$^{bc}$ |
| 4 | | 2.0±0.3$^{b}$ | 6.8±0.6$^{a}$ | 6.7±0.9$^{a}$ | 5.4±1.0$^{a}$ | 6.0±0.8$^{a}$ |
| 6 | | 1.4±0.2$^{c}$ | 6.2±0.8$^{a}$ | 6.3±1.1$^{a}$ | 4.4±0.8$^{b}$ | 5.1±0.7$^{ab}$ |
| 0 | b-value | 8.6±0.3$^{b}$ | 10.3±0.5$^{a}$ | 10.8±0.7$^{a}$ | 10.5±0.9$^{a}$ | 10.4±0.2$^{a}$ |
| 2 | | 8.5±0.2$^{b}$ | 9.9±0.5$^{ab}$ | 10.7±0.8$^{a}$ | 10.3±1.0$^{a}$ | 10.1±0.4$^{a}$ |
| 4 | | 8.3±0.4$^{b}$ | 9.0±0.5$^{ab}$ | 10.2±0.8$^{a}$ | 10.0±0.8$^{a}$ | 10.1±0.7$^{a}$ |
| 6 | | 8.5±0.4$^{c}$ | 9.0±0.4$^{bc}$ | 10.7±0.9$^{a}$ | 10.4±0.7$^{ab}$ | 10.5±0.5$^{ab}$ |
| 0 | Hue angle | 71.4±4.4$^{a}$ | 46.9±1.4$^{d}$ | 52.2±0.2$^{cd}$ | 58.7±1.7$^{b}$ | 55.8±1.4$^{bc}$ |
| 2 | | 72.3±5.7$^{a}$ | 47.0±1.5$^{d}$ | 53.2±2.0$^{c}$ | 59.7±1.8$^{b}$ | 56.5±2.1$^{bc}$ |
| 4 | | 76.3±2.6$^{a}$ | 52.8±1.6$^{c}$ | 56.9±1.6$^{bc}$ | 61.9±2.7$^{b}$ | 59.6±3.1$^{b}$ |
| 6 | | 80.9±1.3$^{a}$ | 55.7±2.7$^{d}$ | 59.8±3.2$^{cd}$ | 67.2±2.4$^{b}$ | 64.3±2.4$^{bc}$ |

"Table 8 (cont'd)"

| Storage (Months) | Color[1] Parameter | Dietary treatments[2] I | II | III | IV | V |
|---|---|---|---|---|---|---|
| 0 | Chroma | 9.1±0.2[b] | 13.8±0.8[a] | 13.7±0.8[a] | 12.3±1.2[a] | 12.6±0.2[a] |
| 2 | | 9.0±0.0[b] | 13.5±0.8[a] | 13.3±1.1[a] | 12.0±1.3[a] | 12.1±0.6[a] |
| 4 | | 8.6±0.3[b] | 11.3±0.8[a] | 12.1±1.2[a] | 11.4±1.1[a] | 11.7±0.9[a] |
| 6 | | 8.6±0.4[b] | 10.9±0.8[a] | 12.4±1.3[a] | 11.3±1.0[a] | 11.6±0.6[a] |

[1] All values represent mean (± standard deviation) of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

[a,b,c,d] Means in the same row bearing same superscript are not significantly different ($p>0.05$).

redness ($a^*$), yellowness ($b^*$) and chroma in fish fed the higher level of α-tocopherol.

According to Schmidt and Cuthbert (1969), the color of sockeye salmon (*Oncorhynchus nerka*) did not show appreciable changes after 105 days of frozen storage (-30°C). However, No and Storebakken (1991) reported that the storage of vacuum packaged-rainbow trout fillets at -20°C and -80°C resulted in increased lightness ($L^*$), redness ($a^*$) and yellowness ($b^*$) and decreased $H(^*)_{ab}$ (hue) values. They speculated that the change in color to a more reddish hue after the frozen storage and thawing of fillets may have been due to changes in light absorption and scattering caused by freeze denaturation of the protein of the trout fillet. Alternatively, the changes may have been caused by exposure of the carotenoids to a more polar, water-rich environment by the release of intracellular liquids. Anderson and Steinsholt (1992) also reported significant increases in color retention in salmon during frozen storage at -13°C and -35°C.

## Changes in fatty acid composition of fish muscle during storage

The results showed a decrease in relative area percentage for di- and polyunsaturated acids as a result of frozen storage (Tables 9, 10 and 11). The predominant saturated fatty acids were palmitic (C14:0), stearic (C16:0) and oleic (C18:0) acids. The predominant unsaturated fatty acids were palmitoleic (C16:1), linoleic (C18:1), linolenic (C18:2),

Table 9. Fatty acid profile of rainbow trout muscle before frozen storage.

| Fatty acid | Percent relative area[1] | | | | |
|---|---|---|---|---|---|
| | I[2] | II | III | IV | V |
| C 13:0 | 0.69 | 0.21 | - | 0.21 | - |
| C 14:0 | 5.05 | 5.10 | 5.53 | 4.79 | 4.61 |
| C 15:0,i | 0.15 | 0.17 | 0.18 | 0.12 | 0.14 |
| C 15:0 | 0.33 | 0.29 | 0.38 | 0.31 | 0.30 |
| C 16:0,i | 0.44 | 0.39 | 0.40 | 0.35 | 0.29 |
| C 16:0 | 24.81 | 22.68 | 24.34 | 21.88 | 20.49 |
| C 16:1 | 9.02 | 9.90 | 11.08 | 10.18 | 10.14 |
| C 17:0 | 0.17 | 0.17 | 0.16 | 0.15 | 0.16 |
| C 17:0 ▲ | 0.22 | 0.16 | 0.35 | 0.34 | 0.28 |
| C 18:0 | 3.34 | 3.36 | 2.94 | 2.74 | 3.59 |
| C 18:1 ω9 | 17.85 | 21.13 | 17.95 | 18.24 | 16.86 |
| C 18:1 ω7 | 2.91 | 2.73 | 2.85 | 2.74 | 2.97 |
| C 18:2 | 8.66 | 9.65 | 9.17 | 10.65 | 10.21 |
| C 18:3 ω6 | 0.32 | 0.33 | 0.36 | 0.38 | 0.35 |
| C 18:3 ω3 | 0.97 | 1.11 | 1.00 | 1.13 | 1.20 |
| C 19:0 ▲ | 1.59 | 1.00 | 1.25 | 1.53 | 1.17 |
| C 20:1 | 5.37 | 5.91 | 6.49 | 6.85 | 7.78 |
| C 18:4 ω3 | 0.34 | 0.30 | 0.42 | 0.45 | 0.61 |
| C 20:3 | 0.38 | 0.32 | 0.30 | 0.32 | 0.40 |
| C 20:4 | 0.45 | 0.32 | 0.32 | 0.33 | 0.26 |
| C 20:5 | 3.11 | 2.53 | 2.65 | 3.04 | 3.34 |
| C 22:1 ω11 | 4.23 | 4.86 | 4.76 | 4.97 | 5.14 |
| C 22:1 ω9 | 0.55 | 0.63 | 0.59 | 0.65 | 0.78 |
| C 22:6 ω3 | 9.05 | 6.75 | 6.53 | 7.65 | 8.93 |
| Saturated | 36.79 | 33.53 | 35.53 | 32.42 | 31.03 |
| Unsaturated | 63.21 | 66.47 | 64.47 | 67.58 | 68.97 |
| Monounsaturated | 39.93 | 45.16 | 43.72 | 43.63 | 43.67 |
| Diunsaturated | 8.66 | 9.65 | 9.17 | 10.65 | 10.21 |
| Polyunsaturated | 14.62 | 11.66 | 11.58 | 13.30 | 15.09 |

[1] Fatty acid relative area percentages are the mean values of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

Table 10. Fatty acid profile of rainbow trout muscle after six months of frozen storage.

| Fatty acid | Percent relative area[1] | | | | |
|---|---|---|---|---|---|
| | I[2] | II | III | IV | V |
| C 13:0 | 3.43 | 4.87 | 0.06 | 0.05 | 0.02 |
| C 14:0 | 4.90 | 5.71 | 5.51 | 5.12 | 4.94 |
| C 15:0,i | 0.04 | 0.09 | 0.15 | 0.13 | 0.09 |
| C 15:0 | 0.30 | 0.33 | 0.34 | 0.33 | 0.33 |
| C 16:0,i | 0.08 | 0.16 | 0.12 | 0.10 | 0.05 |
| C 16:0 | 25.36 | 24.45 | 22.27 | 20.70 | 24.50 |
| C 16:1 | 6.45 | 7.57 | 9.99 | 8.53 | 7.75 |
| C 17:0 | 0.14 | 0.14 | 0.16 | 0.15 | 0.14 |
| C 17:0 ▲ | 0.05 | 0.09 | 0.13 | 0.16 | 0.07 |
| C 18:0 | 3.95 | 3.39 | 3.42 | 3.07 | 3.60 |
| C 18:1 ω9 | 17.33 | 16.11 | 16.69 | 15.46 | 14.60 |
| C 18:1 ω7 | 2.99 | 2.67 | 2.84 | 2.90 | 2.70 |
| C 18:2 | 6.45 | 6.75 | 7.52 | 8.77 | 7.85 |
| C 18:3 ω6 | 0.28 | 0.33 | 0.34 | 0.32 | 0.30 |
| C 18:3 ω3 | 0.60 | 0.69 | 0.91 | 1.03 | 0.91 |
| C 19:0 ▲ | 0.85 | 0.63 | 0.83 | 0.98 | 0.73 |
| C 20:0 | 0.10 | 0.08 | 1.65 | 0.20 | 0.26 |
| C 20:1 | 7.42 | 7.68 | 9.04 | 10.24 | 9.03 |
| C 18:4 ω3 | 0.64 | 0.51 | 0.64 | 1.02 | 0.71 |
| C 20:3 | 0.61 | 0.44 | 0.38 | 0.48 | 0.51 |
| C 20:4 | 0.40 | 0.30 | 0.25 | 0.29 | 0.34 |
| C 20:5 | 2.25 | 2.20 | 1.82 | 2.57 | 2.70 |
| C 22:1 ω11 | 6.56 | 7.57 | 8.24 | 9.24 | 7.99 |
| C 22:1 ω9 | 0.79 | 0.78 | 0.96 | 1.10 | 0.96 |
| C 22:6 ω3 | 8.03 | 6.46 | 5.74 | 7.06 | 8.92 |
| Saturated | 39.20 | 39.94 | 34.64 | 30.99 | 34.73 |
| Unsaturated | 60.80 | 60.06 | 65.36 | 69.01 | 65.27 |
| Monounsaturated | 41.54 | 42.38 | 47.76 | 47.47 | 43.03 |
| Diunsaturated | 6.45 | 6.75 | 7.52 | 8.77 | 7.85 |
| Polyunsaturated | 12.81 | 10.93 | 10.08 | 12.77 | 14.39 |

[1] Fatty acid relative area percentages are the mean values of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

Table 11. Fatty acid profile of rainbow trout muscle dipped in oleoresin rosemary after six months of frozen storage.

| Fatty acid | Percent relative area[1] | | | | |
|---|---|---|---|---|---|
| | I[2] | II | III | IV | V |
| C 13:0 | 0.16 | 0.07 | - | - | 0.05 |
| C 14:0 | 4.75 | 5.57 | 5.34 | 5.53 | 5.06 |
| C 15:0,i | 0.12 | 0.15 | 0.15 | 0.16 | 0.14 |
| C 15:0 | 0.26 | 0.30 | 0.32 | 0.32 | 0.32 |
| C 16:0,i | 0.37 | 0.11 | 0.15 | 0.18 | 0.14 |
| C 16:0 | 21.65 | 20.99 | 21.62 | 20.42 | 20.04 |
| C 16:1 | 9.39 | 10.27 | 11.13 | 9.98 | 10.50 |
| C 17:0 | 0.30 | 0.24 | 0.15 | 0.22 | 0.15 |
| C 17:0 ▲ | 0.15 | 0.18 | 0.14 | 0.16 | 0.14 |
| C 18:0 | 3.55 | 2.99 | 3.36 | 2.82 | 2.95 |
| C 18:1 ω9 | 20.29 | 19.35 | 18.93 | 17.44 | 18.36 |
| C 18:1 ω7 | 2.93 | 2.78 | 2.87 | 2.80 | 2.87 |
| C 18:2 | 8.53 | 9.28 | 8.18 | 9.44 | 9.78 |
| C 18:3 ω6 | 0.32 | 0.31 | 0.32 | 0.30 | 0.33 |
| C 18:3 ω3 | 1.08 | 1.11 | 1.03 | 1.08 | 1.19 |
| C 19:0 ▲ | 1.62 | 1.03 | 0.93 | 1.10 | 1.04 |
| C 20:0 | 0.16 | 0.18 | 0.28 | 0.16 | 0.20 |
| C 20:1 | 7.00 | 8.09 | 8.56 | 9.14 | 8.98 |
| C 18:4 ω3 | 0.42 | 0.40 | 0.58 | 0.64 | 0.63 |
| C 20:3 | 0.42 | 0.38 | 0.32 | 0.39 | 0.43 |
| C 20:4 | 0.43 | 0.28 | 0.28 | 0.29 | 0.27 |
| C 20:5 | 3.01 | 2.52 | 2.27 | 2.73 | 2.53 |
| C 22:1 ω11 | 5.39 | 6.70 | 7.14 | 7.51 | 7.25 |
| C 22:1 ω9 | 0.68 | 0.73 | 0.85 | 0.90 | 0.83 |
| C 22:6 ω3 | 7.02 | 5.99 | 5.10 | 6.29 | 5.82 |
| Saturated | 33.09 | 31.58 | 32.44 | 31.07 | 30.23 |
| Unsaturated | 66.91 | 68.42 | 67.56 | 68.93 | 69.77 |
| Monounsaturated | 45.68 | 47.92 | 49.48 | 47.77 | 48.79 |
| Diunsaturated | 8.53 | 9.28 | 8.18 | 9.44 | 9.78 |
| Polyunsaturated | 12.70 | 10.99 | 9.90 | 11.72 | 11.20 |

[1] Fatty acid relative area percentages are the mean values of three replications and each replication was analyzed in duplicate.

[2] See Table 1 for description of dietary treatments.

eicosaenoic (C20:1), eicosapentaenoic (C20:5), docosaenoic (C22:1) and docosahexaenoic (C22:6) acids. The percent relative areas for mono-, di- and polyunsaturated fatty acids in fish muscle samples at the start of frozen storage ranged from 39.93 to 45.16%, 8.66 to 10.65% and 11.58 to 14.62%, respectively. After six months of storage, the percent relative areas of diunsaturated and polyunsaturated fatty acids decreased to 6.45 to 8.77% and 10.08 to 14.39%, respectively. Oleoresin rosemary provided little protection against the decomposition of diunsaturated fatty acids. The percent relative areas of di- and poly unsaturated fatty acids in fish samples subjected to surface application of oleoresin rosemary ranged from 8.53 to 9.78% and 9.90 to 12.70%, respectively.

The changes in fatty acid composition may have been caused by lipid hydrolysis which has been observed in the neutral and polar lipids during frozen and refrigerated storage (Hwang and Regenstein, 1988; de Koning and Mol, 1990; Ingemansson et al., 1995). Polvi and Ackman (1992) reported the hydrolysis of phospholipids and triglycerides to a moderate extent in muscle of Atlantic salmon fed diets containing 350 mg α-tocopheryl acetate/kg, during frozen storage (-12°C) for 3 months.

Ingemansson et al. (1995) also observed that the major cause of lipid deterioration in light and dark muscle of rainbow trout during frozen storage (-15°C) was hydrolysis. Hydrolysis increased the free fatty acid content by 1 to 10%.

The nutritionally important polyunsaturated n-3 fatty acids (eicosapentaenoic and docosahexaenoic acids) were noticeably increased. The phosphatidylcholine content of the muscle decreased by 25-50%.

In conclusion, supranutritional supplementation of α-tocopherol stabilized lipids during frozen storage. The combination of α-tocopherol with oleoresin rosemary not only inhibited lipid oxidation more efficiently but also protected flesh color. Results suggested that carotenoid breakdown proceeds lipid oxidation. These results points to the beneficial effects of dietary α-tocopherol and surface application of oleoresin rosemary lipid stability during the frozen storage of fish.

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## CHAPTER FIVE

## THE EFFECTS OF α-TOCOPHEROL AND OLEORESIN ROSEMARY ON LIPID OXIDATION AND CHOLESTEROL OXIDE FORMATION IN COOKED RAINBOW TROUT MUSCLE.

### ABSTRACT

The inhibitory effect of dietary α-tocopherol supplementation (100 and 500mg α-tocopheryl acetate/kg diet) and an oleoresin rosemary dip on lipid and cholesterol oxidation in cooked rainbow trout during refrigerated storage was investigated. The thiobarbituric acid-reactive substances (TBARS) numbers of cooked fish muscle increased during storage for all treatments. Dietary α-tocopherol supplementation partially inhibited lipid oxidation. Surface application of oleoresin rosemary further enhanced this protective effect. Cholesterol oxides were formed in all the samples as a result of cooking and storage. The major cholesterol oxidation products (COPS) were identified as 7β-hydroxycholesterol, α- and β-epoxides, and 7-ketocholesterol. Formation of COPS was also reduced by a supranutritional concentration of α-tocopherol and surface application of oleoresin rosemary. Statistical analysis revealed a highly significant ($p<0.01$) inhibitory effect of oleoresin rosemary on COPS formation.

## INTRODUCTION

Lipid oxidation is an important factor in the deterioration of the quality of meat and meat products. The processing of meat and meat products often results in increased oxidative deterioration. Cooking causes the disruption of cellular membranes and hence promotes lipid oxidation (Gray and Pearson, 1987). Oxidation also occurs rapidly during refrigerated storage of cooked meat. Under these conditions, cholesterol can readily undergo autoxidation and produce its oxidized derivatives. It has been suggested that certain cholesterol oxides may possibly cause the interruption of sterol metabolism and exhibit adverse biological effects associated with atherogenicity, cytotoxicity, mutagenicity and even carcinogenicity (Maerker, 1987; Hurrard et al., 1989). These concerns have intensified interest in investigating the extent of cholesterol oxidation in food products of animal origin.

The presence of cholesterol oxidation products (COPS) in various food and food products has been extensively reviewed (Smith, 1981; Finocchiaro and Richardson, 1983; Maerker, 1987; Naresh and Singhal, 1991; Boesinger et al., 1993; Paniangvait et al., 1995). Such compounds have been found in dairy products (Luby et al., 1986; Nourooz-zadeh and Appleqvist, 1988; Sanders et al., 1989; Chan et al., 1993), egg and egg products (Nourooz-zadeh and Appelqvist, 1987; Morgan and Armstrong, 1992; Lai et al., 1995) and meat and meat products

(Bastic et al., 1990; Zulbillaga and Maerker, 1991; Pie et al., 1991; Maerker and Jones, 1992; Monahan et al., 1992,1993; Hwang and Maerker, 1993; Engeseth et al., 1993,1994).

The presence of COPS in traditionally processed marine foods which are extensively consumed in Japan has been established (Osada et al., 1993; Oshima et al., 1993). Similarly, Chen and Yen (1994) reported the presence of COPS in small sun-dried fish (*Spratelloides gracillis* and *Decapterus maruodsi*), a traditional Chinese food. These findings raise questions about the potential safety of marine products which are normally considered beneficial for health.

In general, there are only limited studies on the formation of COPS in fish and marine products. Therefore, the objectives of this study were:

1. To study the effect of cooking and subsequent refrigerated storage on the formation of COPS in rainbow trout; and
2. To investigate the effectiveness of dietary α-tocopherol supplementation and the surface application of oleoresin rosemary in minimizing lipid and cholesterol oxidation.

## MATERIALS AND METHODS

### Reagents

6-Ketocholestanol (6-oxo-5-cholestan) was purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol oxide standards, 7α-hydroxycholesterol (5-cholestene-3β,7α-diol),

7β-hydroxycholesterol (5-cholestene-3β, 7β-diol), α-epoxide (5α,6α-epoxycholestan-3β-ol), β-epoxide (5β,6β-epoxycholestan-3β-ol), 7-ketocholesterol (7-oxo-5-cholesten-3β-ol), 20α-hydroxycholesterol (5-cholestene-3β,20α-diol) and 25-hydroxycholesterol (5-cholestene-3β,25-diol) and cholestane-3β, 5α6β-triol were purchased from Steraloids Inc. (Wilton, NH). Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA plus 1% TMCS) was obtained from Pierce Chemical Co. (Rockford, IL). Oleoresin rosemary (Herbalox seasoning type P) was donated by Kalsec Inc. (Kalamazoo, MI), and α-tocopheryl acetate was obtained from Hoffman La Roche (Basal, Switzerland). Other solvents were of analytical grade.

Diets and feeding trial

Rainbow trout (*Oncorhynchus mykiss*) were fed five different diets for a period of 7 months. The details of experimental design are described in Chapter 1.

The diets are as follows, the values cited being target levels:

Diet I : Commercial control diet (Martin Mills Inc., Elmira, ONT).

Diet II : Commercial canthaxanthin diet (Martin Mills Inc., Elmira, ONT).

Diet III : Diet supplemented with α-tocopheryl acetate (500mg /kg) and canthaxanthin (15 mg/kg).

Diet IV : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (175 mg/kg).

Diet V : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (350 mg/kg).

Diets I and II were commercial rainbow trout diets (floating 40% trout feed grower pellets) and contained 10% herring oil by weight. These diets also contained 100mg α-tocopheryl acetate/kg feed (target level). Diet I served as the control diet in this study. A commercial rainbow trout diet without added herring oil was also purchased from Martin Mills Inc. and used to prepare three supplemented diets (diets III, IV and V). The supplemented diets were prepared by adding α-tocopheryl acetate (500 mg/kg) to herring oil (10% based on the weight of the diet) which was carefully coated on the surface of the feed. Additionally, diet III and diets IV and V contained canthaxanthin (Martin Mills Inc.) and oleoresin paprika (Kalsec Inc.,Kalamazoo, MI), respectively. Diets III, IV and V were prepared by Kalsec Inc.

All fish were slaughtered according to standard commercial practices at the Aquaculture Research Laboratory, Michigan State University at the termination of the feeding trial. Fish were kept on ice and immediately transferred to the Meat Processing Laboratory, Michigan State University, for processing and packaging.

Samples preparation

The slaughtered fish were filletted and their skins were removed. One fillet from each fish was immediately immersed in a 2% solution of Herbalox seasoning type P, oleoresin rosemary

extract (Kalsec Inc., Kalamazoo, MI) in distilled water (w/v) until an approximate pick up of 1% was obtained. The gain in weight was measured by periodic weighing of the fillets after removing excess oleoresin rosemary solution from the surface of the fillets. The other fillet was used as a reference in order to determine the effect of surface application of oleoresin rosemary on lipid and cholesterol stability in cooked fish.

Cooking of samples

Rainbow trout fillets were individually wrapped in aluminum foil and cooked in an oven (Model 186C-2, Market Forge Co., Everette, MA) at 190°C until an internal temperature of 70°C was achieved. The internal temperature was determined using thermocouple thermometer (Model No. 660, Omega Engineering Inc., Stamford, CT).

Storage of samples

Six fish fillets from each treatment were placed individually on polystyrene trays containing adsorbent pads. The trays were overwrapped with a common oxygen-permeable meat stretch-wrap. The samples were kept refrigerated (4°C) for 2 days. Lipid oxidation and cholesterol oxidation measurements were conducted immediately after cooking and after 1 and 2 days of storage.

Measurement of lipid oxidation

The distillation procedure of Tarladgis et al.(1960), as modified by Crackel et al. (1988), was used to measure the extent of lipid oxidation. The thiobarbituric acid reactive-substances (TBARS) were determined spectrophotometrically at 532 nm and were expressed as mg malonaldehyde (MDA)/kg of sample.

Extraction, cleanup and analysis of cholesterol oxidation products

Extraction of lipids: Lipids were extracted using the dry column method of Marmer and Maxwell (1981). A dichloromethane: methanol (9:1 V/V) solvent system was used to elute the lipids and the solvent was evaporated to dryness on a rotary evaporator. The lipids were redissolved in hexane containing 0.005% butylated hydroxytoluene as an antioxidant and transferred to tared vials. Hexane was evaporated under nitrogen and the vials were capped after flushing with nitrogen. Samples were stored at -20°C until required.

Solid phase extraction: COPS in the lipid extract were isolated using a vacuum manifold (Supelco Inc., Bellefonte, PA) and a 3ml capacity Superclean™ LC-Si SPE tube (Supelco Inc.) filled with 300mg silica packing (40μm particles, 60 Å pore). The vacuum manifold was adjusted to provide a flow rate of 0.6±0.1ml/min using a 20kPa vacuum. The SPE tube was prewetted with 3ml hexane to activate the packing material before applying the sample. A 10μl 6-ketocholestanol

(1.5μg/μl) as an internal standard was added to the sample before the solid phase extraction procedure. After adding the sample, the following solvent combinations were applied to the SPE tube when approximately 1mm of the previous solvent remained above the top of the tube frit: 15ml hexane/ethyl ether (95:5, v/v), 30ml hexane/ethyl ether (90:10, v/v) and 20ml hexane/ethyl ether (80:20, v/v). Finally, acetone (5ml) was used to elute the COPS out of the column. The solvent was evaporated under nitrogen and the sample was redissolved in hexane (containing 0.005% butylated hydroxytoluene as an antioxidant). The samples were evaporated to dryness again before storage at -20°C.

Derivatization of COPS to TMS ethers: To form the trimethysilyl (TMS) ether derivatives, samples were derivatized in a 1/2 dram glass vial using 100μl bis (trimethylsilyl) trifluoroacetamide (BSTFA) and mixed with a vortex mixer for 30 sec (Lai et al., 1995). The samples were wrapped in aluminum foil and placed in the dark at room temperature for 50 min before removing the TMS reagent under nitrogen. The residue was redissolved in 100μl hexane.

Gas chromatographic analysis: Analysis of the TMS ethers of COPS was carried out using a Hewlett Packard gas chromatograph (Model 5890A, Hewlett Packard, Avondale, PA) equipped with a flame ionization detector and 15m x 0.25mm (i.d.) DB-1 (0.1μm film thickness) capillary column (J&W Scientific Inc., Folsom, CA). Helium was used as a carrier gas at a split ratio of 15:1. The gas chromatograph was programmed from 170°C to 220°C

at a rate of 10°C/min, then increased to 236°C at a rate of 0.4°C/min. After the peaks of interest (COPS) were eluted, the oven temperature was increased at a rate of 10°C/min to a final temperature of 300°C and held for 25 min or until all lipid residues were eluted out of the column. The temperature of the injection port and detector were held at 275°C and 320°C, respectively. Peak area were integrated with a Hewlett Packard integrator (Model 3392A) and converted to quantity of COPS using the internal standard method.

Recovery of COPS: The recovery study of COPS was performed using the procedure described above with various concentrations (0.15μg/μl, 0.75μg/μl and 1.5μg/μl) of the COPS standards. Each recovery experiment was repeated three times and analyzed in duplicate. The average percent recoveries for α- and β-epoxides, 7β-hydroxycholesterol, 7-ketocholesterol, 20α-hydroxycholesterol, 25-hydoxycholesterol and cholestane-3β,5α,6β-triol were 87.5%, 91.7%, 87.1%, 88.1%, 63.9%, 76.9% and 94.4% with coefficients of variation (CV) of 2.7%, 4.4%, 3.0%, 3.1%, 3.4%, 2.7% and 1.1%, respectively.

## Statistical Analysis

A MSTAT-C microcomputer statistical program (Michigan State University, 1991) was employed to analyze data using a split-plot design with factors A (treatment) and B (replication) applied to the whole plot (fish in rearing tanks) and factors C (storage time) and D (surface application of oleoresin rosemary) applied to the sub-plot (fish during processing and

storage). The Tukey's test was applied to separate the mean values.

## RESULTS AND DISCUSSION

### Effect of antioxidants on lipid stability

Results indicate that the TBARS numbers of cooked rainbow trout muscle samples from all treatments increased during storage (Table 1). Statistical analysis revealed a highly significant interaction ($p<0.01$) between dietary treatments and storage time. After two days of refrigerated storage, a 12.5-13.0 fold increase in the TBARS numbers of muscle samples from rainbow trout fed the commercial canthaxanthin and control diets was observed. On the other hand, the TBARS numbers of muscle samples from fish fed diets III, IV and V (containing 500mg α-tocopheryl acetate/kg diet) increased only 2-3 fold over the same period. These lower TBARS numbers reflect the protective effect of the higher concentrations of α-tocopherol found in the fish muscle (54.1-57.1mg/kg) compared to those from diets I and II (10.5-10.6mg/kg).

The surface application of oleoresin rosemary had a significant ($p<0.05$) effect on the TBARS numbers. Analysis of variance showed a highly significant ($p<0.01$) interaction between surface application and the dietary treatments. The rainbow trout fed the supplemented diets (III, IV and V) initially had TBARS numbers in the range of 0.17-0.27mg MDA/kg. After two days of storage, these numbers increased to

Table 1. Effect of dietary supplementation and surface application of oleoresin rosemary on the development of TBARS (mg malonaldehyde per kg) in cooked rainbow trout fillets during refrigerated storage.

| Dietary Treatment | Storage time, days 0 | 1 | 2 |
|---|---|---|---|
| **Samples without surface application** | | | |
| I | 0.57±0.07$^{ab}$ | 3.64±0.08$^{b}$ | 7.47±1.10$^{a}$ |
| II | 0.62±0.12$^{ab}$ | 3.86±0.59$^{b}$ | 7.74±0.33$^{a}$ |
| III | 0.36±0.04$^{ab}$ | 0.65±0.30$^{c}$ | 1.15±0.51$^{cd}$ |
| IV | 0.39±0.04$^{ab}$ | 0.63±0.30$^{c}$ | 1.21±0.50$^{c}$ |
| V | 0.38±0.09$^{ab}$ | 0.67±0.18$^{c}$ | 1.33±0.28$^{c}$ |
| **Samples with surface application** | | | |
| I | 0.50±0.04$^{ab}$ | 3.60±0.08$^{b}$ | 6.60±0.57$^{b}$ |
| II | 1.19±0.04$^{a}$ | 5.26±1.10$^{a}$ | 7.56±0.90$^{a}$ |
| III | 0.27±0.06$^{b}$ | 0.35±0.05$^{c}$ | 0.69±0.19$^{cd}$ |
| IV | 0.24±0.03$^{b}$ | 0.43±0.14$^{c}$ | 0.83±0.39$^{cd}$ |
| V | 0.17±0.00$^{b}$ | 0.20±0.00$^{c}$ | 0.35±0.11$^{d}$ |

TBARS numbers are the mean values (± standard deviation) of three replications and each replication was analyzed in duplicate. Results are expressed as mg malonaldehyde per kg sample.

Diet I : Commercial control diet (supplemented with 100mg α-tocopheryl acetate/kg feed).

Diet II : Commercial canthaxanthin diet (supplemented with 40mg canthaxanthin and 100mg α-tocopheryl acetate/kg feed).

Diet III: Supplemented diet I (supplemented with 15mg canthaxanthin and 500mg α-tocopheryl acetate/kg feed).

Diet IV : Supplemented diet II (supplemented with 175mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

Diet V : Supplemented diet III (supplemented with 350mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

$^{a,b,c,d}$ Means in the same column bearing the same superscript are not significantly different ($p>0.05$).

0.35-0.83mg MDA/kg sample. However, surface application of oleoresin rosemary did not protect the lipids from oxidation in muscle samples from fish fed the control and commercial canthaxanthin diets. The TBARS numbers of these samples increased from 0.50-1.19mg MDA/kg immediately after cooking to 6.60-7.56mg MDA/kg after two days of storage. These higher TBARS numbers may, in part, be due to the lower concentrations of α-tocopherol in fish the muscle tissue.

There is a paucity of information on the protective effect of dietary α-tocopherol on lipid oxidation in cooked fish. However, the effect of supranutritional levels of α-tocopherol on lipid oxidation in raw fish muscle has been reported. Frigg et al. (1990) reported that TBARS numbers of fillets from rainbow trout (*Oncorhynchus mykiss*) fed a vitamin E- supplemented diet (200mg/kg) were significantly smaller than those of fish fed a control diet. Sigurgisladottir et al. (1994) also reported significantly lower TBARS in fillets from salmon (*Salmo salar*) fed a vitamin E-supplemented diet compared to the control.

The beneficial effects of vitamin E supplementation on other cooked meats has been reported. Lin et al. (1989 a,b) showed that dietary α-tocopherol supplementation (100mg/kg) improved the oxidative stability of raw and cooked broiler meats during refrigerated and frozen storage. Monahan et al. (1990) reported that the stability of cooked pork was improved by supplementing the diet with 200mg/kg α-tocopheryl acetate. Similarly, Engeseth et al. (1993) reported the improvement of

the oxidative stability of cooked veal samples through dietary vitamin E supplementation.

The inhibitory effect of oleoresin rosemary on lipid oxidation in cooked gray trout (*Cynoscion regalis*) flakes during frozen storage has been reported (Boyd et al., 1993). The flakes dipped in a solution of rosemary extract (2.5g rosemary extract/kg fish flakes) had lower TBARS numbers (0.74mg MDA/Kg) than those from the untreated control samples (1.60mg MDA/kg) after frozen storage (-20°C) for 3 months.

Cholesterol oxide formation in cooked fish muscle

Cholesterol oxides, present in all the cooked samples immediately after cooking, increased during storage (Table 2). Statistical analysis indicated that the formation of cholesterol oxides was dependent on both the dietary treatments and the storage time. The major COPS identified included 7β-hydroxycholesterol, α- and β-epoxides, and 7-ketocholesterol. Two other COPS, 20α-hydroxycholesterol and cholestane-triol were also detected in fish fed the control and commercial canthaxanthin diets. All cooked rainbow trout muscle samples contained relatively high concentrations of the β-epoxide and 7β-hydroxycholesterol compared to the other cholesterol oxides.

The total cholesterol oxide content in fish fed the control and commercial canthaxanthin diets increased 3 to 4 fold (from the initial range of 1.14-1.51μg/g to 3.12-5.92μg/g) after two days of storage. Similarly, the

Table 2. Effect of dietary α-tocopherol supplementation on the cholesterol oxide concentrations in cooked rainbow trout muscle during refrigerated storage.

| Dietary Treatment | Cholesterol oxide concentration (μg/g of sample) | | | | | | |
|---|---|---|---|---|---|---|---|
| | β-epoxide | α-epoxide | 7β-OH | 20α-OH | 7-keto | triol | Total COPS |
| **Day zero** | | | | | | | |
| I | 0.48 | 0.22 | 0.24 | ND | 0.20 | ND | 1.14[b] |
| II | 0.63 | 0.34 | 0.43 | ND | 0.11 | TR | 1.51[b] |
| III | 0.55 | 0.21 | ND | ND | 0.18 | TR | 0.94[b] |
| IV | 0.59 | ND | ND | ND | 0.14 | TR | 0.73[b] |
| V | 0.32 | ND | ND | ND | 0.17 | TR | 0.49[b] |
| **Day one** | | | | | | | |
| I | 0.82 | 0.32 | 0.46 | 0.13 | 0.37 | TR | 2.10[ab] |
| II | 0.76 | 0.24 | 0.34 | 0.07 | 0.39 | TR | 1.80[ab] |
| III | 0.52 | 0.28 | 0.32 | ND | 0.16 | TR | 1.28[b] |
| IV | 0.38 | 0.17 | 0.22 | ND | 0.07 | TR | 0.84[b] |
| V | 0.24 | 0.13 | 0.22 | ND | 0.13 | TR | 0.72[b] |
| **Day two** | | | | | | | |
| I | 1.15 | 0.52 | 0.72 | 0.22 | 0.37 | 0.14 | 3.12[ab] |
| II | 2.10 | 1.15 | 1.34 | 0.42 | 0.74 | 0.17 | 5.92[a] |
| III | 0.97 | 0.44 | 0.54 | ND | 0.22 | TR | 2.17[ab] |
| IV | 0.53 | 0.19 | 0.28 | ND | 0.08 | TR | 1.08[b] |
| V | 0.30 | 0.32 | 0.32 | ND | 0.09 | TR | 1.03[b] |

All values represent the mean of two replicates.

[a,b] Means in the same column bearing the same superscript are not significantly different ($p>0.05$).

concentrations of total COPS in fish fed the supplemented diets (diets III, IV and V) increased from the initial range of 0.49-0.94μg/g to 1.03-2.17μg/g over the same period.

On the other hand, fish subjected to surface application of oleoresin rosemary showed less cholesterol oxide formation (Table 3). Statistical analysis revealed a highly significant ($p<0.01$) effect of surface application on the formation of COPS. The surface application also helped to restrict the formation of the cholestane-triol and 20α-hydroxycholesterol. The initial COPS concentrations in the fish fed the control and commercial canthaxanthin diets were in the range of 0.74 to 0.76μg/g. After 2 days of storage, these concentrations increased to 2.19 to 3.35μg/g. In comparison, the fish fed the supplemented diets (III, IV and V) contained smaller COPS concentrations. Initial concentrations ranged from 0.42-0.63 μg/g and increased to 0.85-1.75μg/g after two days of storage. The surface application of oleoresin rosemary inhibited COPS formation by 29.8-43.4% in fish fed diets low in α-tocopherol (diets I and II). The reduction in COPS formation in fish fed diets containing higher levels of α-tocopherol (diets III, IV and V) ranged from 7.7-19.4%.

Formation of COPS in processed fish products has been reported. Chen and Yen (1994) reported four major COPS including 7α- and 7β-hydroxycholesterol, α-epoxide and 7-ketocholesterol in small sun-dried fish (*Spratelloides gracilis* and *Decapterus maruodsi*) which were stored at ambient temperature for 3 months in air. The predominant COP was

Table 3. Effect of surface application of oleoresin rosemary on cholesterol oxide concentrations in cooked rainbow trout muscle during refrigerated storage.

| Dietary[1] treatment | Cholesterol oxide concentration (μg/g of sample) β-epoxide | α-epoxide | 7β-OH | 7-keto | Total COPS |
|---|---|---|---|---|---|
| Day zero | | | | | |
| I | 0.40 | 0.07 | 0.22 | 0.07 | 0.76[bc] |
| II | 0.22 | 0.15 | 0.27 | 0.10 | 0.74[bc] |
| III | 0.22 | 0.23 | 0.13 | 0.05 | 0.63[bc] |
| IV | 0.12 | 0.05 | 0.34 | 0.06 | 0.57[c] |
| V | 0.13 | 0.09 | 0.14 | 0.06 | 0.42[c] |
| Day one | | | | | |
| I | 0.43 | 0.19 | 0.31 | 0.31 | 1.24[bc] |
| II | 0.62 | 0.24 | 0.19 | 0.35 | 1.40[bc] |
| III | 0.31 | 0.20 | 0.41 | 0.07 | 0.99[bc] |
| IV | 0.25 | 0.16 | 0.27 | 0.15 | 0.83[bc] |
| V | 0.08 | 0.03 | 0.50 | 0.06 | 0.67[bc] |
| Day two | | | | | |
| I | 0.70 | 0.28 | 0.49 | 0.72 | 2.19[ab] |
| II | 0.93 | 0.80 | 0.95 | 0.67 | 3.35[a] |
| III | 0.39 | 0.39 | 0.78 | 0.19 | 1.75[bc] |
| IV | 0.34 | 0.17 | 0.52 | 0.14 | 1.17[bc] |
| V | 0.14 | 0.09 | 0.48 | 0.14 | 0.85[bc] |

All values represent the means of two replicates.

[1] See Table 1 for description of dietary treatments.

[a,bc] Means in the same column bearing the same superscript are not significantly different ($p>0.05$).

7α-hydroxycholesterol. The concentrations of COPS in these fish ranged from 4.82μg to 65.7μg/g. Ohshima et al. (1993) reported the formation of COPS in traditional Japanese fish products including salted and dried, boiled and dried, and smoked fish products. The predominant COPS were 7β-hydroxycholesterol and 7-ketocholesterol, while the concentrations of α- and β-epoxides, cholestane-triol and 25-hydroxycholesterol were relatively low. The concentrations of total COPS among different products ranged widely. Concentrations reported were: 8.3μg/g (dry weight basis) in boiled and dried shrimp, 14.3-27.3μg/g in salted-dried northern cod, 6.8 μg/g in smoked keta salmon (*Oncorhynchus keta*) and 188.0μg/g in boiled and dried anchovy. Based on the results of a model system, these investigators also suggested that cholesterol oxidation in fish products proceeds in conjunction with the oxidative decomposition of co-existing polyunsaturated fatty acids.

Similarly, Osada et al. (1993) identified COPS in uncooked and processed marine products such as air-dried sardine, air-dried squid, canned squid, and pickled and spiced Alaskan pollack roe. The major COPS identified in these products included 7α- and 7β-hydroxycholesterol, 5,6α-epoxycholesterol, and 7-ketocholesterol. No COPS were detected in raw fish, while the processed marine products contained 11.0-28.7mg COPS/100g oxidized cholesterol.

The inhibitory effect of natural antioxidants such as α-tocopherol and oleoresin rosemary on the formation of COPS in

marine products has not been reported. However, influence of these antioxidants on COPS formation has been studied in other foods such as pork, veal and eggs (Monahan et al., 1992, 1993; Engeseth et al., 1994; Lai et al., 1995). Monahan et al. (1992) reported that rate of formation of COPS was low in pork from pigs fed high levels of α-tocopherol. Cooked pork from pigs fed the basal level of α-tocopheryl acetate (10mg/kg diet) had total COPS equivalent to 2.7% of the total cholesterol content after two days of refrigerated storage. Cooked pork from pigs fed the supplemented diet (22mg/kg) had 1.6% total COPS over the sample period. Similarly, Engeseth et al. (1994) reported that vitamin E supplementation of veal calves improved the oxidative stability of veal cholesterol.

In conclusion, the combination of dietary vitamin E and dipping of the raw fillets in a solution of oleoresin rosemary before cooking had a protective effect on the oxidative stability of rainbow trout lipids including cholesterol. These results clearly indicate the benefits of such procedures to the fish industry.

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## CHAPTER SIX

# EFFECT OF SELECTIVE ANTIOXIDANTS ON GENERATION OF VOLATILE COMPOUNDS BY LIPOXYGENASE CATALYZED-OXIDATION OF ARACHIDONIC ACID AND RAINBOW TROUT MUSCLE LIPIDS IN A MODEL SYSTEM.

## ABSTRACT

Volatile compounds generated from the lipoxygenase-catalyzed oxidation of arachidonic acid in a model system included 1-octen-3-ol, 2-octenal and 2-nonenal. The volatile compounds generated from the lipoxygenase-catalyzed oxidation of rainbow trout muscle lipids were 1-octen-3-ol and 2-nonenal. Lipoxygenase-catalyzed oxidation of arachidonic acid and fish lipids was partially inhibited by α-tocopherol, capsanthin and oleoresin rosemary.

## INTRODUCTION

Lipid oxidation is well recognized as one of the major causes of quality deterioration in muscle foods during refrigerated and frozen storage. Fish lipids are rich in ω-3 polyunsaturated fatty acids which make them prone to oxidation during storage. Lipid oxidation can directly affect many quality attributes such as color, flavor, texture, nutritive value, and safety (Khayat and Schwall, 1983; Pearson et al., 1983). Flavor changes in fish muscle are not directly due to the presence of *cis, trans* conjugated monohydroperoxides because these compounds are flavorless (Applewhite, 1985). However, decomposition of these labile hydroperoxides generate a complex mixture of compounds which contribute to the off-flavors in rancid foods (Frankel et al., 1981; Frankel, 1984).

Several enzymic and non-enzymic processes can act as mediators of both the initiation and breakdown processes of lipid oxidation in biological tissues (Kanner and Kinsella, 1983; German and Kinsella, 1986; Josephson et al., 1987). Proposed initiators of lipid oxidation in biological systems include oxygen species such as singlet oxygen (King et al., 1975; Kellog and Fridovich, 1977; Foote, 1985) and superoxide radical (Misra and Fridovich, 1972; McCord and Petrone, 1982); hydroxyl radical (Fong et al., 1976; Gutteridge, 1984); perferryl radical (Hochstein et al., 1964; Morehouse et al., 1984); ferryl radical (Koppenol and Liebman, 1984); oxygen-bridge di-iron (Tien and Aust, 1982; Minotti and Aust, 1987);

porphyrin cation radical (Harel and Kanner, 1985), and microsomal enzymes such as peroxidase, lipoxygenase and cyclo-oxygenase (Muftugil, 1985; German et al., 1986; Yamamoto, 1991). These endogenous compounds play an important role in the formation of the primary pool of biological catalysts in muscle tissues.

Lipoxygenase activity has been reported in gill (German and Kinsella, 1986; German and Creveling, 1990), skin (German and Kinsella, 1985) and muscle (Wang et al., 1991; Harris and Tall, 1994) tissues of several species of fish. The presence of 12-lipoxygenase and 15-lipoxygenase in gill and skin tissues of rainbow trout has been confirmed (German and Kinsella, 1985; Hsieh et al., 1988a,b; German and Creveling, 1990). Hsieh and Kinsella (1989) reported the capacity of 12-lipoxygenase in rainbow trout gill tissue to generate short-chain volatile compounds. It has been proposed that the postmortem release of endogenous skin lipoxygenase may constitute a significant source of initiating radicals (German and Kinsella, 1985; Hsieh et al., 1988a).

The choice of antioxidants to stabilize food for human consumption is restricted to only a few substances. Recent attention has focused upon antioxidants such as vitamin E which is a very effective chain-breaking compound. Similarly, rosemary extracts have shown strong inhibitory effects on lipid oxidation. Houlihan and Ho (1985) reported that oleoresin rosemary contains a number of compounds such as carnosol, rosmanol, rosmaridiphenol and rosmariquinone which

possess antioxidant activity similar to or greater than that of butylated hydroxyanisole (BHA).

Because of the possible involvement of lipoxygenase in lipid oxidation during storage, understanding the mechanism of initiation of lipid oxidation in fish may provide the means for improving the flavor stability and quality of fish. This study was undertaken to study the lipoxygenase-catalyzed generation of volatile compounds from arachidonic acid in a model system, and to investigate the effects of several antioxidants on the lipoxygenase-catalyzed generation of volatile compounds from arachidonic acid and lipids present in rainbow trout muscle.

## MATERIALS AND METHODS

### Reagents

Arachidonic acid (20:4n6) was purchased from Sigma Chemical Co. (St. Louis, MO). Standard flavor compounds (1-octen-3-ol, 2-octenal, 2-nonenal and 2-nonanone) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Tenax TA (mesh size 80/100) was obtained from Alltech Associates, Inc. (Deerfield, IL). HPLC grade hexane and 200 proof ethyl alcohol were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ) and Quantum Chemical Corp. (Tuscola, IL), respectively. Bovine serum albumin was purchased from Sigma Chemical Co. All other chemical were of reagent grade quality.

**Diets and feeding trial**

**Rainbow trout (*Oncorhynchus mykiss*) were fed five different diets for a period of 7 months. The details of the experimental design are described in Chapter 1.**

**The diets are as follows, the values cited being targeted levels:**

**Diet I : Commercial control diet (Martin Mills Inc., Elmira, ONT).**

**Diet II : Commercial canthaxanthin diet (Martin Mills Inc., Elmira, ONT).**

**Diet III : Diet supplemented with α-tocopheryl acetate (500mg /kg) and canthaxanthin (15 mg/kg).**

**Diet IV : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (175 mg/kg).**

**Diet V : Diet supplemented with α-tocopheryl acetate (500mg /kg) and oleoresin paprika (350 mg/kg).**

**Diets I and II were commercial rainbow trout diets (floating 40% trout feed grower pellets) and contained 10% herring oil by weight. These diets also contained 100mg α-tocopheryl acetate (target level). Diet I served as the control diet in this study. A commercial rainbow trout diet without added herring oil was also purchased from Martin Mills Inc. and used to prepare three supplemented diets (diets III, IV and V). The supplemented diets was prepared by adding α-tocopheryl acetate (500 mg/kg) to herring oil (10% based on weight of diet) which was carefully coated on the surface of the feed. Additionally, diet III and diets IV and V contained**

canthaxanthin (Martin Mills Inc.) and oleoresin paprika (Kalsec Inc.,Kalamazoo, MI), respectively. Diets III, IV and V were prepared by Kalsec Inc.

All fish were slaughtered according to standard commercial practices at the Aquaculture Research Laboratory, Michigan State University, at the termination of the feeding trial. Fish were kept on ice and immediately transferred to the Meat Processing Laboratory, Michigan State University, for processing and packaging.

Samples preparation

The slaughtered fish were gutted and their skins were removed. One fillet from each fish was immediately immersed in a 2% solution of Herbalox seasoning type P, oleoresin rosemary extract (Kalsec Inc., Kalamazoo, MI) in distilled water (w/v), until an approximate pick up of 1% was obtained. The gain in weight was measured by periodic weighing of the fillets after removing excess oleoresin rosemary solution from the surface of the fillets. The other fillet was used as the reference.

Lipid extraction

Lipids were extracted from the fillets using the dry column method of Marmer and Maxwell (1981).

Lipoxygenase extraction

The enzyme extract was prepared by the method described by Hsieh and Kinsella (1989). Gill tissue was carefully

excised from freshly killed rainbow trout and homogenized at speed 5 in 30mL 0.05 M (pH 7.4) phosphate buffer in a polytron homogenizer (Kinematica AG, Switzerland) for 45 seconds. The gill tissue homogenate was centrifuged at 4°C at 15000g for 15 min in a Sorvall RC2-B centrifuge (Ivan Sorvall Inc., Norwalk, CT). The resultant supernatant fraction was collected and immediately frozen using liquid nitrogen before storage at -70°C. This crude enzyme source was used in the model system without further purification.

Protein determination

The protein concentration of the crude enzyme extract was determined by the Lowry method with bovine serum albumin as the standard protein (Lowry et al., 1951).

Conditioning of Tenax

Tenax (50mg) was packed in a GLT desorption tube (Scientific Instrument Services, Inc., Ringoes, NJ), 4.0 mm I.D, and conditioned at 150°C for 30 hours under nitrogen. The pre-conditioned Tenax was washed with 6ml HPLC grade hexane prior to its use.

Isolation of volatile compounds

The volatile compounds generated from the oxidation of arachidonic acid by rainbow trout gill tissue lipoxygenase were collected using a liquid purge and trap system (Scientific Instrument Services, Inc., Ringoes, NJ). A 20ml

crude enzyme extract (1mg protein/ml) and 20μl arachidonic acid (50μM) were incubated at 25°C for 2 hrs. A 20μl aliquot of a 2-nonanone solution (0.05mg/l), an internal standard, was added to the crude enzyme extract before the addition of arachidonic acid in order to correct the results for recovery. In order to study the effect of different antioxidants, the crude enzyme extract was preincubated with the antioxidant for 10 min at 25°C before the addition of the fatty acid. At the end of the incubation period, the volatiles generated by lipoxygenase activity were purged with nitrogen (40ml/min flow rate) and collected on the Tenax trap for 3 hrs. The trapped compounds were then eluted with 2ml hexane which was later concentrated to 20 μL in a stream of nitrogen.

In order to purge and trap volatile compounds from the reaction mixture of fish lipids and the crude enzyme extract, 30mg/l (final concentration in reaction mixture) of fish lipids were incubated with 20ml crude enzyme extract (1mg protein/ml). The rest of the procedure was similar to that stated above.

The influence of purge and trap system on the recovery of volatile compounds was determined. The recovery experiment was repeated six times and each extract was analyzed in duplicate. The average recovery of the internal standard was 92.3% with 2.1% coefficient of variation (CV).

Effect of heating on enzyme activity

The crude enzyme extract was heated in a water bath at 40°C, 60°C and 80°C for 10 min. The preheated enzyme extract (20ml) was then incubated with arachidonic acid (50μM). The rest of the procedure was similar to that stated above.

Effect of antioxidants on enzyme activity

The crude enzyme extract (20ml) was preincubated with specific antioxidants at 25°C for 10 min. Then, arachidonic acid (50μM) or lipids from rainbow trout muscle (30mg/l, final concentration) were added to initiate the reaction. The rest of the procedure was similar to that stated previously.

Gas chromatographic analysis

Volatile compounds were analyzed using a Hewlett Packard gas chromatograph, (Model 5890A, Hewlett Packard, Avondale, PA) equipped with a flame ionization detector. A fused silica capillary column (HP-20M, Hewlett Packard, Avondale, PA) with a length of 50m and an inside diameter of 0.32mm was used for the separation of the volatile compounds. Helium was the carrier gas and a split ratio of 10:1 was maintained. The GC oven temperature was initially held at 70°C for 5 min, then increased at a rate of 5°C/min to a temperature of 100°C. The temperature was then increased to 120°C at the rate of 2°C. Finally, the temperature was increased to 200°C at the rate of 5°C and held for 23 minutes. The injector and detector temperatures were maintained at 215°C and 220°c, respectively.

Identification of the volatile compounds was based on a comparison of retention times of samples to those of standards. Peak area of each volatile compound was computed by an integrator (Model 3392A, Hewlett Packard, Avondale, PA).

Mass Spectroscopic confirmation of compound identity

A HP 5890A GC equipped with a 5970A mass selective detector and workstation was used for acquisition and analysis of data. The same GC capillary column and GC conditions were used as stated above. The operating conditions were as follows: electron voltage 70 eV; electron multiplier voltage 2000 V; transfer line temperature 280°C; mass analyzer temperature 300°C; ion source temperature 250°C. Identification of the volatile compounds was based on matching their mass spectra with the computerized mass spectral data bases (NISD standard reference data base and the NIST mass spectral data base) of common compounds and also by matching with the mass spectra of authentic standards.

## RESULTS AND DISCUSSION

Lipoxygenase-catalyzed oxidation of arachidonic acid

Earlier work on the lipoxygenase-catalyzed oxidation of arachidonic acid established the generation of three specific volatile compounds, 2-octenal, 1-octen-3-ol and 2-nonenal (Hsieh and Kinsella, 1989). To substantiate these findings, arachidonic acid was incubated with crude enzyme extract from

rainbow trout gill tissue in a model system. Data confirmed the generation of these compounds in the system used in the present study (Table 1).

The identity of these specific volatile compounds was confirmed by mass spectroscopic analysis. The characteristic mass fragments and their relative abundance are shown in Table 2. The characteristic mass fragments of 2-octenal show a pattern of an aldehyde such as m/e 108 (M-18, loss of $H_2O$) and m/e 97 (M-29, loss of $CHO^+$). It has been reported that the mass fragment at m/e 55 (M-71, loss of CH=CHCHO) is a characteristic pattern of an 2-alkenal (Heller and Milne, 1980). Similarly, the characteristic mass fragment at m/e 110 (M-18) in the case of 1-octen-3-ol showed the loss of $H_2O$. The mass fragments at m/e 99 (M-29) and m/e 57 (M-71) arise from the loss of $CHO^+$ and $CHOHCH=CH_2$, respectively. These mass fragments were consistent with those of an authentic standard and with the mass spectral data bases (NIST and NISD). The characteristic mass fragments of 2-nonenal were found at m/e 122 (M-18, loss of $H_2O$), m/e 111 (M-29, loss of $CHO^+$) and m/e 55 (M-85, loss of CH=CHCHO) and were consistent with those of an authentic standard and mass spectral data base (NIST).

To verify that these compound arose from lipoxygenase activity, the enzyme extract was heated at various temperatures (40°C, 60°C and 80°C) for 10 min before its incubation with arachidonic acid. Results indicated some loss of enzyme activity at 40°C as only one compound was detected (Table 1). Heating at 60°C and beyond completely inhibited

Table 1. Effect of heat treatment on lipoxygenase-catalyzed generation of volatile compounds in a model system (25°C) containing arachidonic acid and rainbow trout gill lipoxygenase.

| Treatment | Relative area of volatile compounds | | |
|---|---|---|---|
| | 2-octenal | 1-octen-3-ol | 2-nonenal |
| LOX[1] + AA[2] | 69,756 | 170,022 | 119,257 |
| LOX(heated at 40°C)[3] + AA | 11,611 | ND | ND |
| LOX(heated at 60°C) + AA | ND | ND | ND |
| LOX(heated at 80°C) + AA | ND | ND | ND |

All values represent the mean of duplicate analyses.

[1]Lipoxygenase
[2]Arachidonic Acid
[3]Enzyme heated for 10 min before incubation.

Table 2. Mass spectrometric data for characteristic mass fragments of volatile compounds.

| Compound | MW[1] | Characteristic mass fragments (relative abundance %) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M-18 | M-29 | M-43 | M-44 | M-56 | M-57 | M-70 | M-71 | M-85 | M-97 | M-99 | M-111 |
| 2-Octenal | 126 | 108 (4)[2] | 97 (8) | 83 (31) | - | 70 (50) | - | - | 55 (71) | 41 (100) | 29 (58) | - | - |
| 1-Octen-3-ol | 128 | 110 (4) | 99 (4) | 85 (8) | - | 72 (17) | - | - | 57 (100) | 43 (29) | - | 29 (37) | - |
| 2-Nonenal | 140 | 122 (6) | 111 (6) | - | 96 (11) | - | 83 (33) | 70 (48) | - | 55 (71) | - | 41 (100) | 29 (69) |

[1] Molecular weight
[2] percentage of base peak

the generation of these compounds. These results closely agree with the findings of Hsieh et al. (1988a) who reported that 12-lipoxygenase from rainbow trout gill tissue retained 90% of its activity at 10° and 30°C and 60% of its activity at 0°C. The enzyme retained 80% of its activity when held at 40°C for 10 min. An abrupt decline in enzyme activity was observed when heated at 50°C. The results of the present study and those of Hsieh et al. (1988a) suggest that the high activity of fish lipoxygenase at temperatures close to freezing may play an important role in the initiation of lipid oxidation in fish muscle during storage.

Effect of esculetin on lipoxygenase-catalyzed oxidation of arachidonic acid

The incorporation of 1 μM esculetin into the reaction mixture completely inhibited the generation of 2-octenal and 2-nonenal (Table 3). The presence of 1-octen-3-ol in small amounts was also noticed. These results again confirm the observations of Hsieh and Kinsella (1989) who demonstrated that incorporation of 1μM esculetin into the reaction mixture inhibited the generation of these volatile compounds almost completely.

Esculetin has an oxidation potential of 0.76 V (Scott, 1965; Baumann et al., 1982) and has a catechol structure (Fieser, 1930). In soybean and reticulocyte lipoxygenases, molecules with a catechol structure cause inhibition by forming complexes with the active ferric form of iron (Spaapen et al., 1980; Schewe et al., 1986). Sekiya et al.

Table 3. Effect of esculetin on lipoxygenase-catalyzed generation of volatile compounds in a model system containing arachidonic acid.

| Treatment | Area of volatile compounds | | |
|---|---|---|---|
| | 2-octenal | 1-octen-3-ol | 2-nonenal |
| LOX[1]+ AA[2] | 69,756 | 170,022 | 119,257 |
| LOX + AA + 100nM Esculetin | 12,777 | 3,810 | ND |
| LOX + AA + 1μM Esculetin | ND | 4,708 | ND |
| LOX + AA + 10μM Esculetin | ND | ND | ND |
| LOX + AA + 100μM Esculetin | ND | ND | ND |

All values represent the mean of duplicate analyses.

[1]Lipoxygenase
[2]Arachidonic acid

(1982) reported that a dihydroxy structure in the coumarin skeleton of esculetin was required for the inhibition of platelet 12-lipoxygenase. Similar structural features were required for the inhibition of 5-lipoxygenase in polymorphonuclear leukocytes (Kimura et al., 1985). Esculetin is readily converted to esculin through glycosidation at one of the hydroxy groups of its catechol structure. This compound is not as effective as its precursor in inhibiting lipoxygenase activity (Hsieh et al., 1988b; Hsieh and Kinsella, 1989).

Effect of selected antioxidants on lipoxygenase-catalyzed oxidation of arachidonic acid

Partial inhibition of lipoxygenase activity was observed with β-carotene, α-tocopherol and capsanthin, a major component of oleoresin paprika (Table 4). However, the generation of 2-octenal was apparently enhanced by the addition of β-carotene to the reaction mixture. Sanz et al. (1994) reported that concentrations of β-carotene higher than 14 μM inhibited oxidation by chickpea lipoxygenases. This inhibitory effect may be due to the formation of an irreversible enzyme-β-carotene complex as suggested by Cohen et al. (1985). These data also indicate that the combination of α-tocopherol and capsanthin had a greater inhibitory effect than the individual components. There is a paucity of information on the effect of α-tocopherol and capsanthin on lipoxygenase activity. The addition of oleoresin rosemary to

Table 4. Effect of selected antioxidants on lipoxygenase-catalyzed generation of volatile compounds in a model system containing arachidonic acid.

| Treatment | Area of carbonyl compound | | |
|---|---|---|---|
| | 2-octenal | 1-octen-3-ol | 2-nonenal |
| LOX[1]+AA[2] | 12,064 | 59,395 | 71,761 |
| LOX+AA+100μM β-carotene | 43,607 | ND | ND |
| LOX+AA+100ppm α-tocopherol | 10,623 | 56,868 | ND |
| LOX+AA+100ppm capsanthin | 23,057 | 18,029 | 29,797 |
| LOX+AA+100ppm α-tocopherol+100ppm capsanthin | 23,670 | 1,675 | ND |
| LOX+AA+100ppm α-tocopherol+100ppm capsanthin+100ppm OR[3] | 46,662 | ND | 39,099 |

All values represent the mean of duplicate analyses.

[1] Lipoxygenase
[2] Arachidonic acid
[3] Oleoresin rosemary

the reaction mixture along with α-tocopherol and capsanthin did not show any beneficial effects in this model study.

Effect of selected antioxidants on lipoxygenase-catalyzed oxidation of rainbow trout muscle lipids

Results indicate that gill tissue lipoxygenase was capable of initiating oxidation of lipids extracted from rainbow trout muscle (Table 5). The volatile compounds identified were 1-octen-3-ol and 2-nonenal. Results indicated that generation of these compounds in lipids from fish fed the higher concentrations of α-tocopherol (diets III, IV and V) was suppressed. The generation of these compounds in the model system containing lipids from fish fed diet containing canthaxanthin (diet II) was suppressed compared to the control diet (diet I). It was also noticed that lipids from fish fed the higher levels of oleoresin paprika (diet V) were more stabilized than the lipids from the remaining groups.

Surface application of oleoresin rosemary produced an inhibitory effect in all treatments. A combination of a relatively high level of dietary α-tocopherol and oleoresin rosemary, as in diets III, IV and V, provided the maximum protection against lipoxygenase-catalyzed oxidation. A similar inhibitory effect of α-tocopherol and capsanthin was observed when arachidonic acid was used as a substrate. In this study, the surface application of oleoresin rosemary further enhanced the inhibitory effect of α-tocopherol. It is very likely that significant quantities of the oleoresin rosemary components

Table 5. Effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on lipoxygenase-catalyzed oxidation of rainbow trout muscle lipids.

| Dietary Treatment | Volatile carbonyl compounds (Area) 2-Octenal | 1-Octen-3-ol | 2-Nonenal |
|---|---|---|---|
| Samples without surface application | | | |
| I | ND | 20,655 | 184,434 |
| II | ND | 17,620 | 91,210 |
| III | ND | 6,274 | 48,530 |
| IV | ND | 11,529 | 43,102 |
| V | ND | 732 | 7,851 |
| Samples with surface application | | | |
| I | ND | 23,333 | 38,268 |
| II | ND | 2,491 | 64,942 |
| III | ND | 10,129 | ND |
| IV | ND | 13,103 | ND |
| V | ND | 5,983 | 18,404 |

All values represent the mean of accumulated sample of each treatment analyzed in duplicate.

Diet I : Commercial control diet (supplemented with 100mg α-tocopheryl acetate/kg feed).

Diet II : Commercial canthaxanthin diet (supplemented with 40mg canthaxanthin and 100mg α-tocopheryl acetate/kg feed).

Diet III: Supplemented diet I (supplemented with 15mg canthaxanthin and 500mg α-tocopheryl acetate/kg feed).

Diet IV : Supplemented diet II (supplemented with 175mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

Diet V : Supplemented diet III (supplemented with 350mg oleoresin paprika carotenoids and 500mg α-tocopheryl acetate/kg feed).

possessing antioxidant activity were also extracted along with muscle lipid. The difference in concentration of oleoresin rosemary used in the arachidonic acid study and fish lipid studies may explain the reason for the disparity in the inhibitory effects observed.

Chen et al. (1992) reported that rosemary extracts and their isolated components such as carnosol, carnosic acid and ursolic acid show strong inhibitory effect towards the soybean 15-lipoxygenase enzyme. The rosemary extract displayed $IC_{50}$ (concentration at which 50% of enzyme activity is inhibited) values ranging from 1.3 to 2.6 μg. Carnosol was shown to be more effective as an lipoxygenase inhibitor than carnosic acid and ursolic acid. However, carnosic acid and ursolic acid also displayed an inhibitory effect towards soybean lipoxygenase.

Results presented in Chapters 2-5 clearly demonstrate the protective effect of α-tocopherol, capsanthin and oleoresin rosemary against the oxidation of lipids during refrigerated and frozen storage of raw and cooked rainbow trout muscle. The stabilizing effect of these compounds was also noticed in muscle microsomal membranes when oxidation was stimulated by iron-ascorbate. The inhibitory effect of these compounds on lipoxygenase-catalyzed lipid oxidation further substantiates the fact that lipid oxidation in frozen fish muscle may be, in part, due to lipoxygenase activity.

It is strongly recommended that the fish industry not only supplement fish diets with supranutritional levels of

α-tocopherol but also dip raw and processed fish products in oleoresin rosemary before further handling and storage.

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## SUMMARY AND CONCLUSIONS

A series of studies were designed to investigate the effects of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on color and flesh stability of rainbow trout (*Oncorhynchus mykiss*) muscle during refrigerated (4°C) and frozen storage (-20°C). The effect of dietary α-tocopherol and surface application of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in rainbow trout muscle and microsomes was investigated. The inhibitory effect of these antioxidants on the formation of cholesterol oxides in fish muscle as a result of cooking and subsequent refrigerated storage was also studied. A model study was designed to evaluate the effectiveness of natural antioxidants, in particular, on the lipoxygenase-catalyzed generation of volatile compounds from arachidonic acid and extracted fish lipids.

Muscle and liver concentrations of α-tocopherol increased with increasing levels of dietary α-tocopheryl acetate. Dietary α-tocopherol supplementation had no effect on muscle fatty acid composition. Canthaxanthin pigments were more efficiently deposited in fish flesh (7.9 mg/kg) than those from oleoresin paprika (2.4 to 3.1 mg/kg) and their deposition was not affected by the level of dietary supplementation with

α-tocopheryl acetate. With the increased pigmentation, decreases in lightness ($L^*$) and Hue angle and increases in redness ($a^*$) were observed.

Fish fed diets (III, IV and V) containing 500mg/kg α-tocopheryl acetate/kg feed had smaller TBARS numbers compared to fish fed control and commercial canthaxanthin diets (contained 100 mg α-tocopheryl acetate/kg) during refrigerated storage. A significant ($p<0.05$) interaction between dietary treatments and storage time was observed. Dietary α-tocopherol supplementation also stabilized the flesh color. Lipid and color stability was enhanced by the surface application of oleoresin rosemary.

The TBARS numbers of muscle samples increased during frozen storage in all treatments. The supranutritional level of dietary α-tocopherol provided protection against lipid oxidation. However, surface application of oleoresin rosemary had a significant inhibitory effect on TBARS numbers. Significant reductions in α-tocopherol and total carotenoid concentrations as a result of frozen storage were observed. It seems likely that carotenoid oxidation proceeds lipid oxidation. There was little protective effect of α-tocopherol supplementation on color stability even at the higher feeding level. However, a combination of α-tocopherol and oleoresin rosemary provided stability to redness ($a^*$), yellowness ($Y^*$) and chroma in fish fed the higher concentrations of α-tocopherol.

The TBARS numbers of muscle and microsomes from fish fed diets (III, IV and V) supplemented with the higher level of α-tocopherol were smaller than those of fish fed diets containing lower concentrations of α-tocopherol (diets I and II). A protective effect of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in fish muscle was observed. A significant ($p<0.01$) interaction between dietary treatments and incubation time was observed. The inhibitory effect of dietary α-tocopherol supplementation and surface application of oleoresin rosemary on iron/ascorbate-induced lipid oxidation in fish muscle showed the similar trend as observed in refrigerated storage study. Dietary α-tocopherol supplementation significantly increased the α-tocopherol concentrations of muscle microsomal membranes.

The TBARS numbers of cooked fish muscle increased during storage for all treatments. Dietary α-tocopherol supplementation partially inhibited lipid oxidation. Surface application of oleoresin rosemary further enhanced this protective effect. Cholesterol oxides were formed in all the samples as a result of cooking and storage. The major cholesterol oxidation products (COPS) were α- and β-epoxides, 7β-hydroxycholesterol and 7-ketocholesterol. Formation of COPS was reduced by supranutritional concentrations of α-tocopherol and surface application of oleoresin rosemary.

The specific volatile compounds generated from the lipoxygenase-catalyzed oxidation of arachidonic acid in a model system were 1-octen-3-ol, 2-octenal and 2-nonenal. The

volatile compounds generated from the lipoxygenase-catalyzed oxidation of rainbow trout muscle lipids were 1-octen-3-ol and 2-nonenal. Lipoxygenase-catalyzed oxidation of arachidonic acid and fish lipids was partially inhibited by α-tocopherol, capsanthin and oleoresin rosemary.

Results clearly demonstrated the protective effect of α-tocopherol, capsanthin and oleoresin rosemary against oxidation in lipids, including cholesterol, during refrigerated and frozen storage in both raw and cooked rainbow trout muscle. The stabilizing effect of these compounds was also noticed in muscle microsomal membranes when oxidation was stimulated by iron/ascorbate. The antioxidative effect of these compounds on lipoxygenase-catalyzed lipid oxidation further substantiated the previous observations and also supported the hypothesis on which the present study was based.

Better use of antioxidants in the processing and handling of fish will minimize the oxidation of lipids. It is therefore strongly recommended that the fish industry not only supplement the fish diets with supranutritional levels of α-tocopherol but also dip raw and processed fish products in oleoresin rosemary before their further handling and storage.

## FUTURE RESEARCH

Frozen storage of marine products is a common practice. Factors which can strongly influence the lipid oxidation during storage include muscle and muscle membrane concentrations of α-tocopherol, degree of unsaturation of fatty acids in the muscle and muscle membranes, storage temperature, packaging and length of storage. Lipid oxidation may also be initiated by lipoxygenase enzymes which are known to be highly active even at freezing temperatures. It was observed in this study that α-tocopherol partially inhibited lipoxygenase activity. The degradation of dietary α-tocopherol during frozen storage may explain why dietary α-tocopherol provided little protection against lipid oxidation during frozen storage compared to refrigerated storage. Further research is required to identify ways to protect the α-tocopherol against degradation during storage. It may also be advantageous to identify some other natural antioxidants which can completely inhibit lipoxygenase-catalyzed lipid oxidation.

It is recognized that carotenoid degradation proceeds in an analogous fashion to lipid oxidation. Results of the present study have shown a protective effect of surface application of oleoresin rosemary on lipid and color stability. Further research is needed to investigate the

effect of surface application of α-tocopherol and oleoresin rosemary in combination on the lipid and color stability of both farm-raised and wild fish.

Results of the present study also suggest a synergistic relationship between dietary α-tocopherol and oleoresin rosemary. More detailed studies are necessary to establish and explain this synergism in fish products.