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Enhancing quality and extending shelf life of rainbow trout (*Oncorhynchus mykiss*) by vitamin E supplementation and post- harvest antimicrobial intervention

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**ENHANCING QUALITY AND EXTENDING SHELF LIFE
OF RAINBOW TROUT (*Oncorhynchus mykiss*)
BY VITAMIN E SUPPLEMENTATION
AND POST HARVEST ANTIMICROBIAL INTERVENTION**

Nalini Kamireddy

**Thesis submitted to the
Davis College of Agriculture, Forestry, and Consumer Sciences
At West Virginia University
In partial fulfillment of the requirements
for the degree of**

**Master of Science
in
Animal and Veterinary Sciences**

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**Key words: rainbow trout, vitamin E, acidified sodium chlorite,
storage stability**

ABSTRACT

Enhancing Quality and Extending Shelf Life of Rainbow Trout (*Oncorhynchus mykiss*) by Vitamin E Supplementation and Post Harvest Antimicrobial Intervention

Nalini Kamireddy

Rainbow trout were fed a control (191 mg/kg) or the control diet supplemented with 5000 mg α -tocopherol /kg. Fish were sampled at 2, 3, 4, 5, 7, and 9 weeks of feeding; at each week fillets were evaluated at 0, 7, and 14 d. Supplementation increased fillet α -tocopherol content, and TBARS value were not affected. A feeding by storage time interaction was observed for fat content, psychrotrophic counts, cook yield, and shear force. For antimicrobial intervention, fillets were not treated or were dipped in water, 50 ppm ASC, and 1000 ppm ASC for 30 s. Fillets were stored at 1-2°C for 0, 8, and 15 d. A treatment by storage time interaction was observed for psychrotrophic counts. Aerobic plate counts were not affected by intervention, but increased during storage. Moisture, fat, pH, TBARS, fatty acid composition, TVBN, TMA, color, cook yield, and shear force were not affected by intervention. TBARS decreased during storage. Fatty acids were stable during storage.

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INTRODUCTION

Aquaculture is a growing industry in the United States. The decrease in wild seafood harvest and an increase in consumer demand has contributed to the increase in production of farmed fish. Most of these fish are marketed as fresh or frozen fillets and, post-mortem autolytic and microbiological changes reduce the storage stability of these products. The perishability of fish necessitates development of interventions to extend shelf life and preserve product quality.

Fish lipids are highly unsaturated and prone to oxidation during storage. Supplementation of fish diets with antioxidants may circumvent the deleterious effects of lipid oxidation. Several studies have focused on the post-harvest intervention of fillets with antimicrobials to reduce microbial spoilage. Expanded knowledge of ante and post-mortem factors affecting the storage of fresh and refrigerated fillets will be useful in improving product quality and marketability of these products. Thus, the purpose of this research was to 1) study the effect of storage time and temperature on storage stability of fillets, 2) determine the effect of vitamin E supplementation on product quality and 3) evaluate the use of a post-harvest antimicrobial additive to reduce spoilage of fillets and increase shelf life.

LITERATURE REVIEW

Postmortem changes in fish

After harvest, there is a progressive decline in initial quality due to autolysis and changes resulting from bacterial growth and metabolism (Haard 1992). Both processes may lead to poor functionality of end products (Cheng and others 1979; Scott and others 1988) and reduce shelf life (Huss 1988).

Bacterial changes

Microorganisms are present on the skin and in the gills and intestines of freshly caught fish. Liston (1980) stated a normal range of $10^2 - 10^7$ CFU/cm² on the skin surface. The gills and the intestines contain between 10^3 and 10^9 CFU/g (Shewan 1962). The bacterial flora on freshly-caught fish depends on the environment in which it is caught rather than on the fish species (Shewan 1977). Freshly caught fish and shell fish from warm waters generally carry mesophilic Gram-positive bacteria such as *Micrococcus*, *Coryneforms* and *Bacillus*. On the other hand, cold water species harbor predominately psychophilic Gram-negative microbes including *Moraxella*, *Acinetobacter*, *Pseudomonas*, *Flavobacterium* and *vibrio* genera (Liston 1980; Cann 1977).

After death, the immune system no longer functions allowing bacteria to proliferate freely. During refrigeration or storage on ice, fish harvested from temperate water spoil more quickly than fish caught from tropical waters because of the high loads of psychrotrophic gram-negative spoilage bacteria. These bacteria have a short time lag time before growth and lead to spoilage (Liston 1980; Ward and Baj 1988). Gonzalez and others (1999) determined the initial bacterial flora

associated with brown trout (*Salmo trutta*), wild pike (*Esox lucius*), and aquacultured rainbow trout (*Oncorhynchus mykiss*). Mesophilic counts for skin, gills and intestine were lower than 5×10^5 CFU/cm². An influence of environment on the flora of fish was not observed in this study. The skin of brown trout and gills of rainbow trout carried significant numbers of *Micrococcaceae* that were absent in the water samples. Also, psychrotrophic *Coryneforms* present on the skin and gills of wild pike were not isolated from the surrounding water. Among the three species, gills and intestines of brown trout were the most contaminated, while gills and intestines of rainbow trout had the lowest numbers. Rodriguez and others (2001) reported initial bacterial counts of $5.27 \log_{10}$ CFU/g (30°C) and $4.87 \log_{10}$ CFU/g (25°C) for rainbow trout fillets. Initial counts for salmon fillets were more than $6 \log_{10}$ CFU/g. trout fillets. Trout fillets were acceptable for 7 days; whereas, salmon fillets spoiled after 4 days of storage at 3°C. Surface counts of aerobic psychrotrophic flora exceeded 10^7 CFU/cm² in over-wrapped trout fillets stored at $1 \pm 1^\circ\text{C}$ for 6 days (Gimenez and others 2002). Catfish fillets tumbled with water at 60 rpm for 10 min, showed an increase in psychrotrophic counts with increasing storage time (Kim and others 2000).

The non-protein nitrogen (NPN) content of fish muscle is higher than NPN of muscle from terrestrial animals, ranging from 10% to 40% (Venugopal and Shahidi 1996). The high concentration of these compounds is responsible for the delicate and different flavors typical of seafood. These compounds serve as a substrate for spoilage organisms and are thereby converted to obnoxious smelling volatile bases. These compounds in fish and shelfish are nucleotides, free amino acids, peptides,

guanidino compounds, urea, and quaternary ammonium compounds (Finne 1992). Due to autolytic and bacterial activities, nitrogenous compounds are increased and thereby, produce amines, ammonia (NH_3), aldehydes, hydrogen sulphide (H_2S), mercaptans, indoles, and volatile bases and acids (Love 1980; Thomson and others 1980; Hultin 1984; Sikorski and Pan 1994). Fish contain trimethyl amine oxide (TMAO), and the amount of TMAO depends on species, environment, and in some cases, on fish age (Bystedt and others 1959). Bacterial enzymes convert TMAO to TMA (Babbitt and others 1972). Marine fish contain 1 – 100 mg of TMAO per 100 grams of muscle; whereas, fresh water fish contain 5 – 20 mg/100 g of muscle (Stansby and Olcott 1963). Production of TMA is dependent on species, sex, age, time of year and muscle type (Hughes 1959; Shirai and others 1988).

Volatile bases like trimethyl amine (TMA) and NH_3 are used to measure fish deterioration (Antoine and others 2002). TVBN (total volatile base nitrogen) is a group test that includes TMA, dimethyl amines (DMA) and NH_3 . TVBN increases with degree of spoilage, and it is predominately due to enzymatic activity. Its component, TMA, is a direct result of bacterial activity, and in some cases, its correlation with bacterial counts is poor (Gill 1990). Antoine and others (2002) correlated the production of TVBN with aerobic plate counts ($r=0.71$) and odor scores (0.84) in mahi-mahi (*Coryphaena hippurus*) fillets stored at 7°C for 10 days. On day 3, TVBN values reached the maximum value of 30 mg/100g, a value considered the limit of acceptability (Liston 1982; Farn and Sims 1986) and it correlated well with bacterial counts of 10^6 CFU/ g and odor scores. Rainbow trout

fillets exceeded a value 250 mg/kg on day 4 of storage on ice at $1\pm 1^{\circ}\text{C}$ (Giminez and others 2002).

Lipid oxidation

Fish lipids are highly unsaturated and prone to oxidation (Xiong 1994; Church 1998). Unstable hydroperoxides produced from oxidation of fatty acids or triglycerides are converted to free radicals, that accelerate the rate of autoxidation. Products of these secondary reactions contribute to the oxidized flavor of seafoods (Gray 1978; Khayat and Schwall 1983). Secondary autoxidation products are primarily aldehydes, ketones, alcohols, small carboxylic acids and alkanes. Several of these aldehydes can be determined as thiobarbituric acid reactive substances (FAO 1995).

An increase in TBA value was observed in Black skipjack muscle stored on ice. Initially, a value of 1mg malonaldehyde/100g was observed and this value increased to 5.5 mg malonaldehyde/100g after 24 days (Mazzora-Manzano and others 2000). Ryder and others (1984) reported, for mackerel (*Trachurus novaezelandie*), 8.3 mg malonaldehyde/100g after 7 days of ice storage. Fresh rainbow trout fillets, over wrapped with low-density polyethylene and stored at $1\pm 1^{\circ}\text{C}$ for 20 days, showed an increase in TBARS value during initial stages of storage. However, TBARS decreased when fillets became spoiled (Giminez and others 2002). This decrease may be due to the reaction of malondialdehyde with protein degradation products (Known and others 1965).

Amino acids, heme compounds, and organic acids, catalyze oxidation reactions alone or in association with trace metal ions (Castell and others 1969; El-Zeany and

others 1975; Jurewicz and Salmonowicz 1973). Khayat and Schwall (1983) studied the effect of copper, iron, and hemin on oxidation of Alaskan Pollack flesh homogenate and mackerel oil. They demonstrated acceleration of oxidation by these ions, and they reported the decreasing order of relative effectiveness in absorbing oxygen as: $\text{Fe}^{2+} > \text{hemin} > \text{Cu}^{2+} > \text{Fe}^{3+}$.

Enzyme systems that participate in oxidation of fish lipids include lipoxygenase (German and Kinsella 1985), localized in the skin and gills, and microsomal enzyme systems (McDonald and Hultin 1987). Lipid oxidation may lead to a decrease in nutritional quality, including loss of essential fatty acids, fat-soluble vitamins and essential amino acids, as well as possible toxicity development (Pearson and others 1983).

Changes in texture

Tenderness is the most important palatability attribute and sensory characteristic of fish and other muscle tissues (Ashie and others 1996). Fish and shell fish muscle contain less connective tissue, and the cross-links formed by their collagens are not as extensive as mammals (Suyama and Konosu 1987). Tenderization or flesh softening is associated with the disappearance of Z-disks, disassociation of the actomyosin complex, destruction of connectin, and general denaturation of collagenous tissues (Hatori 1986; Koohmarie and others 1986; Koohmarie and others 1988; Robbins and others 1979; Suyama and Konosu 1987). Færgemand and others (1995) evaluated the effect of post mortem storage time on fillet quality of rainbow trout (*Oncorhynchus mykiss*) stored on ice. Prolonged storage time had a negative effect on fillet texture. Fillets became softer with increasing storage time

(Azam and others 1989; Ando and others 1992). Sato and others (1991) showed that, in rainbow trout, solubility of type V collagen increases significantly during ice storage of fish and that the fillets become soft during chilled storage. Gutted trout fillets stored in ice (4-5°C) were acceptable for six days; subsequent storage resulted in dry texture and a slight bitter flavour (Rodriguez and others 1999).

Ca²⁺ dependent proteases and lysosomal cathepsins cause textural changes during postmortem storage (Koohmarie and others 1986; Koohmarie and others 1988; Jiang and others 1992; Jiang and others 1990). In mammals these autolytic changes are desirable, resulting in more tender meat. However, in fish these autolytic changes have deleterious effects resulting in texture degradation. Postmortem reduction in fish muscle pH affects the physical properties of muscle. As pH drops, the net surface charge on muscle proteins is reduced causing them to partially denature and lose some of their water holding capacity. Loss of water binding ability has a detrimental effect on fish texture. Love (1983) reported that texture of cooked cod becomes soft or sloppy when pH increases to above 7.

Factors influencing fish spoilage

Several factors influence spoilage of fish. However, initial handling techniques, temperature, and pH are perhaps the most important.

Handling

Some post harvest treatments may include stunning, bleeding, scaling, washing, and gutting and heading. Poor post harvest handling practices may promote deterioration. Freshly caught fish are sprayed or washed with clean water to remove surface contamination; removal of surface slime from very fresh fish may affect the

market life of fish as slime may contain antibacterial components such as lysozymes (Haard 1992). Bleeding of white-fleshed fish species often results in more attractive products. It may also minimize off-flavors, improve the quality of surimi prepared from such products, and contribute to an extended shelf life. However, it does not improve post harvest quality in all species (Haard 1992a). Gutted and headed haddock had lower psychrotrophic counts than the non eviscerated fish, although the differences were not significant (Ashie and others 1996). Gutted and headed orange roughy had an increase in shelf life due to delay in degradation of inosine monophosphate rather than a reduction in bacterial activity (Scott and others 1986).

Temperature and pH

Temperature affects the rate of fish spoilage through its impact on bacterial and autolytic enzymes, endogenous to fish. Temperature also affects the rate at which bacteria multiply (Rosinvalli and Charm 1975). Most of the biochemical activities are slowed down at low temperature. Olley and Lovern (1960) showed that protein denaturation and phospholipid hydrolysis is lower at reduced storage temperatures. Hydrolysis at -14°C was 10 times greater than at -22°C . Ashie and others (1996) reported that the temperature coefficient of fish is about 5, between 0 and 6°C , and the shelf life of properly handled and iced fish is about 8 to 12 days for most fish. Temperature can also cause discoloration in fish by influencing the sensitivity of myoglobins to autoxidation (Linnington and Brown 1981). Haard (1992a) reported that, during iced storage, initial color of seafood is changed. The bright shiny appearance of skin becomes dull and faded; the translucent appearance of white

muscle becomes opaque; and the bright red hue of the dark muscle becomes dull or brown. A cooked appearance is caused by rapid post harvest glycolysis and failure to rapidly cool the carcass (Haard 1992).

The pH of live fish is close to 7, and the postmortem pH of fish muscle can vary between 6.0 to 7.1, depending on season, species, and other factors (Simeonidou and others 1998; Church 1998). Postmortem pH decline is caused by lactic acid generated from glycolysis under anoxic conditions. The rate and extent of pH decline influences meat quality (Ashie and others 1996); whereas, during extended storage pH increases due to ammonia and amines produced by bacteria. Bilinski and others (1983) reported that, in spiny dog fish, pH changes are strongly influenced by spoilage temperature. Rapid chilling of dog fish to 0°C delayed the breakdown of nitrogenous compounds, thereby reducing the rate of spoilage. Rodriguez and others (2001) reported that pH increased from 6.42 to 6.61 in trout fillets stored at 3°C for 10 days. Similar results were observed in rainbow trout (Giminez and others 2002).

Antioxidant stabilization of lipids

Antioxidants may circumvent the deleterious effects of lipid oxidation on muscle food quality. Antioxidants are commonly incorporated into fish feeds that contain a high concentration of polyenic fatty acids to prevent lipid oxidation (NRC 1993). Antioxidants can prevent lipid oxidation by reacting with peroxy or alkoxy radicals; this action terminates lipid autoxidation by scavenging free radicals. Several antioxidants such as tocopherols, carotenoids, and ascorbic acid have been used in fish feed to reduce lipid oxidation and color loss in fish. Carotenoids may function as

antioxidants by quenching triplet sensitizers, singlet oxygen, and trapping free radicals (Krinsky 1992,1993). Bell and others (2000) suggested that vitamin E and the carotenoid, astaxanthin, have antioxidant functions in Atlantic salmon (*Salmo salar*). Ethoxyquin, BHT (3,5-di-tert-butyl-4-hydroxytoluene), BHA (2(3)-tertbutyl-4-hydroxyanisole), and propyl gallate are some of the synthetic antioxidants used in fish feed (NRC 1993). Gatlin and others (1992) conducted two feeding trials to study the effect of dietary vitamin E and synthetic antioxidants (ethoxyquin, butylated hydroxyanisole, butylated hydroxytoluene and Endox superTM) on composition and storage quality of channel catfish (*Ictalurus punctatus*). Fillet samples were subjected to forced oxidation by incubating under a stream of oxygen at 40°C for 2 h prior to thiobarbituric acid analysis. Forced oxidation of fillets was reduced in fish fed high levels of vitamin E; none of the synthetic antioxidants reduced oxidation. Neither vitamin E nor the synthetic antioxidants had any effect on growth, and feed efficiency of whole fish or proximate composition and fillet.

Vitamin E requirements

The antioxidant function of vitamin E makes it an essential nutrient in human and other animal diets (Watanabe and others 1981a, 1981b; Drevon 1991). The minimum requirement of vitamin E in rainbow trout ranges from 30 to 100 mg/kg of diet (NRC 1993). Vitamin E is an essential nutrient in salmonid diets (Watanabe 1990), and it is usually supplied as α -tocopherol acetate. Vitamin E is typically added at a level of 300-500 mg/kg diet (Gabaudan and Hardy 2000).

Common signs of vitamin E deficiency are muscular dystrophy involving atrophy and necrosis of white muscle fibers; edema of heart, muscle, and other tissues due

to increased capillary permeability allowing exudates to escape and accumulate; anemia and impaired erythropoiesis; depigmentation; and ceroid pigment in liver (NRC 1993). Vitamin E deficiency signs have been studied in Atlantic salmon (Poston and others 1976), channel catfish (Lovell and others 1984), common carp (Watanabe and others 1981a) and rainbow trout (Cowey and others 1981, 1983; Hung and others 1981; Watanabe and others 1981b). Poston and Livingston (1969) reported that supplementation with very high doses of DL - α -tocopherol (5,000 mg/kg of diet) reduced the concentration of erythrocytes in trout blood. Supplementation with 2500 mg/kg of α -tocopherol did not produce adverse effects in channel catfish (Lovell and others 1984).

Tissue distribution of vitamin E

Vitamin E is a major lipid soluble, free radical scavenging antioxidant *in vivo*. Tocopherols in active biological cells concentrate largely in phospholipid rich cellular regions like the mitochondria and microsomes (Krishnamurthy and Bieri 1963; Gruger and Tappel 1971). Hamre and Lie (1997) suggested that each organ may have a unique combination of mechanisms whereby they take up lipids and tocopherols from plasma. Zhang and others (1996) examined the dietary uptake of vitamin E in laboratory rats supplemented with vitamin E (1mg/kg of diet). Plasma α -tocopherol concentration reached a maximum after 48 h, and the liver was the main organ where the vitamin accumulated. Kidney, heart, muscle and brain levels continued to increase throughout a six week period. The level of α -tocopherol decreased slowly when the supplement was removed from the diet. Catignani and Bieri (1977) reported the presence of a α -tocopherol-binding protein in rat liver, and

the protein (30 kDa) exists uniquely in the hepatocytes of rat liver and regulates plasma tocopherol concentration by preferentially incorporating α -tocopherol into nascent, very low density lipoproteins (Dutta-Roy and others 1994). Tissue distribution of α and γ -tocopherol in Atlantic salmon (*Salmo salar*) was studied by Parazo and others (1998). Their results demonstrated the importance of the liver in selecting α -tocopherol over γ -tocopherol; additionally, a protein analogous to the 30 kDa α -tocopherol binding protein exists in Atlantic salmon liver because the liver appeared to be the most biased organ for accumulating α -tocopherol. During restricted intake of α -tocopherol, loss of α -tocopherol in muscle and liver was greater than in other tissues. Similar results were observed by Cowey and others (1981) in rainbow trout, suggesting the possibility of translocation of tocopherols from the muscle to other critical organs during deprivation.

Vitamin E and oxidative stability of fillets

Fish lipids are rich in omega-3, polyunsaturated fatty acids (Stephan and others 1995), and oxidation of polyunsaturated fatty acids causes rancidity in the fish that often results in deterioration of color, texture and flavor (Frigg and others 1990; Waagbø and others 1993). Leibovitz and others (1990) and Hu and others (1989) demonstrated that enrichment of tissues with α -tocopherol reduces oxidation of fatty acids in tissues subjected to activate oxidation, even if these tissues contain high levels of *n*-3 polyunsaturated fatty acids.

Membrane-bound α -tocopherol stabilizes membrane lipids in broiler and pork muscle, resulting in reduced lipid oxidation during refrigerated and frozen storage (Buckley and others 1988). Similarly, O'Keefe and Noble (1978) found that dietary

levels of α -tocopherol above 400 mg/kg diet were effective in increasing the storage stability of channel catfish fillets.

Frigg and others (1990) studied the effect of dietary vitamin E levels on oxidative stability of trout fillets. Rainbow trout (*Salmo gairdneri*) were fed 0, 50, 100, and 200 IU of supplemental vitamin E/kg feed for 85 days. Levels of α -tocopherol in plasma, liver and fillets were directly related to dietary vitamin E levels. The amount and characteristics of lipids were not influenced by vitamin E. Malondialdehyde (MDA) values were markedly dependent on dietary vitamin E level and α -tocopherol level in the fillet.

Boggio and others (1985) reported a significant increase in tissue α -tocopherol levels of rainbow trout (*Salmo gairdneri*), fed fish oil or swine fat and supplemented with 500 and 1500 mg of α -tocopherol acetate per kg of diet, compared to fish fed 0 and 50 mg/kg diet for 4 months prior to harvest. Eventhough fish fed 1500 mg/kg vitamin E had higher α -tocopherol levels than trout fed 500 mg/kg, these levels did not reduce lipid oxidation after frozen storage at -80°C for 4 months. Lipid oxidation did not decrease in fish fed high levels when compared to 0 and 50 mg/kg vitamin E fed fish after storage at -20°C for 10 months. Dietary α -tocopherol concentration did not affect fatty acid composition; whereas, dietary lipid source altered the fatty acid composition of muscle and viscera. Dietary fat source or α -tocopherol acetate did not affect sensory attributes of trout fillets evaluated fresh or after prolonged storage. Similarly, in turbot (*Scophthalmus maximus*), α -tocopherol supplementation (70 and 320 mg/kg of diet) reduced thiobarbituric acid reactive substances (TBARS) in fresh tissue. Peroxidation of polyunsaturated fatty acids (PUFA) is inhibited more in liver

than in muscle. Storing of muscle at - 20°C for 6 months increased muscle TBARS value, and these values were higher in turbot fed cod liver oil or low α -tocopherol diets (Stephan and others 1995). Vitamin E supplemented (300 mg/kg diet) Atlantic salmon fillets had three times higher α -tocopherol levels than fillets from the non-supplemented group. Vitamin E supplementation did not alter fatty acid composition or lipid levels. Fillet α -tocopherol content was not affected by freezing, storing, or smoking. Sensory parameters were not influenced by lipid quality or dietary supplementation with α -tocopherol (Waagbø and others 1993).

Chaiyapechara and others (2003) conducted a 10 week feeding trial on rainbow trout (*Oncorhynchus mykiss*). Increasing vitamin E concentration lowered TBARS values in fillets stored for 7 days at 4°C and -30°C. Dietary vitamin levels had no effect on L* value. However, an increase in dietary lipid concentration increased whiteness (L*) of fillets, but not the a* and b* values. They suggested that increasing the concentration of vitamin E from 300 to 1500 mg could reduce the “fishy” aroma in fillets of trout fed a 30 % lipid diet.

Chemical preservatives and shelf life extension

The growing demand for fresh fish has necessitated development of new technologies to extend shelf life. Chemical preservatives are applied as dips or additives to delay spoilage of fresh fish. Chemicals used to delay microbial spoilage include sodium hypochlorite, broad spectrum antibiotics, ethylenediaminetetraacetic acid (EDTA), glucose oxidase and organic acids (Haard 1992).

Kim and others (1997) showed that TBA values of Salmon and red grouper fillets treated with 100 and 200 ppm ClO_2 are greater than control and 20 ppm fillets; and a dose dependent response was observed. Chlorine dioxide did not affect fatty acid composition of fillets. There were no differences in monounsaturated and polyunsaturated fatty acids compared to controls. Kim and others (1998) reported that chlorine dioxide did not affect proteins, fats, vitamins and minerals in Salmon and red grouper. Zhang and others (2003) reported that fresh haddock fillets treated with 200 ppm sodium chlorite can be stored for about 18 days at 4°C.

Acidified sodium chlorite (ASC) is approved by the U.S. Food and Drug Administration as a 'secondary direct food additive permitted in food for human consumption'. ASC is approved at a concentration of 500–1,200 ppm for use on poultry, red meat, comminuted meat products and processed fruits and vegetables to reduce bacterial contamination. It can also be used in water or ice at concentrations of 40-50 ppm to rinse, wash, thaw, transport, or store seafood. ASC is an antibacterial agent prepared by mixing sodium chlorite with a generally - recognized - as - safe acid (Anonymous 1999); acids that are typically used include citric acid and phosphoric acid. The antimicrobial activity of acidified sodium chlorite is attributed to the oxidative effect of chlorous acid that is generated when the chlorite ion is converted to its acid form under acidic conditions. Chlorous acid is a metastable oxychlorine species that decomposes to form chlorate ion, chlorine dioxide, and chloride ion (Gordon and others 1972). As chlorous acid is being depleted, residual chlorite produces more chlorous acid and continues the antimicrobial activity until the solution evaporates or dries on the product surface.

The solution is prepared immediately before its application because the reactions occurs instantaneously in the mixed solution (Castillo and others 1999). It is hypothesized that the mode of action of ASC is due to the uncharged chlorous acid; it penetrates bacterial cell walls and disrupts protein synthesis by reacting with sulfhydryl, sulfide, and disulfide containing amino acids and nucleotides. The undissociated acid adversely affects amino acid transport by facilitating proton leakage into cells and thereby increases energy use by cells to maintain internal pH (Warf and Kemp 2001). When ASC comes in contact with organic matter, a number of oxychlorous antimicrobial intermediates are formed. These intermediates are broad spectrum germicides that act by breaking oxidizable bonds on cell membrane surfaces. Su and Morrissey (2003) demonstrated that the antimicrobial activity of ASC was not due to low pH, but primarily due to the generation of bactericidal chemicals, including chlorine dioxide, and hypochlorous acid (HClO₂).

Castillo and others (1999) compared the efficacy of phosphoric acid-activated, ASC and citric acid-activated, ASC as decontaminants on *Escherichia coli* O157: H7 and *Salmonella typhimurium* inoculated beef carcasses. Water wash treatment followed by citric acid-ASC spray significantly reduced *Escherichia coli* O157: H7 and *Salmonella typhimurium* by 4.5 and 4.6 log CFU/ cm² respectively; whereas, water wash alone resulted in a reduction of only 2.9 log units. Nonetheless, 30–50 % of the carcasses treated by ASC yielded countable colonies. Treatment of prewashed poultry carcasses with ASC reduced total aerobes by 90.7%, *Escherichia coli* by 99.6% and total *Coliforms* by 98.5%. A mild whitening of the skin was

observed in carcasses treated with a 1,200 ppm ASC solution (Kemp and others 2000).

Kemp (2001) reported that immersion dipping of three species of snapper for 2 minutes in ASC followed by storage at -3°C in ice or ice/brine solution increased shelf life between 12.9 and 19.1 days depending on the species. Similarly, shelf life of fresh cod was extended by 10 days over the control, untreated cod. Eun and others (2001) dipped whole croaker in different concentrations of ASC for 5 and 10 min. Dipping whole croaker in 50 ppm ASC resulted in a 1-log reduction of psychrotrophic bacteria and total coliforms. A 600 ppm solution resulted in a 1-log reduction of mesophilic bacteria and a 1-log reduction in Gram-negative bacteria. Whereas, a 1000 ppm dip resulted in a 3-log reduction of mesophilic bacteria, and a 2-log reduction of psychrotrophic bacteria. A 1000 ppm ASC dip caused a bleached appearance on whole croaker. There were no differences in these data associated with dipping times. Su and Morrissey (2003) studied the antimicrobial activity of ASC with a focus on *Listeria monocytogenes* in salmon. Total plate counts of skinless fillets ranged from 2.83 to 2.89 CFU/g, and ASC treatment (50 ppm) reduced the initial counts by 0.3 log units. Storage of fillets in ice or at 5° C for 7 days increased total plate counts in fillets. However, counts for acidified sodium chlorite treated samples were 0.34 (5°C) and 0.61(in ice) log₁₀ units lower than the control samples. ASC solution did not affect anaerobic counts. ASC treatment of salmon skin reduced total plate counts by 0.43 log CFU/cm². Compared to the control samples, *Listeria monocytogenes* counts were 0.2 log units lower in fillets stored in ASC ice and 0.62 log units lower in fillets washed with ASC solution and

stored in ASC ice for 7 days. Dipping of salmon fillets in ASC solution at a sodium chlorite concentration of 100 ppm for 1 min resulted in noticeable color loss. Similar discoloration was observed in salmon fillets treated with 100 or 200 ppm of chlorine dioxide (Kim and others 1999).

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CHAPTER 1

TIME AND TEMPERATURE AFFECT STORAGE STABILITY OF RAINBOW TROUT (*Oncorhynchus mykiss*) FILLETS

ABSTRACT

Fresh rainbow trout, *Oncorhynchus mykiss*, fillets were placed on styrofoam trays and over-wrapped with polyvinyl chloride (PVC) film. Fillets were stored at 0 and 4.5°C for 0, 3, 6 and 9 d. Psychrotrophic counts, color and TBARS of raw fillets and shear force of cooked fillets were determined at 0 d and through 9 d of storage. A two by four factorial, randomized complete block design was used. The experiment was replicated three times. Psychrotrophic counts increased as temperature and storage time increased. The a^* -values increased to 3 d, and this value was maintained through 9 d storage. For L^* -values, there was a temperature by time interaction ($p < 0.05$). Fillets stored at 0°C for 0 and 3 d were darker compared to all fillets stored at 4.5°C and fillets stored at 0°C for 6 and 9 days. Fillet b^* -values were higher at 4.5°C than at 0°C. Storage time did not affect b^* -value. Fillets stored at 0°C had a higher pH than fillets stored at 4.5°C; pH increased during storage ($p < 0.05$). Storage temperature did not affect TBARS, but by 3 d storage, TBARS level were higher than at 0 d storage; values decreased through 9 d storage. Kramer shear force was not affected ($p > 0.05$) by storage temperature. However, response peaked at day 6 of storage (199.0 g/g) and declined to 175.9 g/g by day 9.

Keywords: rainbow trout, storage time, storage temperature

INTRODUCTION

The growing demand for fresh fish has necessitated development of new technologies to improve storage stability. After harvest, autolysis and bacterial growth lead to a progressive decline in fish quality (Haard 1992). Freshness of fish is lost even before spoilage occurs (Ehira and Uchiyama 1986). Loss of freshness is mainly due to biochemical and enzymatic changes; whereas, spoilage is due to bacterial action (Vazquez-Orbitz and others 1997). During refrigeration or iced storage, fish harvested from cold waters spoil much quicker than the fish from warm waters due to high loads of psychrotrophic, Gram-negative spoilage bacteria, that decrease the lag time before growth and lead to subsequent spoilage (Liston 1980; Ward and Baj 1988).

Undesirable, deteriorative quality changes continue during processing and storage and reduce shelf life of fish products. Storage temperature is an important factor affecting freshness and quality (Singholt and others 1997). Temperature abuse increases the rate of microbial growth (FAO 1995), and it causes discoloration in fish by increasing the sensitivity of myoglobin to autoxidation (Linnington and Brown 1981). Storage time on ice is an important factor affecting texture of rainbow trout fillets (Færgemand and others 1995). Because rainbow trout fillets are highly perishable, establishing storage limits for fresh trout fillets will facilitate development of appropriate interventions to optimize quality. Hence, the objective of this study was to obtain basic information about the storage stability of rainbow trout fillets stored at different temperatures and times.

MATERIALS AND METHODS

Fish Fillet preparation

Rainbow trout (*Oncorhynchus mykiss*) were obtained from The Conservation Funds, Freshwater Institute. Fish were packed in ice and transported to West Virginia University within 3 h of harvest. Gutting and filleting were performed manually, and fish were subsequently washed in a dilute salt / ice slurry. Fillets were drained for approximately 14-16 h at 0°C. Subsequently, fresh fillets were aerobically packed on styrofoam trays and over-wrapped with polyvinyl chloride (PVC) film. Fillets were stored in the dark at 0 and 4°C for 0, 3, 6, and 9 days.

Sample processing

On one half of the fillet, a 3.81cm dia core was removed at the cranial end; the skin remained on the core for microbial analysis. The remainder of the fillet was skinned, divided into small pieces (excluding belly flap), frozen in liquid N₂, and powdered in a commercial blender (Waring, New Hartford, Conn., U.S.A) for 2 to 3 min. Powdered samples were used to determine thiobarbituric acid reactive substances (TBARS) and pH. The alternate side of the fillet was cooked to an internal temperature of 65°C to measure shear force. Color measurements were recorded prior to cooking this portion of the fillet.

Experimental analyses

pH: Five grams of powdered sample was mixed with 50 mL of deionized distilled water to determine pH. The pH of the slurry was measured using a pH/ion analyzer (Model 350, Corning Inc.; Corning NY, USA) with a general purpose combination probe.

Lipid oxidation: Lipid oxidation in the powdered samples of all fillets was measured by the 2-thiobarbituric acid method (Yu and Sinnhuber 1957). Absorbance was read at 535 nm using a Spectronic 20D spectrophotometer (Milton Roy, NJ, U.S.A). Results were reported as mg malonaldehyde (MDA) per kg of sample.

Psychrotrophic counts: Surface counts of psychrotrophic bacteria were determined on all fillets. A 11.4 cm² core with the skin attached was removed from the cranial end of the fillet. The cored sample was blended with 25 ml of 0.1% peptone water in a Stomacher 400 blender (Tekmar, Cincinnati, Ohio, U.S.A) for 30 sec. A 1 mL aliquot of the homogenate was serially diluted with peptone water and each diluent was spread plated on duplicate plates of plate count agar (Swanson and others 1992). At day 0, a 0.1 mL aliquot was directly spread on the plates. The initial ratio of sample to diluent was 0.912 cm²/mL. Inverted plates were incubated at 25°C for 24 h. Counts were reported as log₁₀ CFU/cm².

Color: Fillet color was measured with a Minolta chromameter (Model CR-300, Minolta Camera Co. Ltd.; Osaka, Japan) calibrated using a standard white plate No.21333180 (CIE L* 93.1; a* 0.3135; b* 0.3198). L* (Lightness), a* (redness), and b* (yellowness) values were measured at the cranial or caudal half of the right or left side of the fillet. The average of these two values was used as the observation for that fillet.

Texture characteristics: A 3 x 8 cm strip was removed from the cranial portion of the fillet above the lateral line. This portion of the fillet was placed in the sample holder with the skin side up, and shear force was measured with a 5-blade Kramer shear attachment of Texture Analyzer (Model TA-HDi, Texture Technologies Corp.;

Scarsdale, NY) at a cross head speed of 125 mm/min. Shear force was expressed as g force/g of sample.

Experimental design

A two by four factorial, randomized complete block design with day as block was used (Appendix 1). Treatment combinations of two temperatures (0 and 4°C) and four storage times (0, 3, 6, and 9 days) were replicated three times on separate days. Data were analyzed using the General Linear Model (GLM) procedure of SAS (SAS Institute, Inc., 1999) to establish main and interaction effects. Significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

pH

Fillets stored at 0°C had higher pH ($p < 0.05$) than fillets stored at 4.5°C (Table 1); Fillet pH increased ($p < 0.05$) to 3 days of storage and thereafter declined to levels higher than values observed at 0 d (Table 2). Rodriguez and others (2001) reported an increase in pH from 6.42 to 6.61 in rainbow trout fillets stored at 3°C for 10 days. The increase in pH during storage may be due to production of NH_3 and amines by bacterial action during storage.

Microbial analysis

Psychrotrophic counts increased as storage temperature and time increased ($p < 0.05$) (Figure 1). Psychrotrophic bacteria grew more quickly on fillets stored at 4.5°C than at 0°C, and significant differences were observed after 3 days of storage ($p < 0.05$). Seafood harvested from cold waters spoil quickly because of high loads of

psychrotrophic, gram-negative spoilage bacteria that decrease the lag time and lead to spoilage during iced and refrigerated storage (Liston 1980; Ward and Baj1988). The counts increased from 2.7 to 6.0 and from 2.9 to 8.8 log₁₀ CFU/cm² from 0 to 9 d for 0 and 4.5°C storage, respectively. Gimenez and others (2002) reported that psychrotrophic counts in rainbow trout fillets stored at 1±1°C for 4 days exceeded the acceptable limit of 10⁷ CFU/cm² where spoilage odors appear (Ordóñez and others 2000). However, Rodríguez and others (2001) reported that rainbow trout fillets stored at 3°C were acceptable for 7 days when the mean aerobic counts (30°C) increased from 5.27 to 8.76.

Lipid oxidation

Temperature did not affect ($p>0.05$) TBARS (Table 1). But by 3 d storage, TBARS were higher than at 0 d storage and this level decreased through 9 d storage ($p<0.05$) (Tables 2). Gimenez and others (2002) also reported similar trends in over-wrapped rainbow trout fillets stored at 1±1°C for 20 days. They suggested that the decline in TBARS during storage after an initial increase may be due to reaction of malonaldehyde with protein degradation products (Known and others 1965); interaction decreases availability of malonaldehyde for calorimetric determination.

Color

L* values were affected by the interaction of time and temperature ($p<0.05$). Fillets stored at 0°C for 0 and 3 d were darker compared to all fillets stored at 4.5°C and fillets stored at 0°C for 6 and 9 days (Figure 2). The a*-values increased to 3 d, and this value was maintained through 9 d storage ($p<0.05$) (Table 2). These values were not affected ($p>0.05$) by storage temperature (Table 1). Color b*-values were

higher for the fillets stored at 4.5°C than at the 0°C ($p<0.05$) (Table 1). Storage time did not affect b^* -value ($p>0.05$) (Table 2). Haard (1992) reported that the bright shiny appearance of skin becomes dull and faded; the translucent appearance of white muscle becomes opaque, and the bright hue of the dark muscle becomes dull or brown during chilled storage.

Shear force

Kramer shear force was not affected ($p>0.05$) by storage temperature (Table 1). However, this response peaked at day 6 of storage (199.0 g/g) and declined by day 9 (175.9 g/g) (Table 2). Fægemand and others (1995) reported that rainbow trout fillets become soft on storage at 0°C for 10 days.

CONCLUSIONS

Storage temperature and time affected fillet quality of rainbow trout. Fillets stored at 4.5°C had higher psychrotrophic counts than fillets stored at 0°C. TBARS were significantly higher on day 3 of storage. These results suggest that fresh fillets over wrapped with PVC should not be stored more than 6 days at 0°C and 3 days at 4.5°C. Basic information on the affect of storage temperature and time on storage stability of fresh rainbow trout would greatly benefit its processing and interventions to improve shelf life.

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Table 1. Effect of storage temperature on muscle pH, TBARS, a* value, b* value, and shear force.

Trait	Temperature, °C	
	0	4.5
Muscle pH	6.32 ^b	6.26 ^a
TBARS, MDA mg/kg	3.2 ^a	3.3 ^a
b* value	7.39 ^a	8.35 ^b
a*value	0.62 ^a	0.56 ^a
Shear force, g/g	176.54 ^a	184.92 ^a

^{ab}Means within a row with different superscripts are different (p<0.05).

Table 2. Effect of storage time on muscle pH, TBARS, a* value, b* value, and shear force.

Trait	Storage time, day			
	0	3	6	9
Muscle pH	6.18 ^a	6.37 ^c	6.31 ^{bc}	6.29 ^b
TBARS, MDA mg/kg	0.8 ^a	4.3 ^b	4.2 ^b	3.7 ^b
b* value	7.78 ^a	7.39 ^a	8.59 ^a	7.70 ^a
a*value	0.09 ^a	0.64 ^b	0.85 ^b	0.80 ^b
Shear force, g/g	172.84 ^a	175.13 ^a	199.03 ^b	175.93 ^a

^{abc} Means within a row with different superscripts are different (p<0.05).

Figure 1. Effect of storage time and temperature on psychrotrophic counts.

^{abcde} Bars with different superscripts are different ($p < 0.05$).

Figure 2. Effect of storage time and temperature on L* value.

^{ab} Bars with different superscripts are different ($p < 0.05$).

APPENDICES

APPENDIX

Statistical program for all responses

```
option LS=80 pagesize=60 nonumber;  
title;  
data response; input wk $ tank $ day $ temp $ response;  
cards;  
proc print;  
proc glm; classes wk day temp response;  
model response=wk day temp temp*day;  
lsmeans day temp temp*day/pdiff;  
run;
```

CHAPTER 2

EFFECT OF VITAMIN E SUPPLEMENTATION ON STORAGE STABILITY OF RAINBOW TROUT (*Oncorhynchus mykiss*) FILLETS

ABSTRACT

Lipid oxidation decreases the storage quality of fresh and refrigerated seafood products. Rainbow trout were fed a commercial diet containing 47% crude protein, 18% crude fat, and 3% crude fiber or the commercial diet supplemented with vitamin E to an α -tocopherol concentration of 5000 mg/kg feed. Fish were fed for 9 weeks, and they were sampled for analyses at weeks 2, 3, 4, 5, 7, and 9. A split-split plot arrangement of treatments in a 2x6x3 factorial, completely randomized design was employed with three replications. At harvest, fish were mechanically stunned and immediately placed on ice in portable coolers for transport not exceeding 3 h. Fish were manually eviscerated and filleted, and fillets were subsequently washed in a dilute salt / ice slurry. Fillets were placed on styrofoam trays and over-wrapped with polyvinyl chloride (PVC) film. Fillets were stored in the dark at 0°C for 0, 7, and 14 days. Fillets were analyzed for α -tocopherol, lipid oxidation (TBARS), pH, cook yield, shear force, psychrotrophic counts, moisture and fat. At week 9, fish in addition to the routine sample, were fasted for three days prior to slaughter and analyzed for α -tocopherol, lipid oxidation, psychrotrophic counts, moisture and fat. Liver samples were obtained from all eighteen fish at the end of week 7 and 9 and also from fish fasted for three days prior to harvest at week 9. At the end of 9 weeks, all fish were returned to the control feed for three weeks. At 12 weeks, six liver samples were collected for α -tocopherol determination. Vitamin E supplementation increased fillet α -tocopherol content ($p < 0.05$). Storage time and fasting at 9 weeks had no effect on fillet vitamin E content ($p > 0.05$). The liver accumulated more vitamin E than muscle. At day 0 storage, TBARS value was not

affected ($p>0.05$) by vitamin E supplementation for any sampling week. Fasted fish generated less ($p<0.05$) TBARS compared to non-fasted fish. For fillet pH, significant ($p<0.05$) diet by storage time and duration of feeding by storage time interactions was observed. Color L^* value was not affected by diet, duration of feeding or storage time interactions ($p>0.05$). Fillets were least red at 14 days of storage ($p<0.05$), and b^* values increased ($p<0.05$) with storage time. A significant ($p<0.05$) duration of feeding by storage time interaction was observed for fat content, psychrotrophic counts, cook yield, and shear force. At day 0, fasted fish had lower ($p<0.05$) psychrotrophic counts than non-fasted fish. Following fasting, a slight increase in moisture and a decrease in fat content was observed on day 14.

Keywords: vitamin E, lipid oxidation, storage stability

INTRODUCTION

Fish lipids are rich in omega-3 fatty acids that are essential for human health. Lipid oxidation is a major concern in post-mortem processing and subsequent storage of fish. Lipid oxidation deteriorates quality of fish flesh, and thus marketability of fish products. Dietary fortification of fish feed with antioxidants increases tissue levels of antioxidants thereby reducing lipid oxidation (Frigg and others 1990; Baker and Davies 1996).

Vitamin E is a lipid soluble antioxidant. In *vivo*, and as a part of an antioxidant defense mechanism, vitamin E protects the cells from reactive oxygen and free radicals generated by autoxidation of membrane lipids and proteins. Among the natural forms of vitamin E, d- α -tocopherol has the highest biopotency (NRC 1993) and it is usually supplemented as α -tocopherol acetate in fish feed. Supplementation of α -tocopherol has been effective in reducing lipid oxidation in rainbow trout fillets (Frigg and others 1990; Chaiyapechara and others 2003). Bai and Gatlin (1993) reported that muscle α -tocopherol concentration, depends on dietary vitamin E concentration and duration of feeding. Prolonged feeding times did not increase vitamin E content in turbot (*Scophthalmus maximus*) (Ruff and others 2003). It is not clear if supplementation of finishing diets with extremely high doses of vitamin E for a short duration can improve the storage stability of fresh, refrigerated fillets. To characterize the impact of vitamin E supplementation on rainbow trout fillet quality, high doses of vitamin E were fed during the last 9 wk of production.

MATERIALS AND METHODS

Diet

A Commercial fish diet (Melick Aquafeeds, Inc., Catawissa, PA, U.S.A) containing 47% crude protein, 18% crude fat, and 3% crude fiber served as the control (C). The vitamin E supplemented diet (VE) was prepared by enriching the control diet with Lutavit E[®] (BASF, Mount Olive, NJ, U.S.A). Lutavit E[®] contained vitamin E acetate oil and silicon dioxide. This supplement was added to the control diet to provide 5000 mg/kg (IU/kg) of DL-alpha-tocopheryl acetate in the finished feed. Control and treated diets were prepared once at the initiation of the trial, and they were stored until the end of the study. Concentrations in the feed were monitored throughout the study (Appendix 1).

Sampling of fish

Initially, six fish from tanks assigned to control and vitamin E supplemented tanks were sampled to determine fish weight, and three fish were sampled to determine initial α -tocopherol concentration of the muscle. At 2, 3, 4, 5, 7, and 9 weeks of feeding, three fish were sampled from each of the control and vitamin E supplemented tanks. At the end of week 9, fish in addition to those sampled routinely, were fasted for three days prior to harvest, and they were sampled from both control and vitamin E tanks. At each week of sampling, fish were mechanically stunned and placed on ice in portable coolers for transport to West Virginia University for processing within 5 h of harvest. Fish were manually filleted and eviscerated; fillets were subsequently washed in a dilute salt / ice slurry at approximately 17 mM. They were aerobically packed on styrofoam trays and over-

wrapped with polyvinyl chloride (PVC) film. Fillets were stored in the absence of light at 0°C for 0, 7, and 14 days. At each sampling week by storage time combination, samples were analyzed for α -tocopherol concentration, lipid oxidation, pH, cook yield, shear force, psychrotrophic counts, moisture and fat. At week 9, fish fasted for three days prior to harvest were also analyzed for α -tocopherol concentration, lipid oxidation, psychrotrophic counts, moisture and fat. Liver samples were obtained from all eighteen fish (3 fish x 2 treatments x 3 replications) at weeks 7 and 9 and also from fish fasted for three days prior to harvest at week 9. At week 9, all fish were realimentated to the control feed for three weeks. After this 3 week period, twelve liver samples were collected for α -tocopherol analyses, six from treated tanks and six from control tanks.

Sample preparation for chemical analyses

On one side of the fillet, a 11.4 cm² core was removed from the cranial portion of the fillet dorsal to the lateral line. The core contained muscle tissue and skin and it was used for determination of microbial levels on the fillet. The remainder of the fillet was skinned, cut into small pieces (excluding belly flap), frozen in liquid N₂, and powdered in a commercial blender (Waring, New Hartford, Conn., U.S.A) for 2 to 3 min. Powdered samples were used to determine proximate composition, lipid oxidation, and α -tocopherol content. The alternate side of the fillet was cooked to an internal temperature of 65°C. Upon cooling to room temperature, a portion of the dorsal musculature was removed to measure shear force and cook yield. Color was measured before the fillets were cooked.

Chemical and physical analysis

Proximate composition: Proximate composition was determined according to AOAC procedures (1990).

pH: Sample pH was measured using a pH/ion analyzer 350 (Corning Inc.; Corning, NY) equipped with a flat surface combination probe. Fillet pH was measured at the cranial and caudal ends of the fillet. The average of the two values was recorded as fillet pH.

α -tocopherol determination: Concentration of α -tocopherol in the muscle, liver and feed was determined according to the simplified extraction procedure of Liu and others (1996). Fillet α -tocopherol content was determined by extracting 1g of muscle. Liver and feed α -tocopherol was measured by extracting 0.25 g of sample. Vitamin E content was quantified using high-performance liquid chromatography (HPLC) with a Perkin Elmer series 4LC and a LC-75 spectrophotometric detector. Peak area of α -tocopherol was recorded and integrated using a Varian 4270 integrator. A Waters C-18 resolve silica, 5 μ m (39x150 mm) column, equipped with a guard column was used for all separations (Waters, Milford, MA, U.S.A). The mobile phase consisted of 96 % iso-octane: 4% tetrahydrofuran (v/v) (Fisher Inc., PA, U.S.A) and was prepared fresh daily. D,L- α -tocopherol (Sigma-aldrich, St.Louis, MO, U.S.A), was used to establish the standard curve for quantification of α -tocopherol.

Lipid oxidation: Lipid oxidation of the powdered samples was measured calorimetrically using 2-thiobarbituric acid (Yu and Sinnhuber 1957). Absorbance was read at 535 nm using a Spectronic 20D spectrophotometer (Milton Roy, NJ, U.S.A). Results were reported as mg malondialdehyde (MDA) per kg of sample.

Psychrotrophic counts: Psychrotrophic bacterial counts were determined on all fish samples at weeks 2, 3, 4, 5, 7, and 9 of feeding on day 0, 7, and 14 of storage; nine-week fasted fish were evaluated on day 0 and 14 of storage. A 11.4 cm² core, with skin attached, was removed from the cranial end of the fillet using a steel corer. The cored sample was blended with 25 mL of 0.1% peptone water in a Stomacher 400 blender (Tekmar, Cincinnati, Ohio, U.S.A) for 30 sec. A 1 mL aliquot of the homogenate was serially diluted with peptone water, and each diluent was spread plated on duplicate plates of plate count agar (Swanson and others 1992). At day 0, a 0.1 mL aliquot was directly spread on the plates. The initial ratio of sample to diluent was 0.912 cm²/mL. Inverted plates were incubated at 25°C for 24 h. Counts were reported as log₁₀ CFU/cm².

Color measurement: Fillet color was measured with a Minolta chromameter (Model CR-300, Minolta Camera Co Ltd.; Osaka, Japan). The instrument was calibrated using a standard white plate No.21333180 (CIE L* 93.1; a* 0.3135; b* 0.3198), and L* (lightness), a* (redness), and b* (yellowness) values were recorded at two randomly chosen locations on the cranial and caudal ends of the fillet. The mean of these two values was used as the color trait observation for that fillet.

Shear force and cook yield: Shear force was measured using a Texture Analyser (Model TA-HDi, Texture Technologies Corp.; Scarsdale, NY) with a 5-blade Kramer

shear attachment at a cross head speed of 127 mm/min. A 3 x 8 cm strip of the cooked fillet was removed from the dorsal musculature, skinned, weighed, and placed beneath the blades with the skin side up. Values were expressed as g force/g of sample. Cook yield was calculated by expressing cooked fillet weight as a percent of raw fillet weight.

Experimental design and statistical analyses

A split-split plot arrangement of treatments in a 2x6x3 factorial, completely randomized design was conducted with three replications (Appendix 2). Factors A, B, and C were diet, duration of feeding, and storage time, respectively. Dietary treatments (control and vitamin E) were the main plot, duration of feeding (2, 3, 4, 5, 7, and 9 wk) was the sub-plot, and storage time (0, 7, and 14 d) was the sub-sub plot. The General Linear Model (GLM) procedure of SAS (SAS Institute Inc., 1999) was used to analyze the data. PDIFF of the lsmeans procedure was used for separating means, and significance was established at $P < 0.05$.

Weight gain was analyzed as a 2x6 factorial, split-plot completely randomized design with dietary treatments as the main plot and duration of feeding as a sub-plot (Appendix 3). Moisture, fat and vitamin E content of the fasted fish (week 9) was analyzed as a 2x2x3 factorial, split-split plot completely randomized design with dietary treatments as the main plot, fasting as a subplot (fasted and not fasted), and storage time as a sub-sub plot (Appendix 4). Psychrotrophic counts on the fasted fish (week 9) were analyzed as a 2x2x2 factorial, split-split plot completely randomized design with dietary treatment as the main plot, fasting as a sub-plot and storage time (0 and 14 d) as a sub-sub plot (Appendix 5). Following 14 d of

refrigerated storage, lipid oxidation in fasted fish (week 9) was analyzed as a 2x2 factorial, split-plot completely randomized design with dietary treatments as the main plot and fasting as a sub-plot (Appendix 6).

RESULTS AND DISCUSSION

The mean, initial fish weight was 602.87 g. Diet did not affect weight gain ($p>0.05$); however, weight increased to 1143.15 g by week 9 ($p<0.05$) (Table 1).

Vitamin E concentration

The initial muscle α -tocopherol concentration was 33.0 mg/kg, and vitamin E concentrations of the control and the vitamin E supplemented feed were 191.1 and 4926.1 mg/kg, respectively. Vitamin E supplementation increased fillet α -tocopherol concentration ($p<0.05$) (Figure 1), and a significant ($p<0.05$) diet by duration of feeding interaction was observed for this response. Fillet α -tocopherol concentration of the supplemented fish increased ($p<0.05$) with prolonged feeding of vitamin E; whereas vitamin content of control fillets did not change ($p>0.05$) (Figure 1). Frigg and others (1990) observed a slight increase in fillet α -tocopherol concentration (15.0 to 19.7 mg/kg) in rainbow trout fed 200 mg of α -tocopherol/kg for 12 weeks. As suggested by Ruff and others (2003), differences in rearing conditions, other dietary factors, method of analysis and size and age of fish could explain differences in vitamin E accumulation in muscle tissue.

Fasting fish for three days prior to harvest did not affect ($p>0.05$) α -tocopherol concentration of the fillet. However, control fillets (34.83 mg/kg) contained less ($p<0.05$) vitamin E than the treated fillets (165.24 mg/kg). At the conclusion of the

feeding trial (9 wk), livers of control and the vitamin E group contained 324.3 mg/kg and 15847 mg/kg α -tocopherol, respectively. After realimentation to the control feed for 3 weeks, vitamin E content of the livers was 479.0 and 6728.9 mg/kg in control and vitamin E supplemented group, respectively. Similar differences in incorporation of vitamin E in the muscle and liver of rainbow trout were reported by Frigg and others (1990) and Cowey and others (1981).

Hamre and Lie (1997) suggested that each organ may have a unique combination of mechanisms whereby they take up lipids and tocopherols from plasma. Catignani and Bieri (1977) reported the presence of an α -tocopherol-binding protein in rat liver; the protein (30 kDa) exists uniquely in the hepatocytes of rat liver and regulates plasma tocopherol concentration by preferentially incorporating α -tocopherol into nascent, very low density lipoproteins (Dutta-Roy and others 1994). Parazo and others (1998) observed the importance of the liver in selecting α -tocopherol over γ -tocopherol. These authors suggested that even though the existence of a 30 kDa protein has not been verified in fish, a protein analogous to the 30 kDa α -tocopherol binding protein exists in Atlantic salmon liver because the liver appeared to be the most biased organ for accumulating α -tocopherol. These authors reported greater reduction of α -tocopherol in the liver and muscle compared to kidney, serum, perivisceral fat, gills, brain, and testes during restricted intake of this vitamin.

Lipid oxidation

A significant ($p < 0.05$) diet by duration of feeding by storage time interaction was found for TBARS (Figure 2). On day 0, there were no differences in TBARS

between the control and vitamin E supplemented fish within each week of feeding ($p>0.05$). This response may be due to sufficient vitamin E in control fillets to limit lipid oxidation or, under the conditions studied, little oxidation occurred. Beneficial effects of high doses of vitamin E are not evident, and this response may be due to a relatively low heme iron content and storage at low temperatures. The antioxidant effect of high doses of vitamin E is not challenged in this scenario as opposed to subjecting product to higher storage temperatures. Additionally, it is likely that following cooking of fillets a different response to vitamin E supplementation would be observed. Control fillets from fish harvested at week 4 showed a significant increase by day 7 ($p<0.05$). By day 14, lipid oxidation increased in control fillets processed at week 2 and 3 and also in vitamin E fillets at week 3 ($p<0.05$). Chaiyapechara and others (2003) observed no significant differences in TBARS for rainbow trout fed 300 mg/kg and 1500 mg/kg of diet and stored for 0 d at 4°C. However, after storage for 7 days at 4°C and at -30°C, TBARS were lower in fish fed either 15% or 30% lipid and vitamin E at 1500 mg/kg compared to the fish fed 30% lipid and vitamin E at 500 mg/kg diet. In turbot (*Scophthalmus maximus*) stored under retail conditions on ice for 9 days, dietary supplementation of vitamin E at 500 and 1000 mg/kg decreased lipid oxidation compared to the low vitamin E diets (100 mg/kg). However, no significant differences were observed between high vitamin E diets. They also reported that by day 7 of storage, lipid oxidation plateaued for fish fed both high and low vitamin E diets. Lipid oxidation increased when the fish were fed more than 9 weeks, which was attributed to the slight increase in fat content after 15 weeks of feeding (Ruff and others 2003). Fish fasted for three days prior to

harvest (9 weeks) produced less TBARS (0.47 mg/kg) than the non-fasted fish (0.7 mg/ K g) ($p < 0.05$).

pH

Significant ($p < 0.05$) diet by storage time and duration of feeding by storage time interactions were observed for pH (Figure 3 & 4). At day 0, pH of control fillets was slightly higher than the fillets of vitamin E fish ($p < 0.05$). In control fillets, pH decreased with storage ($p < 0.05$). At day 0, fillet pH was significantly higher ($p < 0.05$) in fish fed for 2 weeks compared to all other fillets. At day 14, pH of fish fed for 2 weeks decreased ($p < 0.05$), whereas fish fed for 5 and 9 weeks showed an increase in pH ($p < 0.05$). Overall, pH fluctuated between 6.4-6.65. In turbot (*Scophthalmus maximus*), diet did not affect pH. On day 0 of cold storage, pH was higher in fish fed for 15 weeks than in the fish fed for 9 or 12 weeks ($p < 0.01$), and on day 2 of weeks 12 and 15, fish had a higher pH than at 9 weeks (Ruff and others 2003). Foegeding and coworkers (1996) reported a range of 6.2-6.6 for ultimate pH of lean white fish. Nonetheless, these sporadic changes in pH may be of no practical significance because the magnitude of the changes is not enough to alter protein or microbiological quality.

Color

A significant ($p < 0.05$) diet by duration of feeding by storage time interaction was observed for a^* value (Table 2). Fillets became less red on storage. Color b^* values increased from 9.50 to 11.46 on storage ($p < 0.05$), indicating an increase in yellowness of fillets. L^* values were not ($p > 0.05$) affected by diet, duration of feeding and storage time. Chaiyapechara and others (2003) reported that dietary

vitamin E had no effect on color of rainbow trout fed diets containing 300 or 1500 mg/kg α -tocopherol. Robb and others (2000) observed an increase in lightness of rainbow trout fillets with lower post mortem pH and increased hue and chroma in the first four days of slaughter. Ruff and others (2002) reported a direct relationship between b^* value and MDA values in Atlantic halibut and turbot. However, this relationship may not be readily applicable in the present study because MDA value did not vary much during storage. Albeit, subtle changes in fillet microstructure may affect the light reflecting properties of fillets during storage thereby affecting fillet color rendition.

Cook yield and Shear force

During storage, cook yield decreased for fillets fed for 2 and 5 weeks ($p < 0.05$) (Table 3). This decrease may be due to the loss of moisture during cooking. Diet differences were not significant for cook yield ($p > 0.05$). On days 14 and 7 shear force values were numerically higher for fish fed for 4 and 5 weeks, respectively compared to all other fillets. Foegding and others (1996) indicated that relative firmness of cooked fish meats is related primarily to muscle pH and water content. Ando and others (1992) observed softening of rainbow trout during chilled storage. However, in the present study, no clear patterns were observed for the effect of time and treatment on shear force (Table 3).

Psychrotrophic counts

Psychrotrophic counts increased with duration of feeding and storage time ($p < 0.05$) (Figure 5). Initial counts on fillets at week 7 and 9 were significantly higher than other fish ($p < 0.05$). This difference is not readily explainable, because all fish

were grown and processed under controlled conditions throughout the study. Gimenez and others (2002) reported that psychrotrophic counts in over-wrapped rainbow trout fillets exceeded 10^7 CFU/cm² after 6 days of storage at 1 ± 1 °C. In our study, after 14 days of refrigerated storage the psychrotrophic counts ranged from 4.67 - 8.53 log₁₀ CFU/cm². On week 9, fasted fish had significantly lower counts than the non-fasted fish ($p<0.05$) (Table 4). In fasted fish, the viscera might have been relatively clean at processing leading to less opportunity for contaminating the muscle during the filleting operation.

Proximate composition

An interaction of diet, duration of feeding and storage time ($p<0.05$) was observed for moisture content of fillets (Table 5). On day 0, with the exception of week 3 and 9, control fillets had slightly higher moisture content than the vitamin E fillets. When fish were fasted at week 9, moisture content increased in fasted and non-fasted groups during storage ($p<0.05$) (Table 6). Fat content of fish fed 5 weeks decreased significantly on storage ($p<0.05$) (Figure 6). After 9 weeks of feeding, fasted fish had higher fat content than non-fasted fish ($p<0.05$) (Table 6). Reuda and others (1998) reported an increase in lipid content after 7 days of fasting, but fasting for longer duration decreased the lipid content of red porgy (*Pagrus Pagrus* L.). Ruff and others (2003) reported that the moisture, fat, protein, and ash content in turbot (*Scophthalmus maximus*) did not change significantly after feeding vitamin E for 15 weeks.

CONCLUSIONS

Vitamin E supplementation increased fillet α -tocopherol concentration. Lipid oxidation did not change significantly during storage or with supplementation of high doses of vitamin E. Color b^* and psychrotrophic counts were not affected by α -tocopherol content. Supplementation with vitamin E did not preserve fillet a^* value with storage. Based on psychrotrophic counts, PVC over-wrapped fillets should not be stored for more than 7 days at 0°C. Fasting of fish for three days prior to harvest reduced initial psychrotrophic counts, even though these differences were not significant after 14 days of storage at 0°C.

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Table 1. Effect of duration of feeding on weight gain.

Duration of feeding, wk	Weight gain, g
0	602.86 ^a
2	675.08 ^{ab}
3	759.47 ^{bc}
4	805.94 ^{bc}
5	885.64 ^{cd}
7	981.69 ^d
9	1143.15 ^e

^{abcde} Means with different superscripts are different ($p < 0.05$).

Table 2. Effect of diet, duration of feeding, and storage time on a* value.

Diet	Duration of feeding, wk	Storage time, day		
		0	7	14
C	2	-0.43 ^{cd}	-3.37 ^a	-3.30 ^a
VE	2	-1.11 ^{bd}	-1.98 ^{abc}	-2.39 ^{ab}
C	3	-3.94 ^a	-3.00 ^{ab}	-0.09 ^c
VE	3	-3.32 ^a	-1.44 ^{bc}	-0.02 ^c
C	4	-3.28 ^a	-0.02 ^b	-2.33 ^a
VE	4	-1.93 ^a	0.06 ^b	-2.55 ^a
C	5	-0.65 ^{ab}	-2.09 ^a	1.24 ^c
VE	5	0.81 ^{bc}	-1.11 ^a	0.77 ^{bc}
C	7	-0.09 ^b	0.62 ^b	-3.22 ^a
VE	7	-0.08 ^b	0.77 ^b	-2.64 ^a
C	9	-2.33 ^a	-2.32 ^a	4.18 ^b
VE	9	-2.85 ^a	-2.32 ^a	-2.90 ^a

^{abcd}C and VE within each week with different superscripts are different (p<0.05).

Table 3. Effect of duration of feeding and storage time on cook yield and shear force.

Duration of feeding, wk	Cook yield			Shear force		
	Storage time, day			Storage time, day		
	0	7	14	0	7	14
2	81.66 ^e	75.35 ^{ab}	73.61 ^a	178.67 ^a	300.20 ^{bcd}	234.57 ^{abc}
3	77.74 ^{bc}	76.93 ^{ab}	76.17 ^{ab}	198.07 ^a	286.24 ^{bcd}	244.83 ^{abc}
4	75.84 ^{ab}	78.18 ^{bcd}	77.75 ^{bc}	370.77 ^{def}	319.12 ^{cde}	457.41 ^f
5	81.35 ^{de}	77.83 ^{bc}	75.63 ^{ab}	178.57 ^a	405.46 ^{ef}	317.23 ^{cd}
7	83.12 ^e	77.82 ^{bc}	80.83 ^{ce}	225.89 ^{ab}	279.52 ^{bc}	218.28 ^{ab}
9	81.74 ^e	81.30 ^{de}	81.50 ^{de}	197.94 ^a	220.53 ^{ab}	259.63 ^{abc}

^{abcdef} Means within each response with different superscripts are different (p<0.05).

Table 4. Effect of diet, fasting, and storage time on psychrotrophic counts.

Diet	Fasted fish		Non fasted fish	
	Storage time, day		Storage time, day	
	0	14	0	14
C	2.49 ^a	7.72 ^c	3.62 ^b	7.51 ^c
VE	2.29 ^a	7.63 ^c	4.02 ^b	8.23 ^d

^{abcd} Means with different superscripts are different ($p < 0.05$).

Table 5. Effect of diet, duration of feeding, and storage time on moisture content.

Diet	Duration of feeding, wk	Storage time, day		
		0	7	14
C	2	72.73 ^a	73.37 ^{ab}	74.64 ^{cd}
VE	2	73.91 ^{bc}	72.62 ^a	74.41 ^{bd}
C	3	73.45 ^{ab}	74.18 ^b	73.10 ^a
VE	3	72.60 ^a	74.38 ^b	72.56 ^a
C	4	74.00 ^{bc}	72.59 ^a	74.26 ^c
VE	4	72.64 ^a	73.12 ^{ab}	73.52 ^{ac}
C	5	72.23 ^b	73.65 ^{cde}	74.33 ^e
VE	5	70.96 ^a	72.93 ^{bd}	72.68 ^{bc}
C	7	73.03 ^b	73.74 ^{bc}	74.19 ^c
VE	7	70.66 ^a	72.91 ^b	74.21 ^c
C	9	72.18 ^{ab}	73.74 ^{cde}	73.22 ^{be}
VE	9	71.87 ^a	73.00 ^{bcd}	72.96 ^{bc}

^{abcde}C and VE within each week with different superscripts are different (p<0.05).

Table 6. Effect of diet, fasting and storage time on moisture and fat content

Diet	Fasting	Moisture, %			Fat, %		
		Storage time, day			Storage time, day		
		0	7	14	0	7	14
C	F	70.97 ^a	72.92 ^{cde}	73.93 ^f	9.14 ^f	5.88 ^{bc}	3.78 ^a
C	NF	72.18 ^{bc}	73.74 ^{ef}	73.22 ^{def}	5.34 ^{bc}	5.07 ^{ab}	5.11 ^{bc}
VE	F	71.18 ^a	71.54 ^{ab}	72.46 ^{bcd}	8.44 ^{ef}	7.57 ^{de}	4.55 ^{ab}
VE	NF	71.87 ^{ab}	73.00 ^{cdef}	72.96 ^{cde}	5.78 ^{bc}	6.51 ^{cd}	4.74 ^{ab}

^{abcdef} Means within each response with different superscripts are different (p<0.05).

Figure 1. Effect of diet and duration of feeding on vitamin E content.

^{abcde} points on the line with different superscripts are different ($p < 0.05$).

*C-control diet, and VE- 5000mg/kg diet.

**Fish were sampled at 2, 3, 4, 5, 7, and 9 weeks of feeding.

Figure 2. Effect of diet, duration of feeding and storage time on TBARS.

^{abc} Bars in each figure with different superscripts are different ($p < 0.05$).

*C-control diet, and VE- 5000mg/kg diet.

Figure 3. Effect of diet and storage time on pH.

^{abc} Bars with different superscripts are different (p<0.05).

*C-control diet, and VE- 5000mg/kg diet.

Figure 4. Effect of duration of feeding and storage time on pH.

abcde^{gh} Bars with different superscripts are different ($p < 0.05$).

*Fish were sampled at 2, 3, 4, 5, 7, and 9 weeks of feeding.

Figure 5. Effect of duration of feeding and storage time on psychrotrophic counts.

ab c d e f g h i j k Bars with different superscripts are different ($p < 0.05$).

*Fish were sampled at 2, 3, 4, 5, 7, and 9 weeks of feeding.

Figure 6. Effect of duration of feeding and storage time on fat content.

^{abcde} Bars with different superscripts are different ($p < 0.05$).

*Fish were sampled at 2, 3, 4, 5, 7, and 9 weeks of feeding.

APPENDICES

Appendix 1

Vitamin E concentrations of feed

Duration of feeding, wk	Vitamin E concentration, mg/kg	
	C*	VE**
0	191.1	4926.1
2	194.5	4746.1
3	195.7	4464.1
4	177.8	4561.0
5	147.5	5472.7
7	106.6	2602.6
8	91.8	3138.5
9	117.5	3281.2

*C- control diet, **VE- 5000 mg/kg diet.

Appendix 2

Statistical program

```
option LS=80 pagesize=60 nonumber;  
title ;  
data; input week rep day diet $ response;  
cards;  
proc print;  
proc glm; classes diet rep week day;  
model response=diet diet(rep) week week*diet week*diet(rep) day day*diet  
day*week day*week*diet;  
test H=diet E=diet(rep);  
test H=week week*diet E=week*diet(rep);  
lsmeans diet/pdiff E=diet(rep);  
lsmeans week week*diet/pdiff E=week*diet;  
lsmeans day day*diet day*week day*week*diet/pdiff;  
run;
```

Appendix 3

Statistical program for weight gain

```
option LS=80 pagesize=60 nonumber;  
title  
data wholewt; input week rep diet $ wholewt;  
cards;  
proc print;  
proc glm; classes diet rep week ;  
model wholewt=diet diet(rep) week week*diet;  
test H=diet E=diet(rep);  
lsmeans diet/pdiff E=diet(rep);  
lsmeans week week*diet/pdiff ;  
run;
```

Appendix 4

Statistical program for moisture, fat, vitamin E content of fasted fish at week 9.

```
option LS=80 pagesize=60 nonumber;
title ;
data response; input diet $ rep fasted $ day $ response;
cards;
proc print;
proc glm; classes diet rep fasted day;
model response=diet diet(rep) fasted fasted*diet fasted*diet (rep)day day*diet
diet*fasted*day;
test H=diet E=diet(rep);
test H=fasted fasted*diet E=fasted*diet (rep);
lsmeans diet/pdiff E=diet(rep);
lsmeans fasted fasted*diet/pdiff E=fasted*diet (rep);
lsmeans day day*diet diet*fasted*day/pdiff;
run;
```

Appendix 5

Statistical program for psychrotrophic counts of fasted fish at week 9

```
option LS=80 pagesize=60 nonumber;
title ;
data response; input diet $ rep fasted $ day $ response;
cards;
proc print;
proc glm; classes diet rep fasted day;
model response=diet diet(rep) fasted fasted*diet fasted*diet (rep)day day*diet
diet*fasted*day;
test H=diet E=diet(rep);
test H=fasted fasted*diet E=fasted*diet (rep);
lsmeans diet/pdiff E=diet(rep);
lsmeans fasted fasted*diet/pdiff E=fasted*diet (rep);
lsmeans day day*diet diet*fasted*day/pdiff;
run;
```


Appendix 6

Statistical program for TBARS of fasted fish at week 9

```
option LS=80 pagesize=60 nonumber;  
title ;  
data tbars; input diet $ rep fasted $ response;  
cards;  
proc print;  
proc glm; classes diet rep fasted;  
model response=diet diet(rep) fasted diet*fasted;  
test H=diet E=diet(rep);  
lsmeans diet/pdiff E=diet(rep);  
lsmeans fasted diet*fasted/pdiff;  
run;
```

CHAPTER 3

EFFICACY OF ACIDIFIED SODIUM CHLORITE IN EXTENDING STORAGE STABILITY OF RAINBOW TROUT (*Oncorhynchus mykiss*) FILLETS

ABSTRACT

Fresh rainbow trout were obtained from a local farm, mechanically stunned, packed in ice, and transported to West Virginia University within 3 h of harvest. Gutting and filleting were performed manually, and fillets were subsequently washed in a dilute salt/ ice slurry and rinsed under running tap water to remove blood and waste. These fillets were drained overnight at 4°C to remove excess water. The ASC solution was prepared by mixing equal volumes of Sanova Activator[®] and Sanova[®] base. The activator and base were mixed immediately prior to treatment application. At room temperature, fillets were dipped in water for 10 s and then drained for 10 s before treatment with ASC. Pre-dipped fillets were placed in an agitating ASC solution for 30 s and allowed to drain for 10 s. The ratio of fish-to-solution was 1:3 (W/V). Subsequently, fresh fillets were aerobically packed on styrofoam trays and wrapped with polyvinyl chloride (PVC) film. Fillets were stored at 1-2°C for 0, 8, and 15 days. Four intervention treatments, control (no treatment), water, 50 ppm ASC, and 1000 ppm ASC and three storage periods resulted in 12 treatment combinations. A randomized complete block design with a split-plot arrangement of treatments was replicated on three separate days. Significance was established at $p < 0.05$. A significant interaction ($p < 0.05$) of treatment and storage time was observed for psychrotrophic counts. Aerobic plate counts were not affected ($p > 0.05$) by intervention; however, a significant increase in aerobic plate counts was observed during storage ($p < 0.05$). Fillet pH, moisture, fat, TBARS, fatty acid composition, TVBN, TMA, color, cook yield, and shear force were not affected ($p > 0.05$) by antimicrobial intervention. TBARS decreased ($p < 0.05$) during storage.

Generally, percent fatty acids were stable during storage ($p>0.05$); however, C15 and C20:2 decreased ($p<0.05$). Fat content, TMA, L^* , b^* , and cook yield increased ($p<0.05$) during storage. Fillet pH, moisture, TVBN, a^* value, and shear force did not change ($p>0.05$) with increasing storage.

Key words: acidified sodium chlorite, rainbow trout, microbial counts, lipid oxidation, fatty acids

INTRODUCTION

Fish are highly perishable, and post-mortem autolytic and microbiological changes reduce the storage stability of these products. This perishability necessitates development of interventions to extend shelf life and preserve product quality. Application of antimicrobials is one way of reducing microbial spoilage in fish. Chemicals such as sodium hypochlorite, broad spectrum antibiotics, ethylenediaminetetraacetic acid, glucose oxidase, organic acids (Haard 1992), and chlorine dioxide (Kim and others 1999) have been used to delay microbial spoilage in fish.

Acidified sodium chlorite (ASC) is an antimicrobial agent approved by the U.S. Food and Drug Administration as a secondary direct food additive permitted in food for human consumption. ASC is approved at 500 to 1,200 ppm for use on poultry, red meat, comminuted meat products and processed fruits and vegetables to reduce bacterial contamination; whereas, in seafood, ASC is approved at a concentration of 40 to 50 ppm. ASC can be used to rinse, wash, thaw, transport, or store seafood. ASC is an antibacterial agent prepared by mixing sodium chlorite with a generally - recognized - as - safe acid (Anonymous 1999). The antimicrobial activity of acidified sodium chlorite is attributed to the oxidative effect of chlorous acid, generated from the conversion of chlorite ion into its acid form under acidic conditions. Chlorous acid is a metastable oxychlorine species that decomposes to form chlorate ion, chlorine dioxide, and chloride ion (Gordon and others 1972). Bacterial contamination on beef and poultry was reduced by treating the carcass with 1200 ppm of acidified sodium chlorite (Castillo and others 1999; Kemp and

others 2001). Su and Morrissey (2003) reported that treatment of salmon fillets with 50 ppm ASC reduced the total plate counts (viable cell counts) of fillets. The objective of this research is to study the efficacy of ASC, at approved and higher concentrations, in extending the storage stability and quality of rainbow trout fillets.

MATERIALS AND METHODS

Processing of fish

Fresh rainbow trout were obtained from a local farm, mechanically stunned, packed in ice and transported to West Virginia University within 3 h of harvest. Gutting and filleting were performed manually, and fillets were subsequently washed in a dilute salt/ ice slurry and rinsed under running tap water to remove blood and waste. Following processing, fillets were drained overnight at 4°C to remove excess water.

Preparation of ASC solution

The ASC solution was prepared by mixing equal volumes of Sanova Activator® and Sanova® base (Alcide Corporation, Redmond, WA, U.S.A). The activator and base were obtained in separate containers and stored at room temperature. A final concentration of 50 ppm ASC was obtained by mixing 0.66% citric acid (Sanova Activator®) and 0.01% sodium chlorite (Sanova® base). For 1000 ppm, 0.76% citric acid (Sanova Activator®) and 0.2% sodium chlorite (Sanova® base) were used. The activator and base were mixed immediately before application of treatments. The sodium chlorite concentration of the ASC solution was determined by titration with sodium thiosulphate before and after dipping the fillets.

Treatment of fillets

Four intervention treatments, control, water, 50 ppm ASC, and 1000 ppm ASC and three display days resulted in 12 treatment combinations. Fifteen fillets were randomly assigned to each treatment. At room temperature, fillets were dipped in water for 10 s and then drained for 10 s on oven trays before treatment with ASC. Pre-dipping of fillets in water was done immediately before applying the treatments, to ensure uniform surface tension moisture. For each batch of 15 fillets, ASC solution was freshly prepared and the ratio of fish-to-solution was 1:3 (W/V). Pre-dipped fillets were dipped in agitating ASC solution for 30 s and allowed to drain on oven trays for 10 s. Agitation was accomplished by placing the tub containing the ASC solution on a shaker table. For the water treatment, pre-dipped fillets were dipped in agitating tap water (1:3 (W/V)) and allowed to drain on oven trays for 10 s. Fillets that did not receive any treatment were used as the control. Following treatment, fillets were aerobically packed on styrofoam trays and wrapped with polyvinyl chloride (PVC) film. Fillets were stored at 1-2°C for 0, 8, and 15 days. Five fillets from each treatment were sampled at each display period to conduct analyses.

Chemical and physical analyses

Moisture and Fat: Moisture and fat were determined according to standard AOAC procedures (1990).

pH: The pH of ASC solutions and muscle were measured using a pH/ion analyzer 350 (Corning Inc.; Corning, NY) equipped with a general surface combination probe. The pH of ASC solutions was measured before and after dipping the fillets. On each display day, muscle pH was randomly recorded on one of the five fillets assigned to

that display period. One reading was taken at the cranial and caudal halves of the fillet. These two values were averaged and recorded as fillet pH.

Microbial analysis: Surface counts of psychrotrophic (PPC) and aerobic bacteria (APC) were determined on all five fillets of each treatment. A 11.4 cm² core was taken from the center of the cranial portion of the fillet using a sharp-edged metal corer. The cored sample was blended with 25 mL of 0.1% peptone water in a Stomacher 400 blender (Tekmer, Cincinnati, Ohio, U.S.A) for 30 s. A 1 mL aliquot of the homogenate was serially diluted with peptone water and each diluent was spread plated on duplicate plates of plate count agar (Swanson and others 1992). At day 0, a 0.1 mL aliquot was directly spread on the plates. The initial ratio of sample to diluent was 0.912 cm²/mL. Inverted plates were incubated for 24 h at 25°C and 37°C for psychrotrophic and aerobic bacteria, respectively. Counts were reported as log₁₀CFU/ cm². These counts represent microflora present on the flesh and skin sides of the fillet because the core passed through the fillet.

Total volatile base nitrogen (TVBN) and Trimethyl amines (TMA):

TVBN and TMA content was measured by a steam distillation method (Malle and Tao 1987).

Thiobarbituric acid Reactive Substances (TBARS): One fillet from each treatment was skinned (excluding belly flap), cut into small pieces, frozen in liquid N₂, and powdered in a commercial blender (Waring, New Hartford, Conn., U.S.A). TBARS in the powdered samples were measured calorimetrically using 2-thiobarbituric acid (Yu and Sinnhuber 1957). Absorbance was read at 535 nm using a Spectronic 20D

spectrophotometer (Milton Roy, NJ, U.S.A). Results were expressed as mg malonaldehyde (MDA) per kg of sample.

Fatty acid analysis: Total lipids were extracted according to the rapid method of total lipid extraction and purification of Bligh and Dyer (1959). Fatty acid methyl esters (FAME) were prepared according to Fritsche and Johnston (1990) (Appendix 1). A Varian 3800 gas chromatograph (Varian Inc; Palo Alto, CA, U.S.A) equipped with a Varian 4800 analyzer (Varian Inc., Palo Alto, CA, U.S.A) and a flame ionizing detector was used to separate and identify the fatty acids; a split ratio of 10:1 was used. The column was a W Cot fused silica capillary (FAME) column, 50 mm long and 5 μ m i.d (Varian Inc, Palo Alto, CA, U.S.A) and nitrogen as carrier gas was employed. Temperatures of injector and detector were 275°C and 300°C, respectively. Initial oven temperature was 140°C held for 5 min, then increased to 200°C at the rate of 4°C/min and held for 6 min. Final oven temperature was increased to 220°C at the rate of 3°C/min and held for 15 min. Total run time was 48 min. Fatty acid methyl esters were identified by comparing retention times with those of FAME 37 standard (Sigma-Aldrich, St.Louis, MO, U.S.A). Individual fatty acid values were expressed as a percent of total fatty acids measured. Samples were injected once for analysis.

Color: Fillet color was measured with a Minolta chromameter (Model CR-300, Minolta camera Co Ltd.; Osaka, Japan). The instrument was calibrated using a standard white plate No.21333180 (CIE L* 93.1; a* 0.3135; b* 0.3198). L* (lightness), a* (redness), and b* (yellowness) values were recorded at one randomly

chosen location on the cranial and caudal portions of fillet. The mean of these two values was used as the observation for that fillet.

Shear force and cook yield: Shear force was measured using a Texture Analyzer (Model TA-Hdi ; Texture Technologies Corp., Scarsdale, N.Y., U.S.A) with a 5-blade Kramer shear attachment at a cross speed of 127 mm/min. A 3 x 8 cm strip of the cooked fillet was removed, skinned, weighed and placed beneath the blades with the skin side up. Values were expressed as g force/g of sample. Cook yield was calculated by expressing cooked fillet weight as a percent of raw fillet weight.

ASC solution concentrations: Sodium chlorite content of the treatment solutions was determined before and after dipping the fillets in ASC as described in Appendix 2.

Experimental design

Four intervention treatments, control, water, 50 ppm ASC, and 1000 ppm ASC, and three display days resulted in 12 treatment combinations. A randomized complete block design with a split-plot arrangement of treatments was replicated on three separate days (Appendix 3). The General Linear Model (GLM) procedure of SAS (SAS Institute, Inc., 1999) was used to establish main and interaction effects. The PDIFF of the lsmeans procedure was used to separate means. Significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

The pH of 50 and 1000 ppm solutions increased from 2.53 and 2.67 to 2.68 and 2.75, respectively, after dipping the fillets in ASC for 30 s. Sodium chlorite concentrations of solutions were in the range of 52-56 and 997-1011 ppm. Following

dipping, concentrations were 49-51 and 989-997 ppm. The pH of fillets treated with ASC was not different ($p>0.05$) from the pH of the control fillet, and increasing storage time did not affect ($p>0.05$) fillet pH (Table 1 & 3). Fillet moisture and fat content was not affected ($p>0.05$) by treatment (Table 1). Kim and others (1998) showed that treatment of salmon and red grouper fillets with chlorine dioxide did not significantly affect the moisture, fat and protein of fillets when compared to the non-treated control. Fat content increased ($p<0.05$) during storage, but the moisture content of the fillets remained unchanged ($p>0.05$) (Table 3).

Microbial counts

A significant ($p<0.05$) treatment by day interaction was observed for psychrotrophic counts (Figure 1). Initial counts (day 0) were significantly lower ($p<0.05$) for fillets treated with 50 ppm of ASC compared to all other treatments; no differences ($p>0.05$) were observed between water and 1000 ppm treatments. Psychrotrophic counts increased ($p<0.05$) during storage. Fillets dipped in 1000 ppm ASC had significantly lower ($p<0.05$) counts than all other treatments at 8 and 15 d. Aerobic plate counts were not ($p>0.05$) affected by treatment (Table 2), but they increased from $1.94 \log_{10}$ to $5.02 \log_{10}$ CFU/cm² ($p<0.05$) as time at 1-2°C increased (Table 4). Similar results were observed by Su and Morrissey (2003) for salmon fillets dipped in 50 ppm of ASC for 1 min and stored on ice or at 5°C for 7 days. In their study, ASC treatment reduced initial total plate counts by 0.3 log units, however a gradual increase in counts was observed for all samples during storage. They demonstrated that the bactericidal activity of ASC is primarily due to the generation of chlorine dioxide and chlorous acid and not related to low pH of the

ASC solution. By suspending the *Listeria monocytogenes* cocktail (6.91 log CFU/mL) in 9 volumes of Butterfield's phosphate buffer that had been adjusted to pH 3.03 and 3.30 with 1 N hydrochloric acid they demonstrated this mechanism of ASC action. The survival of the cells was determined immediately after mixing (15 s) and after 1, 2, 3, and 5 min by the total plate count method. *Listeria monocytogenes* survived well in acidic phosphate solutions and decreased only slightly after 5 min, from 5.91 to 5.87 and 5.82 log CFU/mL at pHs 3.30 and 3.03, respectively. These authors believed that the low bactericidal activity of ASC was due to its interaction with fish muscle protein, resulting in less interaction between ASC and bacterial cell walls. On the other hand, Warf and Kemp (2001) reported that the antimicrobial activity of ASC is due to the uncharged chlorous acid; it penetrates bacterial cell walls and disrupts protein synthesis. In our study low initial counts on the fillets and short contact time (30 s) between fillets and ASC solution may have contributed to our observation that ASC has limited antimicrobial action.

Total volatile base nitrogen (TVBN) and Trimethylamines (TMA) content

TVBN and TMA were not affected ($p>0.05$) by treatment intervention ($p>0.05$) (Table 2). TMA values increased during storage ($p<0.05$) (Table 4). A trend of increasing TVBN values was observed; however this response was not affected by storage ($p>0.05$) (Table 4). TVBN is widely used to indicate fish quality and spoilage (Pivarnik and others 1998; Duflos and others 1999). Initial TVBN values were higher than the values reported for rainbow trout. TVBN values of rainbow trout fillets stored at $1\pm1^{\circ}\text{C}$ for 4 days exceeded 25 mg N/100g of muscle (Gimenez and others 2002) which is comparable to the values observed in the present study. Mazorra-

Manzano and others (2000) reported an insignificant change in TVBN values with storage time for black skipjack stored at 1 to 3°C for 24 d; these values ranged between 25.5 to 34.3 mg N/100g muscle during storage. TVBN is more useful in assessing degree of freshness than in evaluating changes during the first stages of spoilage (El Marrack-chi and others 1990).

An increase in TMA with storage time was reported for rainbow trout fillets stored at 4° C (Rodriguez and others 1999). However, TMA values observed in our study were higher than these reported values. Production of TMA is dependent on species, sex, age, time of year and muscle type (Hughes 1959; Shirai and others 1988). Freshwater fish do not contain trimethylamine oxide (TMAO) which produces TMA by bacterial action. In our study, high levels of TMA in fillets might have accumulated from the diet which is formulated with aquatic protein and fat sources. Marine fish typically contain 1-100mg of TMAO per 100g of muscle (Stansby and Olcott 1963). Rodriguez and others (1999) suggested that TMA value is affected by bacterial flora, feed characteristics and method of storage, and it may not be useful for evaluating freshness of rainbow trout.

Lipid oxidation and fatty acid composition

ASC formed by acidification of chlorous acid decompose into chlorine dioxide, chlorate ion and chlorite ion (Gordon and others 1972). Treatment of fillets with ASC did not affect ($p>0.05$) TBARS or measurable fatty acid content of the fillets (Table 1 & 5). Kim and others (1997) reported that TBARS were higher for salmon and red grouper fillets dipped in 100 and 200 ppm chlorine dioxide for 5 min compared to the control (untreated) and 40 ppm fillets. However, 100 ppm and 200 ppm treatments

were not different from each other. In addition these authors also reported that fatty acid composition of fillets was not affected by chlorine dioxide treatment. Comparing our results with the aforementioned study, no effect ($p>0.05$) of ASC on fillet TBARS may be associated with differences in contact time. In addition to different exposure times (5 min VS 30 s), evaporation of treatment solution on the fillet surface, and interaction of ASC or its decomposed components with other organic compounds in the fillet may have caused minimal interaction of chlorine dioxide with lipids reducing the likelihood of ASC-induced lipid deterioration. A decrease ($p<0.05$) in TBARS value was observed on day 8, and this value was constant through 15 days of storage. The decrease in TBARS may be due to result of malondialdehyde reacting with fish proteins (Known and others 1965); this interaction decreases availability of malonaldehyde for calorimetric determination. Percent fatty acid content of fillets did not change ($p>0.05$) during storage, except for C15 (Pentadeconoic acid) and C20:2 (Cis -11, 14 - eicosadienoic acid) which were slightly lower ($p<0.05$) on day 8 and day 15 (Table 6). The ratio of $\omega 3$ to $\omega 6$ was 0.8 and did not change ($p>0.05$) with treatment intervention or storage time (Table 5 & 6).

Color

Treatment with ASC did not cause ($p>0.05$) color changes (Table 1). L^* and b^* values increased ($p<0.05$) during storage (Table 3); whereas, a^* value remain unchanged ($p>0.05$) (Table 3). Eventhough 1000 ppm treated fillets appeared paler than the control fillets, the differences were not significant ($p>0.05$). Similar results were reported by Su and Morrissey (2003) in that dipping salmon fillets and whole salmon in 50 ppm ASC for 1 min did not result in visible color loss. Similar increases

in L^* value during storage were observed in our earlier study (chapter 1). However these changes are not clearly understood and need to be studied further, because there is limited literature available in this area of fish fillet color variable.

Nonetheless, Robb and others (2000) observed increased lightness of rainbow trout fillets with lower post mortem pH. In addition, hue and chroma increased during the first four days of slaughter.

Cook yield and shear force

Cook yield and shear force were not affected ($P>0.05$) by intervention treatments (Table 1). Cook yield increased during storage ($p<0.05$) and shear force was not affected ($p>0.05$) (Table 3). Eventhough, treatments did not cause a significant change in cook yield, dipping of fillets in ASC followed by immediate cooking may have caused some denaturation of proteins on the surface, resulting in more water loss during cooking thereby decreasing the cook yield. However the pH equilibrium between surface and deeper portions of the muscle after 8 d and 15 d may have caused the proteins to regain their native form and thereby improve the water holding capacity of the muscle and thereby increase cook yield.

CONCLUSIONS

Treatment of fillets with 50 and 1000 ppm ASC reduced psychrotrophic counts of fillets, but had no affect on aerobic plate counts. Dose dependent responses were not observed for lipid oxidation or fatty acid composition. ASC did not affect fillet color when compared to water and control treatments. Based on these results, 50 ppm ASC performed equally as well as 1000 ppm ASC. Hence, it may not be practical to apply high concentrations of ASC to rainbow trout fillets when a similar affect could be obtained at the approved concentrations (50 ppm).

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Table 1. Effect of ASC treatment on muscle pH, moisture, fat, TBARS, color, cook yield and shear force of fillets.

Trait	Treatment			
	Control	Water	50 ppm ASC	1000 ppm ASC
Muscle pH	6.11 ^a	6.39 ^a	6.29 ^a	6.31 ^a
Moisture, %	75.64 ^a	75.32 ^a	75.54 ^a	76.13 ^a
Fat, %	5.22 ^a	5.44 ^a	5.43 ^a	5.15 ^a
TBARS , mg MDA/100g	0.6 ^a	0.8 ^a	0.8 ^a	0.9 ^a
L* value	48.76 ^a	49.17 ^a	50.53 ^a	50.80 ^a
a*value	-1.62 ^a	-2.01 ^a	-2.31 ^a	-2.37 ^a
b*value	8.47 ^a	7.57 ^a	7.65 ^a	6.16 ^a
Cook yield, %	78.59 ^a	77.89 ^a	77.45 ^a	77.47 ^a
Shear force, g/g of sample	266.43 ^a	263.75 ^a	249.55 ^a	290.33 ^a

^aMeans within a row with different superscripts are different (p<0.05).

Table 2. Effect of ASC treatment on APC, TVBN, and TMA of fillets.

Trait	Treatment			
	Control	Water	50 ppm ASC	1000 ppm ASC
APC, log ₁₀ CFU/cm ²	4.08 ^a	3.76 ^a	3.54 ^a	3.43 ^a
TVBN, mg N/100g muscle	24.88 ^a	26.98 ^a	23.40 ^a	22.54 ^a
TMA, mg N/100g muscle	22.64 ^a	23.42 ^a	20.47 ^a	19.88 ^a

^aMeans within a row with different superscripts are different (p<0.05).

Table 3. Changes in muscle pH, moisture, fat, TBARS, color, cook yield and shear force during storage.

Trait	Length of storage at 1-2°C		
	0	8	15
Muscle pH	6.30 ^a	6.32 ^a	6.22 ^a
Moisture, %	75.85 ^a	75.71 ^a	75.42 ^a
Fat, %	5.00 ^a	4.98 ^a	5.95 ^b
TBARS, mg MDA/100g	0.9 ^a	0.7 ^b	0.7 ^b
L* value	48.13 ^a	51.14 ^b	50.18 ^b
a* value	-1.80 ^a	-2.38 ^a	-2.05 ^a
b* value	4.19 ^a	9.10 ^b	8.37 ^b
Cook yield, %	73.18 ^a	81.23 ^b	79.15 ^b
Shear force, g/g of sample	310.84 ^a	212.91 ^a	278.79 ^a

^{ab}Means within a row with different superscripts are different (p<0.05).

Table 4. Changes in APC, TVBN, and TMA of fillets during storage.

Trait	Length of storage at 1-2°C		
	0	8	15
APC, log ₁₀ CFU/cm ²	1.94 ^a	4.14 ^b	5.02 ^c
TVBN, mg N/100g muscle	23.44 ^a	24.69 ^a	25.22 ^a
TMA, mg N/100g muscle	20.87 ^a	20.96 ^{ab}	22.97 ^b

^{abc}Means within a row with different superscripts are different (p<0.05).

Table 5. Effect of ASC on percent fatty acid* content of fillets.

Fatty acid	Treatment			
	Control	Water	50 ppm ASC	1000 ppm ASC
C14:0	3.74	3.86	3.79	3.87
C15:0	0.18	0.23	0.24	0.26
C16:0	20.71	20.90	20.72	20.66
C16:1	17.09	16.22	17.79	18.12
C17:0	0.23	0.26	0.28	0.29
C17:1	0.12	0.17	0.21	0.14
C18:0	3.67	3.79	3.72	3.75
C18:1n9	8.57	9.48	9.36	10.33
C18:2n6	8.11	8.19	7.02	7.64
C18:3 n3	1.18	1.32	1.19	1.15
C18:3 n6	0.01	0.03	0.02	0.02
C20:1	0.81	0.83	0.74	0.80
C20:2	0.66	0.68	0.73	0.78
C20:3 n3	0.01	0.03	0.01	0.02
C20:3 n6	0.08	0.2	0.11	0.19
C20:4 n6	0.63	0.60	0.50	0.56
C20:5 n3	10.03	9.37	8.89	9.44
C22:2	24.99	25.20	24.86	22.74
C22:2	10.03	9.37	8.88	9.44
C22:6 n3	24.99	25.20	24.86	22.74
C24:1	0.10	0.18	0.15	0.17
sat/unsat	0.39	0.39	0.40	0.39
ω3/ω6	0.78	0.79	0.80	0.77

No differences were found ($p>0.05$).

*Values are relative percent of total fatty acids from three replications.

Table 6. Changes in percent fatty acid* content during storage.

Fatty acids	Length of storage at 1-2°C		
	0	8	15
C14:0	4.11 ^a	3.77 ^a	3.57 ^a
C15:0	0.32 ^b	0.20 ^{ab}	0.16 ^a
C16:0	21.07 ^a	20.43 ^a	20.72 ^a
C16:1	17.92 ^a	17.96 ^a	16.03 ^a
C17:0	0.36 ^a	0.23 ^a	0.19 ^a
C17:1	0.15 ^a	0.16 ^a	0.17 ^a
C18:0	3.93 ^a	3.60 ^a	3.66 ^a
C18:1n9	12.26 ^a	10.06 ^a	6.18 ^a
C18:2n6	7.04 ^a	7.23 ^a	8.99 ^a
C18:3 n3	1.35 ^a	1.18 ^a	1.10 ^a
C18:3 n6	0.05 ^a	0.02 ^a	0.01 ^a
C 20:0	0.03 ^a	0.43 ^a	0.01 ^a
C20:1	0.83 ^a	0.76 ^a	0.79 ^a
C20:2	0.93 ^b	0.67 ^{ab}	0.55 ^a
C20:3 n3	0.20 ^a	0.01 ^a	0.03 ^a
C20:3 n6	0.22 ^a	0.12 ^a	0.09 ^a
C20:4 n6	0.51 ^a	0.61 ^a	0.61 ^a
C20:5 n3	9.53 ^a	8.37 ^a	10.40 ^a
C22:2	0.89 ^a	0.90 ^a	0.86 ^a
C22:6 n3	24.15 ^a	23.14 ^a	26.05 ^a
C24:1	0.19 ^a	0.13 ^a	0.13 ^a
sat/unsat	0.38 ^a	0.39 ^a	0.40 ^a
ω3/ω6	0.80 ^a	0.77 ^a	0.78 ^a

^{abc} Means within each row with different superscripts are different (p<0.05).

*Values are relative percent of total fatty acids from three replications.

Figure 1. Effect of ASC and storage time on psychrotrophic counts.

abcdeghi Bars with different superscripts are different (p<0.05).

APPENDICES

APPENDIX 1

Muscle Lipid Extraction and Fatty Acid Methylation (Also use for feed sample with animal base)

Sample preparation

Sample can be stored by either one of these methods:

1. **Intact muscle form.** Fillets (excluding belly flap) are skinned, vacuum packed and kept at -20°C . When lipid extraction is performed, frozen fillets are partially thawed and processed the same as powdered sample preparation.
2. **Powder form.** Fillets are skinned, cut into small pieces (excluding belly flap), frozen with liquid N_2 , and powdered in a Waring blender for 2-3 min. BHT is added before blending to prevent fat oxidation. Powdered samples are kept in screw cap plastic tubes at -80°C .

Instruments

1. Waring blender and liquid nitrogen
2. Hood
3. 60°C water bath
4. 90°C water bath or heating block
5. Manifold and nitrogen gas
6. Centrifuge
7. Vortex

Chemicals

1. Trizma/EDTA buffer:
50 mM Trizma HCl: 7.880 g per 1000 mL dd water
1 mM EDTA-disodium salt: 0.372 g per 1000 mL dd water
Mix above stock solutions, adjust pH to 7.4 with 5 N then 1 N NaOH, then filter using a 0.2 micron filter storage unit. Buffer is stored at 4°C .

2. C:M:A (chloroform: absolute methanol: glacial acetic acid – 2:1:0.015 by volume)
e.g. 400:200:3 mL (use glass cylinder)
3. 2:1 (chloroform:methanol by volume) e.g. 400:200 mL (use glass cylinder)
4. 4:1 (chloroform:methanol by volume) e.g. 400:100 mL (use glass cylinder)
5. 4% (w/v) H₂SO₄ in anhydrous methanol. prepare fresh. Do not keep the left-over solution
6. Chloroform
7. Anhydrous Na₂SO₄
8. 1-PS Phase separation filters (diameter 9 cm). This filter type takes water out of solution, thus filtered solution is water free.
9. Glasswool

Lipid extraction step

1. Weigh 0.5 g powdered sample + 5 mL Trizma/EDTA (use plastic pipett) in 50-mL screw cap plastic tube.
2. Vortex for 60 sec.
3. Transfer 5 mL of homogenate in 50-mL Teflon lined screw cap glass tube, add 5 mL Trizma/EDTA and 40 mL C:M:A (use glass cylinder).
4. Vortex vigorously twice (15 sec x 2).
5. Hold at room temperature for 10 min.
6. Centrifuge at 900 *g* (4000 rpm), 10°C for 10 min.
7. Transfer all lower layer with glass Pasteur pipet over 1-PS filter. Collect filtered sample in 50-mL Teflon lined screw cap glass tube. (Pre-rinse filter paper 3 times with 5 mL 2:1 C:M each (use glass cylinder) to remove trace silicone residue, and put away filtrate.)
8. Add 25 mL 4:1 C:M (use glass cylinder) to the upper layer, vortex for 15 sec, and centrifuge at 900 *g* (4000 rpm), 10°C for 10 min.
9. Again transfer all lower layer over 1-PS filter. Rinse filter paper with 5 mL 2:1 C:M after the filtrate has gone. (Filtrated sample can be stored at 0-5°C for 1-3 days.)
10. Blow down under regular nitrogen gas (to dry sample) in 60°C water bath.
Nitrogen gas outlet should be close to liquid surface. Temperature must not be

higher than 60°C. Set hot plate at 4. When temperature goes to 55°C, set it back to 2. Watch Temperature Often.

Methylation step

11. Add 4 mL of 4% H₂SO₄ solution (use glass pipett). (cap tightly-no leaking)
12. Heat in water bath or heating block at 90°C for 60 min. Set water bath at 100°C, cover with foil.
13. Cool to room temperature then add 3 mL dd water (to stop reaction).
14. Add 8 mL chloroform (use glass pipett), then vortex for 30 sec.
15. Centrifuge at 900 g (4000 rpm), 10°C for 10 min.
16. Transfer the bottom layer (chloroform layer) through a Na₂SO₄ filled glass Pasteur pipet into 50-mL Teflon lined screw cap glass tube. (Fill Pasteur pipet with glass wool first, then with around 1 inch of Na₂SO₄ layer.)
17. Blow down under nitrogen gas in 60°C water bath. Nitrogen gas outlet should be close to liquid surface (same as #10).
18. Resuspend dried sample in 3 mL of isooctane and keep at -20°C or inject into GC.

APPENDIX 2

Procedure to determine sodium chlorite concentration of ASC solution

1. Weigh 25-30 g of ASC solution into a teated 250 mL beaker. Record weight to the nearest 0.01 g.
2. Dilute sample with 100 mL of deionized distilled water.
3. Add 2.0 g (1.8-2.2g) of potassium iodide crystals.
4. Add stir bar, and mix until crystals dissolve.
5. Add 10 mL of hydrochloric acid sol. N/2(Red/brown color appears)
6. While stirring, titrate with sodium thiosulphate N/10 until brown color fades to pale yellow.
7. Add 2 mL of starch indicator, 0.5%
8. Continue titration with sodium thiosulphate N/10 to a clear end point
9. Record volume of sodium thiosulphate used in mL (V) to the nearest 0.01 mL.
10. Calculation: $(V) \times 2261 / \text{Sample wt} = \text{ppm NaClO}_2$

APPENDIX 3

Statistical program for all responses

```
option LS=80 pagesize=60 nonumber;  
title ;  
data; input block day treat $ response;  
cards;  
proc print;  
proc glm; classes treat block day;  
model response=block treat|day block(treat);  
test H=treat E=block(treat);  
lsmeans treat/pdiff E=block(treat);  
lsmeans day treat*day/pdiff;  
proc sort; by treat;  
proc means mean stderr; by treat;  
proc sort; by day;  
proc means mean stderr; by day;  
proc sort; by treat day;  
proc means mean stderr; by treat day;  
run;
```


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